



# **9<sup>TH</sup> ANNUAL CONFERENCE OF THE METABOLOMICS SOCIETY**

## **FULL ABSTRACTS**

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# PLENARY SPEAKER ABSTRACTS

PL-1

## THE CELLULAR UPTAKE OF PHARMACEUTICAL DRUGS: A PROBLEM NOT OF BIOPHYSICS BUT OF SYSTEMS BIOLOGY

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A fundamental question remains as to whether xenobiotic drugs cross cellular membranes mainly (or exclusively) by 'passive' (transporter-independent) diffusion across cellular membranes, or whether they normally (or exclusively) 'hitchhike' rides using the carriers normally involved in the metabolism of natural metabolites. The former would involve a biophysical mechanism, based mainly on lipophilicity, while the latter requires a mechanistic understanding of which carriers are involved, and is thus a problem of network or systems biology. In other words [1], is carrier-mediated transport of pharmaceutical drugs the exception or the rule?

A huge amount of literature [1-5, and references therein], that I shall summarise, indicates that there is no serious evidence for transbilayer-mediated transfer of pharmaceutical drugs across biological membranes, while there is abundant and increasing evidence for the carrier-mediated route. A recent approach in yeast illustrates this experimentally [6], while the digital availability of principled metabolic network models [7,8] allows one to determine [9], consistent with this, that successful pharmaceutical drugs are much more like metabolites than are the 'Lipinski-compliant' molecules typically available in drug discovery libraries. This suggests that cellular drug uptake is more or less exclusively transporter-mediated, and that knowledge of both the metabolome and of the transporters used by individual xenobiotics will be of much value in designing better drugs [10].

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## SOLVING THE BIOCHEMICAL JACOBIAN – AN EQUATION TO SYSTEMATICALLY LINK THE GENOTYPE TO THE MEASURED METABOLOMIC PHENOTYPE

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Systems biology is the approach to combine molecular data, genetic evolution, environment and species-interaction with the computer-assisted understanding, modeling and prediction of active biochemical networks. The idea relies strongly on the existence of complete genome sequences and the development of new technologies for the analysis of molecular data. Here, projection of metabolomics data into genome-wide metabolic networks combined with metabolic modeling emerge as important technologies for improving gene annotation processes (Weckwerth, 2011b). Using quantitative proteomics and metabolomics we begin to investigate the genome-scale molecular phenotype and the interrelation of the metabolome, the proteome and its environment (Weckwerth, 2011a). Metabolomics and proteomics data integration strategies and modeling approaches will be discussed for model systems as well as ecosystems. For these approaches an extended metabolomics platform comprising GC-MS and LC-MS is presented (Scherling et al., 2010; Doerfler et al., 2012; Mari et al., 2012). However, before data reveal their interrelation, extended statistical and mathematical concepts are required for the integrative analysis of multifactorial phenomena (Weckwerth, 2003). The detection of significant correlations between the different components based on principal components analysis or related techniques is the basis for biological interpretation (Morgenthal et al., 2005; Weckwerth and Morgenthal, 2005; Weckwerth, 2008; Wienkoop et al., 2008). We have extended this idea and developed an approach which connects systematically the predicted genotype with the statistical features of metabolomics data (Sun and Weckwerth, 2012). By using this approach, recently, we were able to calculate the differential biochemical Jacobian from perturbed metabolomics data for the first time (Doerfler et al., 2012). Results and implications of this approach will be discussed.

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PL-3

**COMPREHENSIVE METABOLOMIC ANALYSIS FOR STUDY OF HUMAN DISEASES:  
INTEGRATION OF PLATFORMS AND TOOLS IN THE NIH WEST COAST METABOLOMICS  
CENTER**

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The NIH Common Fund has created three regional resource cores to increase the capacity for metabolomic research for NIH-funded projects. At UC Davis, the West Coast Metabolomics Center integrates more than 30 mass spectrometers and 5 NMR instruments in eight laboratories, focusing on glycomics, complex lipids, eicosanoids and lipid mediators, imaging, primary metabolism and identification of unknowns. In addition, integration with genomic data including pathway mapping and statistics is part of research advancements and fee-based services. In its first year, the West Coast Metabolomics Center has hosted three symposia on cancer metabolism, microbial metabolism and a hands-on workshop for training the next generation of scientists. The yearly pilot project funding competition for external clinical and biomedical scientists has been decided, and the projects will be highlighted briefly.

Platforms and example studies conducted by the West Coast Metabolomics Center are presented to give insights into the approach taken by our Center. We will elaborate how metabolomic data could formulate hypotheses that are subsequently validated by genomic data in pharmametabolomic studies, we will show how discoveries in glycomics led to different treatments of newborns in the neonatal care unit at the UC Davis hospitals, and we demonstrate how novel biomarkers in lung cancer enable diagnosis of the disease up to one year before patients present to the clinic, giving more time for treatment for these patients.

## **DROSOPHILA, METABOLOMICS AND FUNCTIONAL GENOMICS – A MATCH MADE IN HEAVEN**

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Compared to classic models like yeast, multicellular organisms present the challenge of complexity but also the opportunity to understand function of the whole organism in the context of its constituent parts. *Drosophila* is an ideal 'bridge' organism, with a compact, sequenced genome, and powerful genetic resources and reverse genetic technologies that allow gene function to be elucidated in the context of the whole organism. Previously, we published a tissue and life-stage specific transcriptional atlas for *Drosophila* (FlyAtlas.org), providing insights into what individual tissues contribute to overall function. We have also shown that *Drosophila* is amenable to global metabolomic analysis, using the Orbitrap mass spectrometer to identify around 500 compounds in each run. This approach has been particularly useful in studying fly models for human inborn errors of metabolism, such as xanthinuria. We have also shown that, just like mRNA levels in FlyAtlas.org, baseline metabolomes differ between tissues in larval and adult fly, providing useful insights into pathways of particular importance in specific tissues. To take our analysis to a further level, we have developed a model of core metabolism in MATLAB/COBRA, and used flux balance analysis to identify gaps. Presently, we are engaged in filling these gaps experimentally by reverse genetics, and extending the model.

## ROLE OF POST-TRANSCRIPTIONAL REGULATION IN THE CONTROL OF CARBON METABOLISM

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Metabolism is a basic cellular function that sustains survival, growth and adaptation of living organisms. As such metabolism is submitted to intense regulation by highly sophisticated global regulatory networks coordinating the physiological and metabolic responses. If metabolic and transcriptional regulations of metabolism have been studied in details, the role of post-transcriptional regulation in the control of carbon metabolism has been poorly investigated. The Carbon Storage Regulator (Csr) system is a post-transcriptional regulation system that controls a broad range of physiological mechanisms (including formation of biofilm, motility and virulence) and is a global regulator of central metabolism in the bacterium *Escherichia coli*. To get comprehensive understanding of the role of Csr in metabolic adaptation, we have investigated mutants altered for the various components of the Csr system. The results show that mutants altered for CsrA, the main component of this system shave decreased growth efficiencies on a broad range of physiologically relevant carbon sources. Detailed investigations of the metabolomes and fluxomes of mutants and wild-type cells grown on various carbon sources revealed significant and nutrient-dependent re-adjusting of central carbon metabolism, indicating a significant role of CsrA in the control of carbon metabolism.

**PL-6**

## **METABOLISM AND CANCER: WHY SHOULD WE CARE?**

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As a result of increased bioenergetic demands and the need to grow and proliferate, cancer cells have unique metabolic traits compared to normal cells. At the same time, cancer cells are exposed to more extreme conditions of metabolic stress due to the uncontrolled growth of the tumour away from the vascular system that provides oxygen and nutrients to its cells. Therefore, cancer cells have developed defence mechanisms that are selected under conditions of stress and cells that survive this strongly selective environment have a more aggressive phenotype. Targeting these survival mechanisms may help eliminate cancer growth and specifically induce cancer cell death.

Our work utilizes analytical chemistry and system biology approaches to study metabolic transformation. We investigated cells deficient in the mitochondrial tumour suppressor fumarate hydratase (FH). FH is a tricarboxylic acid (TCA) cycle enzyme and a tumour suppressor which is lost in some severe cases of renal cell cancer. Using genetically-modified primary mouse renal cells we collected metabolomics data and applied a computational model, generated to study their unique metabolome. We identified several important metabolic pathways which are specific and crucial for the survival of cells deficient in FH. These include the heme biosynthesis and degradation pathway as well as mechanisms of alleviating TCA cycle carbon stress. These technologies are not only important for understanding the basic biochemistry of cancer cells but they can inform us on future clinical management of cancer and may lead to new therapeutic approaches to target cancer-specific metabolic pathways.

PL-7

## APPLICATION OF METABOLOMICS TO PLANT BREEDING BY GENETIC INTERVENTION

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Tomato fruit and its products are one of the most widely consumed fruits and vegetables in the world. They are important components of the human diet supplying essential micronutrients. In addition to being an important economic crop, tomato is also a model for all crop plants. Excellent genetic resources exist in tomato culminating in the recent release of the tomato genome sequence ([www.solgenomics.net](http://www.solgenomics.net)). The depth and breath of chemical diversity found in tomato has also contributed to the development of metabolomics/metabolite profiling expertise in tomato and other Solanaceae. In this presentation the role of metabolomics in:

- (i) The large-scale multi-platform metabolite analysis of natural variation in tomato that exists in the form of the *Solanum pennellii* near isogenic introgression (II) will be described. Collectively this data generated has provided, a valuable metabolite resource, a rapid means of associating trait to metabolite and in some cases effector gene and revealed valuable insights into the organisation of metabolism in tomato fruit and other plant systems.
- (ii) The metabolomic characterisation of genetic engineering approaches for high value isoprenoids in *Solanaceae* illustrating the perturbations that arise across metabolism and how changes in the composition of metabolites can impact on the plastids generated and their internal ultrastructure.

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PL-8

**ANALYTICAL, IMAGING, MINIATURE AND PREPARATIVE MASS SPECTROMETRY IN METABOLOMICS**

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A minor revolution is occurring in mass spectrometry with the growing recognition that it is possible to simplify sample preparation and still obtain high quality chemical information on complex real-world samples. At the core of this development is a set of ambient ionization experiments in which ionization is performed in air, outside the mass spectrometry, on samples in their native form. Starting with the use of a charged spray (desorption electrospray ionization, DESI) in 2004, implementation included plasmas, lasers and thermal agents. The fundamentals of DESI and the paper spray method are reviewed and their applications to metabolomics are illustrated. These include DESI imaging applied to disease diagnosis and paper spray for the quantitative analysis of therapeutics and metabolites in whole blood. Comparisons with traditional LC/MS/MS methods of trace quantitative analysis are made to validate these rapid methods. Remarkable reaction rate enhancements are observed in the spray based ambient ionization methods that allow increased sensitivity and specificity in metabolite determination in biofluids. An overview of mass spectrometry instrumentation is followed by a discussion of the current state of development of miniature mass spectrometers, especially their use as portable instruments for in-situ applications.

PL-9

**STRESS & DRUGS AND ROCK & ROLL: TOWARDS AN UNDERSTANDING OF MICROBIAL ADAPTATION**

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Human pharmaceuticals are readily detected in wastewater treatment plants, rivers and estuaries. Whilst levels are not yet high enough to cause immediate harm to aquatic life, it is widely acknowledged that there is insufficient information available to determine whether exposure to low levels of these substances over long periods of time is having an impact on the microbial ecology of these environments. In order to investigate the effect on the metabolic potential of the microbial community we have been adopting a metabolomics approach using various analytical platforms including vibrational spectroscopic approaches for generating metabolic fingerprints, gas chromatography-mass spectrometry (GC-MS) for metabolic profiling and direct infusion (DIMS) and liquid chromatography mass spectrometry (LCMS) for lipid profiling. Analysis of environmentally relevant microbes and algae will be presented. We shall show that Propranolol had significant effects on the lipid components of *Pseudomonas putida* cells, and in particular we detected changes in the acyl chains of cardiolipins (the degree of saturation) which may have changed in order to maintain correct membrane fluidity (so called homeoviscous adaptation). Propranolol is a chiral pharmaceutical and we shall also show that some bacteria respond differently to the two enantiomers and this results in different effects on cellular phenotype. Finally, we turn our attention to exploiting metabolomics for probing real complex environmental systems and investigate phenol-degrading bioreactors containing yet to be defined microbial communities.

**PL-10**

## **METABOLOMICS IN SCHIZOPHRENIA**

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A major challenge in medicine is to understand genetic, molecular and cellular mechanisms underlying common mental disorders including schizophrenia, which involve complicated genetic and environmental determinants. Schizophrenia is a common mental disorder, affecting 0.5-1% of the population. It mostly presents with several episodes and tends to become chronic. The underlying molecular mechanism of schizophrenia is poorly understood. Metabolomics can provide valuable information about disease pathogenesis and result in metabolic signatures that could be developed as biomarkers for disease and progression. Comparative studies in plasma could help map peripheral changes in metabolism in schizophrenia and enable a more accessible way for biomarker development. Using targeted metabolomics we quantified and compared 103 metabolites in plasma samples from 216 healthy controls and 265 schizophrenic patients, including 52 cases without antipsychotic medication. Compared with healthy controls, levels of five metabolites were found significantly altered in schizophrenic patients (p-values ranged from  $2.9 \times 10^{-8}$  to  $2.5 \times 10^{-4}$ ) and in neuroleptics-free probands. These metabolites include four amino acids (arginine, glutamine, histidine and ornithine) and one lipid (PC ae C38:6) and are suggested as candidate biomarkers for schizophrenia. Furthermore we constructed a molecular network connecting these five aberrant metabolites with 13 schizophrenia risk genes. Our result implicated aberrations in biosynthetic pathways linked to glutamine and arginine metabolism and associated signaling pathways as genetic risk factors, which may contribute to patho-mechanisms and memory deficits associated with schizophrenia. This study illustrated that the metabolic deviations detected in plasma may serve as potential biomarkers to aid diagnosis of schizophrenia.

PL-11

**Deciphering Highly Dynamic Chemically Mediated Interactions of Microalgae Using a Combined Metabolomics / Bioassay Approach**

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It is well established that unicellular algae in biofilms and in the plankton have established means to interact with other organisms using chemical signals. Algal exudates and metabolites stored in the cells can e.g. mediate feeding activity of herbivores and interactions with conspecific algae via pheromones. But also the surrounding microbial community can be influenced by released metabolites. We introduce an approach to address such chemically mediated interactions based on a comparative metabolomics approach. Metabolomic surveys indicate that diatoms exhibit a high plasticity of metabolic activity in response to environmental factors and biotic interactions. Metabolite concentration changes dramatically in response to limiting nutrients or osmotic stress and a pronounced circadian variability can be observed as well. But our results also indicate that the regulation of biosynthetic pathways in microalgae is highly dependent on the ecological context of the cells. We used bioassays to demonstrate how the variable chemical profiles of the algae are causing pronounced variability of the chemical interaction with the environment. Such comparative metabolomics approaches enabled the identification of the first diatom pheromone, novel diatom defense metabolites and of compounds influencing diatom-diatom interactions. Consequences for future investigations of diatom chemical interactions and ocean functioning are discussed.

**PL-12**

**HIGH THROUGHPUT METABOLOMICS OF THE ENTIRE *E. coli* DELETION LIBRARY**

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Given that we know the structure of metabolic networks relatively well, our focus is on understanding how metabolic fluxes emerge from the interactions of thousands of genes, proteins and metabolites (1). In particular we ask how these network-wide fluxes are managed and which of the multiple overlapping regulatory mechanisms and the metabolic feedback into these regulatory networks actually control flux under a given condition (2, 3). Since the throughput of <sup>13</sup>C-based flux experiments for systematic large-scale studies remains still limited, we developed a novel method for high-throughput intracellular metabolomics (4). Here I will discuss the application of this method to all 8600 clones of the *E. coli* genome-deletion (KEIO) library (5), and how those results can be exploited for functional assignment of novel enzymatic functions and in learning from metabolomics data about active regulation mechanisms.

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**PL-13**

**METABOLOMICS PROFILING IN DIABETES AND HEART DISEASE**

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We seek to apply metabolomics for understanding of mechanisms underlying the pandemic metabolic diseases of our era--diabetes, obesity, and cardiovascular disease. We have used these tools to define mechanisms underlying development of peripheral insulin resistance and glucose intolerance in animals and humans. For example, we have recently identified perturbations of branched chain amino acid (BCAA) catabolism in multiple cohorts of insulin resistant humans compared to normally insulin sensitive controls. Our studies and those of others have demonstrated the prognostic power of this signature to predict incident diabetes and intervention outcomes. These metabolites are also uniquely sensitive to the most efficacious interventions for obesity and diabetes. We have translated these findings to rodent models to demonstrate a contribution of BCAA to abnormalities in mitochondrial metabolism that contribute to the insulin resistant state, as well as to behavioral abnormalities associated with obesity. We have also identified novel metabolic signatures of imminent cardiovascular events, including BCAA and a cluster of short-chain dicarboxylated acylcarnitines, and are integrating genomic and metabolomics analyses in large cohorts of human subjects to identify pathways involved in the production of these metabolites and their relationship to risk of cardiovascular disease. These examples will serve to illustrate the potential of comprehensive metabolic profiling methods for providing insights into metabolic disease mechanisms.

**TOWARDS PERSONALISED MEDICINE OF CANCER BASED ON MS-BASED METABOLOMICS**

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In the 21st medicine a growing interest is to develop an individually tailored therapeutic approach by using the disease information characterized at the molecular level to improve diagnoses and produce better medical outcomes. Biomarkers are the foundation for personalised medicine. Metabolomics is a tool to study all endogenous small molecules in a biological system, it can provide the metabolic state (phenotype) of an individual and find the potential biomarkers.

In our lab a comprehensive metabolomics platform has been established by using chromatography-mass spectrometry and capillary electrophoresis-mass spectrometry techniques. More than 10 analytical methods have been developed. With these methods, great attention has been paid to the early diagnosis and personalized treatment of hepatocellular carcinoma and oral squamous cell carcinoma. Several metabolites have been identified as combinational biomarkers for cancer diagnosis, evaluation of the operation and chemotherapy efficacy, and prediction of tumor recurrence after surgery. We also found that serum metabolites were sensitively responsive to TPF induction chemotherapy, and the metabolites were beneficial to the evaluation and prechemotherapy prediction of induction chemotherapy outcomes, illustrating the metabolomics potentials for personalised induction chemotherapy.

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**PL-15**

**METABOLOMIC ANALYSIS OF PARASITIC PROTOZOA**

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Metabolomics allows the simultaneous identification of many hundreds of metabolites within a given system. We have applied untargeted LC-MS based metabolomics approaches (based on Orbitrap technology) to numerous systems including trypanosomes and leishmania. In studying modes of action of trypanocidal drugs we show how metabolomics can point to drug targets e.g. ornithine decarboxylase in treatment with eflornithine and also drug resistance mechanisms (loss of drug uptake associated with eflornithine resistance). In differentiation of slender bloodstream form to short stumpy form trypanosomes we note increases in carnitine levels as slender forms transform to stumpy forms and both the pentose phosphate pathway and cellular thiol levels increase. In Leishmania we have identified a mechanisms for resistance to amphotericin B by taking a polyomic based approach comparing wild-type and resistant cells at the level of genome, proteome and metabolome, with changes to sterol metabolism shown to underlie resistance related to alterations to the parasite's CYP51 enzyme. Novel unexpected pathways of metabolism have also been identified by studying the distribution of heavy atom isotopes of various substrates. In addition to identifying many aspects of parasite biochemistry we have also investigated how host's respond to parasite infection and reveal important markers of infection.



# ORAL ABSTRACTS

O1A-1

## **LARGE-SCALE SURVEY OF METABOLITE CONCENTRATIONS IN HUMAN, CHIMPANZEE, MACAQUE AND MOUSE TISSUES SUGGESTS TRADEOFF BETWEEN HUMAN MUSCLE AND BRAIN**

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Currently there is limited knowledge about metabolic changes specific to humans, as well as about the overall extent of metabolic divergence among mammalian species. In this study we conducted a large-scale analysis of metabolite concentrations in three brain regions (prefrontal cortex [PFC], primary visual cortex [V1] and cerebellar cortex [CBC]) and two non-neural tissues (skeletal muscle and kidney) of humans, chimpanzees, macaques and mice using six mass spectrometry-based metabolomics approaches. In a total of 365 tissue samples we assayed 17,087 metabolite peaks, 2,469 of which were recognized as metabolites based on metabolite standards or metabolite database matching. In addition, transcript concentrations were measured in a subset of 120 tissue samples using RNA-sequencing (RNA-seq).

In all tissues, metabolite concentrations diverged rapidly among species: 74% of metabolic peaks differed among the four species and 44% - among primates. Rate of metabolic divergence on the human lineage varied substantially among tissues: in V1 and kidney human- and chimpanzee-specific divergence were equal and scaled with the species phylogenetic distances. By contrast, in PFC and muscle human-specific divergence exceeded chimpanzee-specific one by over 4- and 7-fold. This metabolic change was confirmed by expression of corresponding metabolic enzymes and affected pathways associated with cognitive functions, energy metabolism and human metabolic diseases such as diabetes. Using macaques as a model system to test effects of stress, diet and physical activity on metabolome, we assessed that less than 2% of observed species-specific changes could be attributed to these environmental factors.

We hypothesize that rapid metabolic evolution of human PFC and muscle shaped unique human cognitive and physical abilities. While cognitive functions clearly distinguish humans from other primates, no systematic comparison of the muscular strength of humans and other primates exists. We performed behavioural tests in humans, chimpanzees and macaques and demonstrated significantly inferior human muscular strength compared to the other two species. Altered muscle metabolism might have conditioned the evolution of the highly energy-demanding human brain.

O1A-2

**METABOLOMICS AND SYSTEMS BIOLOGY OF GENOTYPE-BY-DIET INTERACTIONS UNDERLYING METABOLIC SYNDROME IN DROSOPHILA**

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Diseases linked to Metabolic Syndrome (MetS) such as type-2 diabetes and cardiovascular disease are rapidly increasing due to the influences of a modern Westernized-life style, but the genetic, environmental, and physiological mechanisms linking the symptoms of MetS remain to be elucidated. Large-scale studies to systematically assess how genotype interacts with the environment to cause complex disease are very difficult in humans, but such studies are relatively tractable in genetic model systems such as *Drosophila melanogaster*. *Drosophila* bears great homology to mammals in many aspects of relevant physiology and organ systems including insulin signalling, heart function, innate immunity, and response to aerobic exercise. Previously, we have shown that there is a very substantial contribution of genotype-by-environment interactions to the phenotypic variation observed for MetS-like symptoms (e.g. body weight, triglyceride storage, blood sugar) in a naturally genetically variable population of *D. melanogaster*. We pursued the mechanistic link between diet and genotype leading to MetS-like disease using two basic approaches. Initially, we performed GC-MS/MS metabolomic and whole genome transcriptomic profiling of the genotype-by-diet interactions underlying MetS-like phenotypes. Using 20 wildtype isogenic lines across four diets, we integrated the data across physiological levels to develop a systems biology framework for understanding these traits. We demonstrate clear correlations between metabolomic and gene expression profiles and MetS-like symptoms as they vary across diet. Module-based analysis of co-expressed metabolites allows us to identify genetic pathways contributing to variation in the MetS-like symptoms. As a second approach, we are mapping the genetic basis of variation in dietary influences of a normal versus high fat diet on metabolomic profiles (as determined by both GC and LC-MS/MS techniques) and the associated MetS-like phenotypes, using the *Drosophila* Synthetic Population Resource. Multiple genetic loci are associated with these traits and within those loci are both familiar and novel candidate genes for involvement in Metabolic Syndrome. Overall, this study demonstrates the power of model systems to leverage experimental tractability coupled with metabolomics methods to elucidate complex disease pathways.

**O1A-3**

**METABOLOMIC ANALYSIS OF PEANUT ALLERGY IN A MOUSE MODEL**

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Hypersensitivity or allergy to peanuts is an increasingly problematic health concern around the world, involving approximately 1-2% of children in North America. There are no useful clinical biomarkers for peanut allergy and no known mediators for development of this allergy or for onset of anaphylaxis induced by exposure to peanuts. We report the first application of a comprehensive metabolomics approach to serum in mice during the time courses of (a) the development of sensitization to peanuts and (b) the period following intraperitoneal injection of peanut extract in peanut-sensitized and non-sensitized mice.

Mouse serum samples (30  $\mu$ L) were added to MeOH:EtOH:H<sub>2</sub>O (2:2:1, 120  $\mu$ L) then centrifuged to afford a clear supernatant ready for LC-ESI-MS analysis (2  $\mu$ L injection). The LC method employed a ZIC-HILIC column followed by a RP Amide column (both 2.1 x 50 mm) under HILIC gradient elution conditions (200  $\mu$ L/min); MS analysis was performed on a Bruker micrOTOF II time-of-flight mass spectrometer (10,000 mass resolution FWHM). This method, which allowed the separation and analysis of both polar and non-polar metabolites, was shown to have a significantly less ion suppression across the chromatogram compared to that observed using either column separately. All treatments were performed as seven replicates. The resulting chromatograms (including pooled samples) were processed using XCMS and CAMERA to afford 3500 features. These data were analyzed using OPLS-DA and ANOVA methods.

Statistical analyses of the 3500 metabolic features across these time-course experiments revealed significant changes across the metabolome, particularly in the purine catabolism pathway. Subsequent experiments in two mouse models of peanut allergy showed conclusively that uric acid functions as a mediator of both peanut sensitization and peanut anaphylaxis in mice. Numerous other changes were observed across the metabolome. This work highlights the use of metabolomics as a discovery tool for the confirmed identification of a molecule that serves as a mediator of an important disorder.

#### O1A-4

### **THE COMBINED USE OF DROSOPHILA AND YEAST AS MODEL ORGANISMS FOR THE IDENTIFICATION OF UNKNOWN MITOCHONDRIAL PROTEIN FUNCTION**

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The human genome has approximately 20,000-25,000 protein coding genes, many of these have no known function. To fully develop the potential of functional genomics the identity of these unknown genes is imperative. We have implemented a metabolomics approach to identify the function of mitochondrial genes using a combined LC/GC-MS approach. To greatly simplify the metabolomics analysis, the model organisms *S. cerevisiae* and *Drosophila* are employed. Many genes in these organisms have high human homology and are interchangeable between species. In addition these model organisms have well established genetic and biochemical protocols. Our strategy for this work encompasses both genetics and metabolomics. Genes which have high homology in humans, yeast and fly that have no known function are identified and gene deletion mutants are constructed in both model organisms. We have developed ideal growth conditions and extraction methods for metabolomics analysis for yeast in batch culture and for the fly at different stages of its life cycle. We use two instrument platforms for metabolomics profiling, a GC-TOF-MS for the non-targeted profiling of the low molecular weight metabolome, and a LC-QTOF-MS for the non-targeted LC-MS analysis of both the higher molecular weight metabolome and the lipidome.

A recent example (1) of this approach is the discovery of a mitochondrial pyruvate carrier required for pyruvate uptake. Two proteins were identified, Mpc1 and Mpc2, as essential for mitochondrial pyruvate transport in yeast, *Drosophila*, and humans. Using the metabolomics based approach yeast and *Drosophila* mutants lacking MPC1 display impaired pyruvate metabolism, with an accumulation of upstream metabolites and a depletion of tricarboxylic acid cycle intermediates. Loss of yeast Mpc1 results in defective mitochondrial pyruvate uptake, while silencing of MPC1 or MPC2 in mammalian cells impairs pyruvate oxidation. Human genetic studies of three families with children suffering from lactic acidosis and hyperpyruvateemia revealed a causal locus that mapped to MPC1, changing single amino acids that are conserved throughout eukaryotes. These data demonstrate that Mpc1 and Mpc2 form an essential part of the mitochondrial pyruvate carrier.1) Bricker et. al. Science. 2012. Jul 6;337(6090):96-100.

**O1B-1**

**METABOLOMICS AND FLUXOMICS IN MAMMALIAN TISSUE: METHOD OPTIMIZATION AND INSIGHTS INTO METABOLITE FLUX DURING EXERCISE**

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Mammalian tissue such as skeletal muscle, liver, and adipose are widely-studied targets of metabolomics experiments, since alteration in function of major organs is a key feature of diseases including cancer and diabetes. Metabolomics of organ tissue introduces particular challenges regarding tissue collection and data normalization which have not been as thoroughly studied as many other aspects of sample preparation. We present experimental results from LC-MS and GC-MS analysis of rodent tissue collected under various modes of commonly-used anesthesia. Different modes of anesthesia resulted in significant alterations in central carbon metabolism including changes in hexose phosphates, lactate, TCA cycle intermediates, and nucleotide phosphates. Based on these findings we have developed refined methods for tissue collection and extraction for metabolomics studies. We have applied these methods to the study of metabolite concentrations and metabolic flux during exercise. In these experiments, carbon-13 enriched stable-isotope tracers were administered to rodents immediately prior to treadmill exercise. Mass isotopomer analysis allowed detection of tracer incorporation into downstream metabolites and enabled assessment of relative flux under different exercise conditions. These experiments demonstrate the feasibility of these methods for studies of animal models of disease and their potential for studies involving human subjects.

## O1B-2

### A METHOD TO MEASURE METABOLIC FLUX WITH HEAVY ISOTOPE LABELLING AND MASS SPECTROMETRY

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A tool for measuring the changes of metabolic flux would be of great value for better understanding of plant metabolism.

We have developed a method to label six week old *Arabidopsis thaliana* plants with heavy carbon dioxide (<sup>13</sup>CO<sub>2</sub>) and used combined gas- and liquid- chromatography – mass spectrometry (GC- and LC-MS) methods to follow the incorporation of the <sup>13</sup>C into the metabolic network. Following the shift from non-labelled to labelled molecules makes it possible to get an overview of the metabolic flux of the primary carbon metabolism.

A knockout single mutant lacking the most highly expressed isoform of mitochondrial malate dehydrogenase, mMDH1, was used for proof of concept. Labelling was done during two hours in the middle of the photoperiod, when the metabolism is at its most stable state. Three different CO<sub>2</sub> conditions were investigated; low (< 150 ppm), ambient (400 ppm) and high (> 1000 ppm).

Although not displaying any visible growth differences with wild type plants, the mutant displayed a distinct metabolic phenotype, which was strengthened under low CO<sub>2</sub> conditions. These results support previous reports that malate dehydrogenase has an important function in photorespiratory metabolism. This also demonstrates how monitoring metabolic flux, using the method presented, can be a helpful tool in the task to better understand plant metabolism.

O1B-3

**CROSS-LABELED  $^{13}\text{C}$ -STEARATE FATE DETECTION IN THE  $[1,2-^{13}\text{C}_2]$ -D-GLUCOSE DERIVED ISOTOPOLOME IMPROVES SYSTEM WIDE ASSOCIATIONS WHEN COMPARED WITH EXTERNAL  $[\text{U}-^{13}\text{C}_{18}]$ -STEARATE INCUBATION IN ROSIGLITAZONE TREATED HEPG2 CELLS**

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Uniquely labeled atoms link their tracer-carrying substrate with diverse metabolic pathway products within the metabolome, which benefit biological studies and drug research. Isotopome-wide association studies (IWAS) are based on similar principles and pursue similar goals as studies of genome-, proteome- and transcriptome-wide associations. Specifically, metabolic tracers yield products in which the replacement and positions of atomic substitutions are correlated in the organism's metabolome, as the speed with which these labeled stable isotopes assume their positions in product molecules reveal highly significant relational associations within drug-responsive metabolic pathways. We herein demonstrate that  $[1,2-^{13}\text{C}_2]$ -D-glucose derived cross labeled  $^{13}\text{C}$ -stearic acid improves system wide associations between ribonucleic and fatty acids in 1 and 10 microM rosiglitazone treated HepG2 cells, in comparison with associations obtained when external  $[\text{U}-^{13}\text{C}_{18}]$ stearate was used. Our study shows that internally cross-labeled  $^{13}\text{C}$ -stearate from the single  $[1,2-^{13}\text{C}_2]$ -D-glucose tracer readily serves as the precursor of other fatty acids and RNA ribose, while external  $[\text{U}-^{13}\text{C}_{18}]$ stearate pends transport and substrate availability constraints. More specifically, internally  $^{13}\text{C}$  labeled stearic acid from  $[1,2-^{13}\text{C}_2]$ -D-glucose showed an improved, by about 20%, set of correlation coefficients via carbon exchange between  $^{13}\text{C}$  stearate fractions and  $^{13}\text{C}$  RNA ribose after rosiglitazone treatment, in comparison with that obtained with external  $[\text{U}-^{13}\text{C}_{18}]$ stearate (0.5 mM) incubation for 72 hours. Based on our study single tracer isotopome wide associations offer significant advantages over parallel multiple  $^{13}\text{C}$  tracer metabolic flux analyses because 1) drug responsive associations are observed in the same experiment among various substrate-product pools, 2) internally cross-labeled precursors more readily enter metabolic channels and therefore 3) less time and resources are required to generate physiological information in comparison with versatile multiple tracer substrate experiments. In conclusion, targeted  $[1,2-^{13}\text{C}_2]$ -D-glucose assisted metabolomics for network-wide pathway associations involving carbohydrate, nucleotide, lipid, and amino acid metabolism during drug treatment recognizes important substrate-product relationships on a system wide scale in the same experiment.

**O1B-4**

**KINETIC MODELING APPLIED TO THE ANALYSIS OF <sup>13</sup>C TRACER DISTRIBUTION IN METABOLITES: NEW LIFE FOR THE OLD TOOL**

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Kinetic modeling is a classical method widely used for the analysis of biochemical data in order to assist in understanding the mechanisms of enzyme reactions and/or regulation of metabolic pathways. Whereas it proved to be very informative being applied for the in vitro data analysis, it is less fruitful in the analysis of metabolism of living cells, because of limitation in the amount of input information necessary for the reliable analysis. Tracing metabolites through the network of reactions in the cell using <sup>13</sup>C isotopes is presumably the method that can provide the information necessary for a reliable kinetic analysis. Here the method of adapting the kinetic modeling to the analysis of time course of isotopic isomer distributions is presented. The steps of such an adaptation are i) extension of the set of metabolic fluxes to account for all the isotope exchanges, and ii) transformation of reactions between substances into reaction between isotopomers. Advantages of the kinetic approach: i) extraction the most complete information from the dynamics of labeling, ii) evaluation of the characteristics of metabolism more profound than just flux distribution, such as metabolism compartmentation, parameters of enzymatic reactions, regulatory mechanisms. Several examples of the application of kinetic modeling for <sup>13</sup>C tracer data analysis are considered.

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O2A-1

**THE Myc/p53-DEPENDENT TUMOUR SUPPRESSOR miR-22 REGULATES MULTIPLE METABOLIC PATHWAYS IN CANCER CELLS**

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The reprogramming of tumour metabolism is a fundamental hallmark of cancer and is actively driven by alterations in tumour suppressors and oncogenes such as MYC and p53. MicroRNAs (miRNAs) have been shown to play a key role in producing common tumour metabolic phenotypes such as the Warburg effect and glutaminolysis in response to MYC overexpression. From an integrative analysis of miRNA, mRNA, and metabolite profiles from the NCI-60 cancer cell panel we observed that miRNAs negatively regulated by the oncogene MYC was highly influential on cell metabolism. Within this module the p53-dependent tumour suppressor, miR-22, showed the most profound association to variation in metabolic phenotype. Ectopic expression of miR-22 had a pleiotropic effect on metabolism in breast cancer cells, suppressing glycolysis, promoting *de novo* fatty acid synthesis and glycogen metabolism, and targeting one-carbon metabolism. We identified post-transcriptional repression of ATP citrate lyase and fatty acid elongase 6 to be a possible mechanism by which miR-22 reduces the flow of glucose to fatty acid synthesis. We also found that miR-22 acts as a negative regulator of one-carbon metabolism by directly targeting expression of the mitochondrial enzyme MTHFD2, which was a predictor of breast cancer outcomes. Inverse correlations were observed in breast tumours between miR-22 expression and expression of MTHFD2 and other putative target genes in glycogen metabolism. Our data establish miR-22 as a regulator of multiple facets of metabolism, a role that could contribute to its proposed function as a tumour suppressor.

**O2A-2**

**THE GLUTAMINE METABOLISM NETWORK IN MELANOMA**

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Glutamine is an important nutrient, for both nitrogen and carbon metabolism, in many cancer cells that are incapable of synthesizing their own glutamine. In previous studies, we demonstrated that glutamine is a major carbon source for melanoma cell metabolism. In particular, after conversion to glutamate and 2-oxoglutarate, it acts as an anaplerotic feedstock for the tricarboxylic acid (TCA) cycle, and therefore has a role in energy metabolism and for biosynthesis. The importance of glutamine was confirmed in a panel of melanoma cell lines, where removal of glutamine from culture medium stopped growth and led to cell death (whereas glucose removal had variable effects on growth and did not kill cells). The glutamine concentration required for cell growth, and whether it could be replaced by other nutrients, was assessed by titrating glutamine in culture medium in the presence of a broad range of medium supplements. A combination of methylated (cell-permeable) 2-oxoglutarate, and non-essential amino acids or asparagine, reduced the minimum glutamine requirement for optimal growth of Lu1205 cells from 1.0 mM to 0.25 mM. This pointed to aspartate/ asparagine synthesis as a critical drain (or cataplerotic output) from the TCA cycle in melanoma cells. <sup>13</sup>C-metabolite tracing and quantification of metabolite fluxes by GC-MS indicated that >10% of glutamine taken up by cells was channelled into the TCA cycle, and that the terminal destination of ~5% of glutamine was aspartate/ asparagine in proteins. In contrast to other cancer cell types, there was little conversion of glutamine (via malate and pyruvate) into lactate (classical "glutaminolysis") in melanoma cells. The significance of glutaminase (GLS1) activity, which catalyzes the initial conversion of glutamine to glutamate, and other steps in glutamine metabolism in melanoma cells have been explored by gene knockdown or enzyme inhibition. The ultimate aim of these studies is to determine whether glutamine metabolism is a potential target for anti-melanoma therapy, and, if so, which enzymes within the glutamine metabolic network are the most promising targets.

## O2A-3

### ANALYSIS OF THE HYPOXIA METABOLOME

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Intra-tumoral hypoxic microenvironment leads to the activation of the hypoxia inducible factor, HIF-1. Cancer cell metabolism markedly differs from normal cells and the Warburg effect represents the best known and studied example of oncogenic metabolism reprogramming in cancer cells. Metabolomics studies and the optimization of new platforms can help to explain these metabolic changes. Colorectal cancer HCT116 wild-type and *hif-1α* knockout (-/-) cells were cultivated in normoxia or hypoxia for 24 hours. Highly sensitive measurement of lipophilic and polar metabolite profiles was performed by nanoflow-based LC-MS platforms using reverse- and in-phase chromatography.

Reproducibility and stability of nanoflow-based LC-MS platforms were checked by quality controls (QCs). Over 24 runs the pattern of the total ion chromatogram (TIC) showed a substantial stability and repeatability for both RPC18 and HILIC configurations. The number of molecular features detected in each of the 24 QC run was comparable in RPC18 and HILIC. Instrument response stability was checked using nominal masses with similar retention time over the 24 QCs runs.

Targeted metabolomics at high detection specificity allowed us to assess hydrophilic compounds and the role of HIF-1 $\alpha$  and/or hypoxia on glycolysis pathway-related metabolites. ATP and ADP had comparable levels when wild-type and *hif-1 $\alpha$* <sup>-/-</sup> cells were cultivated in normoxia and for wild-type cells grown in hypoxia; hypoxic *hif-1 $\alpha$* <sup>-/-</sup> cells had significantly lower levels of ATP and ADP.

We established a nanoflow LC-MS platform based on reversed- and in-phase chromatography allowing us to investigate the cancer cell metabolome including lipophilic and polar metabolites in as few as  $2.5 \times 10^5$  of cells, thus allowing analysis of clinical samples such as tissue biopsies. Metabolomics microarray analysis shows that many metabolites change independently of HIF, and in particular lipophilic metabolites are independent of HIF-1 $\alpha$  in hypoxia. Glycolytic intermediates are significantly increased in hypoxic conditions in the absence of HIF-1 $\alpha$  implying greatly reduced flux.

## O2A-4

### LIPIDOMIC RESPONSE TO HYPOXIA PROFILED BY 2-DIMENSIONAL GAS CHROMATOGRAPHY/MASS SPECTROMETRY

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Background: Cellular transformation from healthy to cancerous states is accompanied by reprogramming of metabolic pathways. This altered metabolism might support the unrestrained proliferation of cancer cells, leading to the formation of a tumor mass characterized by a low tissue oxygen tension. This oxygen limitation, termed hypoxia, induces a cellular response involving the activation of numerous hypoxia-induced genes and the activation of the hypoxia inducible factor, HIF-1 $\alpha$ .

Along with the dysregulation of glucose and glutamine metabolism, it has been proposed that enhanced *de novo* lipid biosynthesis and a more general lipogenic phenotype could be an important component of the metabolic reprogramming in cancer cells.

Methods: Colorectal cancer HCT116 wild-type cells were cultivated in normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>) for 24 hours in the presence of glucose (2000 mg/L). At 80% confluency, 2 $\times$ 10<sup>5</sup> cells were harvested, lysed and extracted in a Water/Methanol/Chloroform mixture. The **lipidic** fraction was used for analysis. We used GCxGC-MS to maximise the measurement of complex lipophilic molecules from cells.

A non-polar column was coupled via a micro connector to a polar column to ensure orthogonality of separation. A modulated cycle of cryo-trapping and heating was used following separation on the first column, which allowed separation in the second column/dimension over a 6 second period. This gave rise to a two-dimensional data matrix (fig 1) that was analysed on an electron impact (EI) Single Quadrupole QP2010 Ultra Mass Spectrometer (Shimadzu). MS data sets were processed and analysed using GC Image (Shimadzu) and ChromeSquare software packages.

Results: HIF-1 $\alpha$  was strongly induced by hypoxia in HCT116 cells, measured by western blot. Additionally, hypoxic HCT116 cells showed altered morphology with lower cell volume and cell diameter. There was no significant difference in the proportion of cells in G1, S and G2 cell cycle phases, but the cell proliferation rate was increased under hypoxic conditions.

Hypoxia induced a clear difference in the lipidomic profile of HCT116 cells detected by GCXGC-MS analysis. Lipophilic profiling by GCXGC-MS identified 461 GCxGC features (representing 132 different compounds) in normoxic HCT116, and 518 GCxGC features (representing 123 different compounds) in hypoxic HCT116 cells.

Conclusions: Cells cultivated in hypoxia demonstrate phenotypic differences compared to normoxia. Cell volume, diameter and total protein content were significantly reduced after 24 hours in 1% O<sub>2</sub>, while the proliferation rate is increased.

Two columns dimensional separation performed by GCXGC-MS allowed us to investigate the cancer cell lipophilic fraction polar metabolites in 2.5 $\times$ 10<sup>5</sup> of cells. Metabolomics profile shows that many lipophilic metabolites changed in hypoxia and that when overexpressed HIF-1 $\alpha$  could play an important role in the metabolism of the lipophilic fraction in HCT116 cancer cells.

O2A-5

**USE OF LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS) METABOLOMICS TO STUDY PLASMA BIOMARKERS: CASE STUDY USING A POTENT, SELECTIVE PAN-CLASS I PHOSPHATIDYL-INOSITOL-3-KINASE (PI3K) INHIBITOR**

Joo Ern Ang<sup>1</sup>, Rupinder Pandher<sup>1</sup>, Victoria Revell<sup>2</sup>, Sarah Davies<sup>2</sup>, Yasmin Asad<sup>1</sup>, Alan Henley<sup>1</sup>, Melanie Valenti<sup>1</sup>, Gary Box<sup>1</sup>, Ben Holmes<sup>2</sup>, Alfred Thumser<sup>2</sup>, Alexis de haven Brandon<sup>1</sup>, Suzanne Eccles<sup>1</sup>, Paul Workman<sup>1</sup>, Debra Skene<sup>2</sup>, Johann de Bono<sup>1</sup>, Florence Raynaud<sup>1</sup>

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Background: Class I phosphatidyl-inositol-3-kinases (PI3Ks) regulate multiple cellular processes that are often dysregulated in cancer. For example, amplification and overexpression of *PIK3CA* and deletion or mutation of *PTEN* are common in human cancers. Potent and selective PI3K inhibitors, such as GDC-0941, show promise in early phase clinical trials; the development of these drug candidates can potentially be accelerated with the use of novel, minimally-invasive biomarkers.

Methods: LC-MS metabolomic profiling was performed on plasma from pre-clinical models including xenograft harbouring hyperactivated PI3K and non-tumour-bearing mice. Plasma metabolites were subsequently assessed in 41 patients enrolled in a Phase I clinical trial of GDC-0941; the impact of time-of-day and timed meals as potential confounders of these results were additionally studied in a separate group of 12 healthy volunteers maintained under stringently-controlled laboratory conditions.

Results: We identified plasma metabolites, including amino acids, components of  $\beta$ -oxidation and phospholipids, that were consistently modulated with the application of GDC-0941 in murine models and human subjects with time-dependent dose-response relationships. Plasma metabolites that were significantly different between tumour and non-tumour-bearing mice were detected. Importantly, the time-of-day and food intake impact significantly on the level of these putative biomarkers.

Conclusions: We demonstrate the potential of LC-MS metabolomics as a powerful tool in oncology drug development to discover novel circulating pharmacodynamics and diagnostic biomarkers in plasma; further validation of these data is planned. Time-of-day and food intake need to be considered in future studies involving such an approach.

**O2B-1**

## **HIGH THROUGHPUT TREE PROFILING – A NEW DIMENSION IN PLANT METABOLOMICS**

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Research into woody bioenergy crops is looking towards metabolomic profiling as an aid to select and improve biomass yield and quality for a number of industrial applications. As well as improvements in biomass yield and calorific value (for the direct combustion for power generation), researchers are also looking towards such crops as non-petrochemical sources of feedstock for the synthesis of fuels and industrial chemicals.

Furthermore, future scenarios of the large scale planting of willow as a bioenergy crop, need to consider several aspects of the carbon cycle, including deposition and retention of carbon in the soil, via the roots and leaf litter, as well as greenhouse gas mitigation. Metabolomics has much to offer in the study of carbon flow in such crops.

We report on the establishment of a metabolomics screening system for the bioenergy crop willow, for which Rothamsted Research maintains the UK National Collection and has also developed large-scale genetic mapping populations that are growing in the field in several locations in the UK. Tree/shrub species such as willow (and relatives such as poplar and aspen) present a new set of problems to the plant metabolomics community. In particular the interplay of metabolism across very different tissue types (leaves and woody stems), the large plant size and developmental differences at different seasons and locations on the plant structure, make sampling especially important.

We will report on the development of high throughput willow genotype screening by combined NMR and direct infusion ESI-MS, a project that required completely new solutions to deal with tissues containing large amounts of calcium ions and organic acids. Progress in annotation of the extractable metabolome of bioenergy genotypes e.g. *Salix viminalis* will be reported as well as application of the screen to the study of diurnal rhythms in metabolism, effects of field nitrogen and drought conditions will be highlighted. The results will be discussed in the context of carbon flow within the plant and in relation to biomass, agronomy and secondary product biosynthesis.

## O2B-2

### INTEGRATED METABOLOMIC AND TRANSCRIPTOMIC PROFILING ILLUSTRATES SUCCESSIVE PHASES OF INCREASING GENE EXPRESSION ASSOCIATED WITH CHILLING-RELATED APPLE PEEL CELL DEATH

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Superficial scald is a chilling-related storage disorder of apple caused by the death of peel epidermal and hypodermal cells and associated discoloration. It is controlled using postharvest antioxidant (diphenylamine; DPA) and ethylene action inhibitor (1-methylcyclopropene; 1-MCP), and/or controlled atmosphere (CA) storage treatment. We are using integrated metabolomic and transcriptomic profiling to reveal metabolic processes necessary to develop for diagnostic tools, breeding markers, and novel treatments to mitigate economic impact of the disorder. 'Granny Smith' apples were stored for up to 6 months at 1 °C and apple peel sampled periodically. Scald was reduced in apples treated with DPA or 1-MCP compared with untreated apples. GC and LC-MS protocols were employed to profile over 600 discrete identified and unidentified metabolite MSTs and RNA-seq to evaluate the expression of over 36,000 gene models. Initial evaluation of these data using multi-block PLS-DA indicated that changes in both the metabolome and transcriptome were treatment dependent. Changes preceded visible scald symptoms in untreated fruit and a succession of increasing levels of 3 scald-associated metabolites [2,6,10-trimethyldodeca-2,7(E),9(E),11-tetraen-6-ol (CTOL; 12 w prior), then acylated steryl glycosides (ASGs; 8 w prior), then methanol (MeOH; coinciding with symptoms)] representing different metabolic events during storage. Pearson's correlation (expression-product) networks of individual metabolite changes and summarized transcript data (k-means clustering) revealed clusters of gene models associated with successive increases in the levels of each model metabolite. Gene ontology analysis of early CTOL co-expressed genes are linked with stress response while later MeOH (cell-death) genes include cellular catabolism, cell death, and disassembly. Analysis of individual genes within highly correlated clusters highlights links with metabolites including among MeOH, methyl esters, and putative pectin methylesterase transcripts indicating that cell-death associated middle lamella degradation and an altered volatile aroma profile may be linked.

**PROFILING OF SPATIAL METABOLITE DISTRIBUTIONS IN WHEAT LEAVES UNDER NORMAL AND NITRATE LIMITING CONDITIONS**

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Nitrogen and carbon assimilatory pathways must be co-ordinated in higher plants in photosynthetic as well as in non-photosynthetic tissue. Physiological differences between the basal to mid-section and on to the mature tip region of wheat primary leaves confirmed that there was a change from heterotrophic to autotrophic metabolism. This difference between the basal, mid and terminal 20 mm sections of the developing wheat leaf (*Triticum aestivum*) was further studied using metabolic fingerprinting and profiling in order to produce a detailed description of the metabolic response of the wheat leaf as it develops photosynthetic capacity while growing in the presence or absence of nitrate in the nutrient solution. Fourier Transform Infrared (FT-IR) spectroscopy confirmed the suitability and phenotypic reproducibility of the leaf growth conditions used. Principal Component – Discriminant Function Analysis (PC-DFA) revealed distinct clustering between base or tip leaf sections and from plants grown in the presence or absence of nitrate. More detailed analysis of the metabolite profile using Gas Chromatography-Time of Flight / Mass Spectrometry (GC-TOF/MS) combined with multivariate chemometric approaches, univariate data interpretation, and an extremely novel approach within the metabolomics field known as Bayesian Network (BN) based correlation analyses confirmed that the metabolite composition in the base and tip of the primary wheat leaf was distinct. Furthermore this study indicates that such changes represent the different metabolic processes operating in young and mature wheat leaf cells. The operation of nitrogen metabolism also impacted on the levels of amino acids, organic acids and carbohydrates within the wheat leaf. This research represents both a well validated approach to plant metabolic profiling from the technical aspect as well as revealing significant changes in metabolite distributions with respect to leaf position, nitrate supplementation, and the development of photosynthetic capacity.



## O2B-4

### DIURNAL COMPOSITIONAL CHANGES OF TOMATO FRUIT AND LEAF

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The Fruit Integrative Modelling project is an Eranet EraSysBio+ project which aims at describing and modelling the influence of environmental factors on tomato fruit central metabolism during its development. In this project, high-throughput biochemical phenotyping, MS and NMR spectrometry have been used to characterise fruit and leaf metabolites and estimate their levels for tomato plants (*Solanum lycopersicum* cv Moneymaker) cultivated in a greenhouse. Within this frame, one experiment focussed on the effect of harvest time on tissue composition throughout a day and night cycle. First, expanding fruits, and the mature leaves close to the harvested fruit cluster, were harvested on two different representative days. Analyses of pericarp tissue and foliar limb were performed on water/methanol/chloroform extracts using GC/EI-ToF-MS after derivatization, methanol/water extracts using LC/ESI-QToF-MS and ethanol/water extracts for robotized biochemical phenotyping and 1H-NMR quantitative profiling. Metabolite data were processed using univariate, multivariate and clustering analyses. For source leaves, metabolite changes were related to well-known physiological processes. For green fruits, several metabolites were shown to change throughout the day. The second step of this study was the combined data analysis of the compositional changes of mature leaves close to the harvested fruit cluster with that of the fruit pericarp.

**THE MOLECULAR ARMS RACE BETWEEN *CLADOSPORIUM FULVUM* AND TOMATO AT THE METABOLOME LEVEL**

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The molecular arms race between plants and pathogens involves an array of molecules, from defence-related proteins to secondary metabolites. In this presentation we will discuss recent data of our metabolomics study on the interaction of both resistant and susceptible tomato plants with leaf mold pathogen *Cladosporium fulvum*. Using transgenic tomato plants that expresses the fungal effector protein, we were able to mimics the highly localized response of resistant tomato plants to the effector of the fungus. The recognition of the expressed effector by the matching tomato resistance (Cf) protein resulted in systemic hypersensitive response (HR) throughout the plant. This neat system can be used to study and verify the changes in the metabolome that are found in the leaf mesophyll cells of resistant plants upon *C. fulvum* infection. Using untargeted metabolomics technologies, we identified an important classes of small molecules such as hydroxycinnamic acid amides (HCAAs) and benzenoids that are induced in resistant tomato plants, as part of the hypersensitive response (HR), to *C. fulvum* infection. In contrast to resistant plants, the HR is not induced in susceptible plants upon *C. fulvum* infection, enabling the pathogen to proliferate in the leaf apoplastic space. The apoplstic fluid metabolome analysis revealed how the fungus manipulates the host defence system, through detoxification of preformed defence compounds such as glycoalkaloids. Furthermore, *C. fulvum* enriches its carbon pool by converting plant sugars in to sugar alcohols. Other than serving as a carbon storage compounds, the sugar alcohols play a role in suppression of the host reactive oxygen species (ROS)-associated defence mechanisms.

## O3A-1

### MASS SPECTROMETRY ANALYSIS OF SIGNALLING AND METABOLIC LIPIDS

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We have developed, validated and adapted unique normal phase HPLC separation, hyphenated with high resolution and accurate mass (HR/AM) mass spectrometry approaches to analyse almost all known lipids including 27 classes of neutral lipids, phospholipids and sphingolipids from a single Folch extract of any cellular or tissue samples (<http://www.babraham.ac.uk/lipidomics/lipidomics.html>). In contrast a number of lipidomics labs utilise direct infusion nanoelectrospray (nESI) HR/AM mass spectrometry approaches to analyse lipids; this approach can quickly generate reliable data for major lipids from various samples without difficulty. However, the direct infusion approach without chromatographic separation of lipid classes cannot produce accurate data for minor and trace but biologically-important signalling lipids, such as PA, LPA, PIP2 and PIP3, particularly from samples with significant matrix interferences, such as adipose tissue. In addition this approach can provide false measurements of LPA and PA because of in source transformation of PC/PS to PA, LPC/LPS to LPA, and potential head group loss/change of other classes of lipids. Consequently we have adopted nanoESI-HR/AM mass spectrometry method only for the analysis of the major lipids and have adopted our unique normal phase LC-HR/AM mass spectrometry methodology for minor and trace lipids analysis, and have adopted specific extraction and methylation methods for PIP2 and PIP3 analysis.

The above approaches have been routinely used for analysing lipids from over 5000 samples, addressing a range of biological questions, generating much publishable data. For example, our lipidomics data shows the influence of DGK- $\alpha$  on particular DAG and PA species; that depletion of SREBP alters the cellular lipid spectrum and causes loss of mono unsaturated fatty acids; ectopic *Runx* reduces intracellular ceramides and increases extracellular S1P in NIH 3T3 fibroblasts; PLD1 rather than PLD2 is the active enzyme in the generation of PA from PC in neutrophils; Mosaic overgrowth with fibroadipose hyperplasia, thought to be caused by somatic activation mutations in *PIK3CA* is indeed reflected in an increases in PIP3 generation. In addition the study of resected colorectal tumour tissues has identified lipid changes indicative of metabolic changes in the tumour.

## O3A-2

### A PROTOTYPE MICROFLUIDIC MS PLATFORM FOR METABOLOMICS

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The need for a fast, comprehensive and sensitive analysis of the hundreds of biological samples challenges both the chromatographic separation and mass spectrometry.

We used a prototype microfluidic device optimized for MS analysis of metabolites in complex biological extracts. The devices are fabricated from resistant ceramic materials that permit operation at high pressure with sub 2 micron particles, leading to highly efficient LC separations of small molecules. By integrating microscale LC components into a single platform design, the devices avoid problems associated with capillary connections and the need to keep the system free of leaks, blockages, and excessive dead volume. Such integrated microfluidic devices are suitable for metabolomics analyses with performance comparable to analytical scale LC-MS analysis. We separated metabolites using 150  $\mu\text{m}$  ID x 100 mm devices packed with 1.7  $\mu\text{m}$  particles at flow rates of 2-3  $\mu\text{l}/\text{min}$ .

Metabolomics analyses were conducted using small volumes of standards and extracts from typical biological samples including plasma, brain, heart or liver. Mobile phases and analysis times were similar to regular LC methods using analytical-scale columns. Data was collected using MS systems (Q-ToF and triple quadrupoles) operated in both negative and positive mode in the data-independent acquisition mode. Untargeted metabolomics analyses were conducted using Q-ToF mass spectrometers with an alternate low and elevated collision energy method to acquire both precursor and product ion information in a single analytical run. Metabolic profiles were processed using multivariate and pattern-recognition tools to group the observed changes in lipids, which were identified using online database search. Such microfluidic-based metabolomics analyses lead to equivalent results to using analytical-scale columns, with an overall reduction in solvent consumption of > 200 x. Potential applications include large-scale metabolite profiling and low-abundance metabolite analyses in biological materials.

O3A-3

**MULTI-SEGMENT INJECTION-CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY FOR METABOLOMICS: HIGH SAMPLE THROUGHPUT MEETS HIGH DATA QUALITY?**

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Separation science plays a key role for enhancing the performance of mass spectrometry (MS)-based metabolomic studies. However, sample throughput is limited when using conventional separation platforms with gradient elution involving a "single" sample injection. In this case, major efforts are devoted to quality assurance and data pre-processing to correct for long-term instrumental drift that are time-consuming and/or subject to bias. To address this challenge, we have introduced a new approach for multiplexed analysis based on multi-segment injection (MSI)-CE-MS that enhances sample throughput while improving data quality. A unique advantage of CE is that separations use a homogeneous aqueous buffer system, thus allowing for multiple sample plugs to migrate under similar electrophoretic conditions. We demonstrate that up to seven distinct sample plugs can be analyzed simultaneously within a "single capillary" while maintaining isomeric resolution without deleterious ion suppression. In effect, MSI-CE-MS offers the sample throughput analogous to direct injection-ESI-MS while retaining the benefits of a high resolution separation, including greater selectivity and better quantitative reliability for complex biological samples. MSI-CE-MS was used as a high throughput platform for untargeted profiling of polar metabolites and their isomers derived from plasma filtrates without complicated sample handling. Time-resolved metabolomic studies were also performed to examine the putative health benefits of high intensity interval training (HIT) among a group of obese/overweight female subjects as a therapeutic tool for type 2 diabetes prevention. Plasma markers of differential treatment responses to HIT intervention were identified among subgroups of non-diabetic yet high risk subjects. MSI-CE-MS offers a unprecedented approach for enhancing sample throughput up to an order of magnitude while simplifying data pre-processing requirements that is urgently needed for biomarker discovery and personalized medicine.

**SPATIALLY-ENCODED 2D NMR STRATEGIES FOR FAST QUANTITATIVE METABOLOMICS**

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Two-dimensional Nuclear Magnetic Resonance (2D NMR) forms a powerful tool for the targeted analysis of metabolic samples, thanks to its capacity to simultaneously identify and quantify major metabolites in biological samples. However, its use for quantitative purposes is far from being trivial, not only because of the associated experiment time, but also due to its subsequent high sensitivity to hardware instabilities. The latter highly affect the analytical performance of 2D experiments (repeatability, linearity), thus affecting the accuracy and precision of quantitative analysis. Recent papers described the development and optimization of 2D NMR experiments for quantitative analysis of metabolic samples.<sup>1</sup> Reducing the experiment duration appears as indispensable to reach a high quantitative performance. In this context, we recently focused our attention on the development of fast quantitative 2D NMR approaches applied to the measurement of major metabolites in breast cancer cell line extracts, allowing the discrimination between different cancer cell lines.<sup>2</sup>

On the other hand, the NMR community has developed a number of approaches departing from the classical parametric incrementation scheme of 2D NMR, in order to drastically reduce the duration of 2D NMR experiments. In particular, the last 10 years have witnessed large efforts geared at developing the so-called “ultrafast 2D NMR” methodology, capable of providing a complete 2D correlation in a single scan, i.e. in a fraction of a second.<sup>3</sup> Based on this approach, we developed a quantitative “multi-scan single shot” (M3S) strategy,<sup>4</sup> capable of measuring absolute metabolite concentrations in complex mixtures with a high precision in a reasonable time. The analytical performance of this methodology was compared to the one of conventional 2D NMR. 2D COSY spectra were obtained in 10 minutes on model metabolic mixtures, with a precision in the 1-4% range (versus 5-18% for the conventional approach).<sup>5</sup> This much higher precision is due to the excellent immunity of the M3S approach towards hardware instabilities. The M3S approach also shows a better linearity than its conventional counterpart. It ensures that accurate quantitative results can be obtained provided that a calibration procedure is carried out. The M3S COSY approach was then applied to measure the absolute metabolite concentration in three breast cancer cell line extracts, relying on a standard addition protocol. M3S COSY spectra of such extracts were recorded in 20 minutes and gave access to the absolute concentration of 14 major metabolites, showing significant differences between cell lines.<sup>5</sup> The concentrations measured are coherent with the biological processes in cancer cells and are in good adequacy with metabolic studies performed in our group by Isotopic Ratio Mass Spectrometry (IRMS).<sup>6</sup>

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## O3A-5

### **THE USE OF STANDARD REFERENCE MATERIALS (SRMs) AND CONTROL MATERIALS (CMs) FOR METABOLOMICS QUALITY CONTROL AND STABILITY ASSESSMENTS**

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The interpretation of results from metabolomics experiments depends heavily on the quality of the data since many multivariate analysis techniques may be unpredictably sensitive to analytical noise and other experimental factors that occur in sample processing.

For studies which may cover extended time frames or which include numerous individuals in sample processing, the issue of data consistency should be addressed in the experiment design so that the results are interpreted in the proper context. For example, if the biological system being studied could have a large amount of innate biological variability within the individual treatment groups, knowing the relative size of the analytical variability will allow a more significant differentiation of class.

Traditionally, in analytical chemistry, measurement techniques have included analysis of materials where the analytical results are 'known' so that an assessment of precision and trueness could be made. These are analytical quality control (QC) materials.

Here, we report on the systematic use in NMR-based metabolomics of matrix matched control materials (CM) and standard reference materials (SRM). CMs are often developed within a project for QC using pooled body fluids or tissues, but they may not be the most appropriate material for subsequent projects. CMs represent a relevant measurement of intra-project sample and data quality. For comparison of QC results across varied projects, SRMs represent an approach that can lead to increased confidence in the methods and practice in a laboratory and provide a consistent matrix for method development or refinement. Development of SRMs by National Metrology Institutes (NMI) for metabolomics studies would be beneficial for interproject or interlaboratory studies. However, the extensive catalogue of biological SRMs developed at NIST for other analytical measurements is a rich source of high quality materials suitable for metabolomics studies.

## O3A-6

### **CAN WE TRUST UNTARGETED METABOLOMICS: RESULTS OF THE METABO-RING INITIATIVE, A LARGE-SCALE MULTI-INSTRUMENTS INTER-LABORATORY STUDY**

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The Metabo-Ring initiative brings together 5 Nuclear Magnetic Resonance (NMR) and 11 different Mass Spectrometers (MS), to assess the reliability of the untargeted metabolomic approach to obtain the same biological information. This was estimated by measuring the proportion of common spectral information extracted from the different MS and NMR platforms.

Biological samples obtained from 2 different experimental tests were analyzed by partners using their own in-house protocols. Test #1 referred to a high biological contrast experiment, obtained by spiking the urines of 14 healthy adult volunteers with 33 metabolite standards. Test #2 concerned a low biological contrast experiment situation comparing the plasma of rats supplemented or not with vitamin D. Spectral information from each instrument was assembled into separate statistical blocks. Correlations between blocks (e.g. instruments) were examined using the RV coefficients (similar to the R2 coefficient of determination) and the structure of the common spectra by Common Components and Specific Weights Analysis. In addition, in test#1 an outlier individual was blindly introduced to evaluate its detection by the various platforms.

Despite large differences in the number of spectral features produced by the instruments, the heterogeneity in the analytic conditions and the data post-processing, the spectral information both within (NMR and MS) and across methods (NMR vs MS) was highly converging (from 64% to 91% on average). No effect of the MS configuration (TOF, QTOF, Orbitrap) was noticed. An outlier individual was best detected and characterized by MS instruments.

In conclusion, metabolomics profiling brings a consistent information within and across instruments of various technologies even without prior standardization.



O3B-1

## DEVELOPMENT OF METABOLITE PROFILING DATABASE FOR KNOCK-OUT MUTANTS IN ARABIDOPSIS (MeKO)

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Despite recent intensive research efforts in functional genomics, the functions of only a limited number of *Arabidopsis* (*Arabidopsis thaliana*) genes have been characterized experimentally. Improving gene annotation remains a major challenge in plant systems biology. Because metabolite profiling can characterize the metabolomic phenotype of a genetic perturbation of plant metabolism, it provides clues to the function of genes of interest. In this regard, we chose 50 *Arabidopsis* mutants including a set of characterized and uncharacterized mutants, which resemble wild-type plants. We analyzed the plants using gas chromatography–mass spectrometry (GC–MS). To make the dataset available as an efficient public functional genomics tool for hypothesis generation, we developed the MeKO database, which allows evaluation of whether a mutation affects metabolism during normal growth. This database includes images of mutants, statistical data analyses, and data on differential metabolite accumulation. Non-processed data, including chromatograms, mass spectra, and experimental meta-data, follow the guidelines of the Metabolomics Standards Initiative (MSI) and are freely downloadable. Proof-of-concept analysis suggests that the MeKO database is highly useful for gene annotation and for generation of hypotheses for genes of interest. MeKO is publicly available at <http://prime.psc.riken.jp/meko/>.

O3B-2

## **METABOLOMICS AS A TOOL TO CHARACTERIZE BIOCHEMISTRY OF THE MEDIATOR COMPLEX IN PLANTS**

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The Mediator is a multiprotein complex thought to be a center to integrate environmental signals and activate targeted gene expression. In yeast, the Mediator comprises 25 subunits that can be grouped into three modules, the head, middle and tail, according to their position in the multiprotein complex. The global functions of Mediator in different metabolic pathways have so far not been studied in higher eukaryotes.

Metabolomic profiling of Arabidopsis mutant lines for 13 different subunits was performed by LC-TOF MS. A systematic identification of different classes of metabolites was based on high-resolution measurement by Orbitrap-MS and Mass frontier 7.0 Software. The OPLS-DA of each mutant showed that all 13 mediator mutants displayed changes in specific classes of metabolites that differed significantly from wild type. The predictive loading vectors from the OPLS-DA models were subjected to hierarchical cluster analysis and heat-map visualization. The heat map showed three distinct clusters representing the three different domains of the multiprotein complex. Interestingly, metabolomic profiles of mutants of four subunits for which the position in the complex was previously unknown mapped to these clusters. E.g. the *med23* clustered together with the mutants of the Middle, *med28* and *med34* with Tail and *med25* with Head domain. Subsequent biochemical experiments confirmed the presence of *med25* in the Head domain.

In this study the metabolomic approach has not only revealed novel metabolite-phenotype but provided a solid base for further studies on Mediator subunit function in integration of signals from different environmental conditions.

### O3B-3

#### **METABOLOMICS AS TOOL TO CHARACTERIZE GENES INVOLVED IN THE SYNTHESIS OF BIOACTIVE SESQUITERPENES**

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Sesquiterpene lactones are a major class of terpenoids particularly abundant in plant species of the Asteraceae family, which include both crops and traditional medicinal herbs like feverfew (*Tanacetum parthenium*) and *Inula britannica*. Sesquiterpene lactones have various biological activities and uses for humans, including antimicrobial, anticancer and anti-inflammatory properties. In feverfew, the most abundant sesquiterpene lactone is parthenolide, which is presumed to be formed from costunolide via a cytochrome P450 enzyme. We study the biosynthesis of pharmaceutically active sesquiterpenes, such as parthenolide, and the possibility to produce these sesquiterpenes in a controlled manner using heterologous expression platforms.

LCMS and GCMS profiling platforms were used to screen different tissues and developmental stages of feverfew plants, in order to get insight in the spatial and temporal accumulation of sesquiterpenes including parthenolide. The strongest accumulating tissue was subsequently used to prepare an EST library to identify sesquiterpene synthases, which lead to the isolation of the feverfew germacrene A synthase (*TpGAS*) gene, involved in the first biosynthetic step from FPP towards sesquiterpene lactones. Expression analysis of both the gene and parthenolide indicated that the glandular trichomes are the secretory tissues where parthenolide biosynthesis occurs.

By expressing *TpGAS* in combination with the previously cloned chicory germacrene oxidase (*CiGAO*) and 5 yet unknown CYP71 P450 sequences, we were able to identify *CiCOS*, the gene encoding the enzyme responsible for the biosynthesis of costunolide, the presumed precursor of parthenolide. Reconstruction of the costunolide pathway by expressing all 3 genes in yeast indeed resulted in the production of costunolide. However, transient expression of the genes in *Nicotiana benthamiana* resulted in only trace amounts of free costunolide. Using an untargeted comparative metabolomics approach followed by identification of differential mass signals, we were able to show that *in planta* costunolide mainly accumulated as novel glutathione and cysteine conjugates, likely due to the action of endogenous GSH-transferases.

O3B-4

**METABOLITE PROFILING OF VACCINIUM BERRY STANDARD REFERENCE MATERIALS BY GC-MS**

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*Vaccinium* berries, including cranberries, blueberries, and bilberries, are believed to provide a number of health benefits. Consumption of these fruit has been associated with a reduced risk of cardiovascular disease, infections, and cancer. Hence, there has been significant interest in identifying the specific chemical constituents that may promote good health. In addition, dietary supplements and related products containing these berries or their extracts are now widely available. NIST recently introduced a suite of Standard Reference Materials (SRMs) for *Vaccinium* berries, and the suite includes dried fruit, berry extracts, and oral dosage forms. In previous work, an untargeted metabolite discovery analysis of these berry SRMs was performed by LC-MS and yielded putative identifications of more than 70 metabolites. However, unambiguous assignments were sometimes hindered by the limited information provided by collision-induced dissociation (CID). In the current work, these SRMs were subjected to nontargeted metabolite profiling by GC-MS with derivatization. The resulting EI spectra were searched against the NIST mass spectral libraries and compared with literature reports. Identifications were further validated through retention index matching and comparison with the previous LC-MS results. Nearly 100 metabolites were identified in this suite of six berry SRMs through GC-MS profiling. Of these, 37 had also previously been identified by LC-MS. Thus, additional confidence in these identifications can clearly be gained through the use of multiple analytical platforms, and the total number of metabolite identifications was expanded. In addition, the richer EI spectra obtained in the GC-MS analyses were particularly valuable for identification of closely related compounds that were indistinguishable by CID.

O3B-5

**PLANT LIPIDOMICS LEADS TO THE IDENTIFICATION OF A NOVEL LIPID CLASS PLAYING AN ESSENTIAL ROLE IN MITIGATION OF PHOSPHORUS DEPLETION**

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Phosphorus (P) supply is one of the major factors responsible for reduced crop yields. Thus, plants utilize various adaptive mechanisms against P depletion, including lipid remodeling. Here, we report the involvement of a novel plant lipid glucuronosyldiacylglycerol (GlcADG) in the lipid remodeling against P depletion. Lipidomic analysis of Arabidopsis plants cultured in P-depleted conditions revealed inducible accumulation of GlcADG. Investigation using a series of sulfolipid sulfoquinovosyldiacylglycerol (SQDG) synthesis-deficient mutants of Arabidopsis (*ugp3*, *sqd1*, and *sqd2*) determined the biosynthesis of this lipid shares the pathway of SQDG synthesis in chloroplasts. Under P-depleted conditions, the Arabidopsis *sqd2* mutant, which did not accumulate both SQDG and GlcADG, was the most severely damaged of the three SQDG-deficient mutants. As GlcADG was still present in the other two mutants, this result indicates that GlcADG plays a role in stress mitigation under P limitation. P deprivation usually leads to significant decreases of phospholipids in Arabidopsis plants. However, levels of phosphatidylinositol and phosphatidylethanolamine were almost maintained in P-starved *sqd2* mutants, suggesting that GlcADG deficiency lead to a de-regulation of phospholipid metabolism. GlcADG was also found in rice, and its concentration significantly increased following P limitation, suggesting a shared physiological significance of this novel lipid against P depletion in plants.

O3B-6

**IDENTIFYING NOVEL SALINITY TOLERANCE MECHANISMS BY SPATIAL ANALYSIS OF LIPIDS IN BARLEY ROOTS**

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We are facing the challenging task to meet the growing demand for food which must occur in an environment of a changing climate with increasing environmental stresses such as drought, extreme temperatures, nutrient deficiencies and mineral toxicities. Less land available to cultivate crops, declining water quality and prioritisation of biofuel production at the expense of food production further exacerbates the situation. A combination of climate change and poor agricultural practices signifies that 50% of current arable land is at high risk of increased salinity and hence unusable by 2050. Here we aim to develop and apply new tools to unravel how plants respond to the perception of salt stress. Evidence is accumulating that lipid signalling is an integral part of the complex regulatory networks in the responses of plants to salinity. Modifications of membrane lipids occur through the activity of phospholipases, lipid kinases and phosphatases such as phospholipase D and diacylglycerol kinase that produce different classes of lipid and lipid-derived messengers. These provide spatial and temporal regulatory functions crucial for cell survival, growth and differentiation and for an appropriate response of the plant to environmental stimuli. We are using modern lipidomics technologies to compare the root plasma membrane (PM) compositions of different barley genotypes with contrasting salinity tolerance levels upon salt stress. Our aim is to investigate the link between PM composition and functionality in aspects of salinity response by examining whether observed changes in lipids are involved in either the alteration of fluidity, or in lipid-based downstream signalling. We are also using MALDI-FT-MS based imaging technologies to monitor spatial distributions of lipids across root sections of salt treated tolerant and intolerant barley genotypes. These novel findings will lead to a better understanding of the role of lipids, lipid composition and signalling for plant salt tolerance.

## O4A-1

### **METABOLIC PHENOTYPING BY <sup>1</sup>H-NMR SPECTROSCOPY DETECTS LUNG CANCER VIA A SIMPLE BLOOD SAMPLE**

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**Introduction:** Lung cancer is the leading cause of cancer death worldwide. There is an urgent need of effective methods to detect lung cancer. Accumulating evidence shows that the metabolism of cancer cells differs from that of normal cells. Disturbances in biochemical pathways which occur during the development of cancer provoke changes in the metabolic phenotype.

**Objective:** To determine the metabolic phenotype of lung cancer by <sup>1</sup>H-NMR spectroscopy.

**Methods:** Fasting venous blood samples of 78 patients with confirmed lung cancer (before any treatment) and 78 controls are collected and analyzed by <sup>1</sup>H-NMR spectroscopy. The integration values of 110 spectral regions, representing the metabolite concentrations, are analyzed by a Mann-Whitney test to identify those which differ significantly between lung cancer patients and controls. Next, orthogonal partial least squares discriminant analyses (OPLS-DA) are performed to investigate whether the metabolic composition of blood plasma discriminates between lung cancer patients and controls.

**Results:** The integration values of 28 out of 110 spectral regions are significantly different for lung cancer. These regions include signals of several amino acids, citrate, lactate and lipids. These 28 significantly different integration values are used to build a statistical classifier by means of OPLS-DA multivariate statistics. Via this classifier model, lung cancer can be detected with a sensitivity of 86% and a specificity of 95%.

**Conclusion:** Metabolic phenotyping of blood plasma by <sup>1</sup>H-NMR spectroscopy detects lung cancer with a high degree of sensitivity and specificity. At present, lung cancer patients and controls are recruited to validate these promising results in a larger population study.

## O4A-2

### FROM METABOLIC DIFFERENCES TO GENETIC DIFFERENCES VIA QUALITATIVE METABOLIC NETWORK ANALYSIS

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Identifying genes that are responsible for differences in metabolism could have important applications such as gene ranking for genome-wide association studies and drug target identification. We have developed a qualitative computational method that uses a curated metabolic network (YeastCyc) and LC/MS and GC/MS data of measured metabolite differences to identify genes that are likely to be causal for the metabolic differences.

The computational method first produces a table that predicts the changes in metabolite concentrations that would result from increasing or decreasing the activity of each metabolic gene. Second, based on this table and measured differences in metabolite concentrations between experimental and control samples, it computes a score that evaluates the potential for each gene to be a causative factor for those metabolic differences. The scores are based upon the quality of the match between the measured metabolic changes and the predicted effects in the table. The genes are then ranked by the scores, and the top-ranked genes are deemed to be mostly likely candidates for causing the differences in metabolites.

We validated the algorithm using data from two experiments in *S. Cerevisiae*. The first experiment compared the concentrations of metabolites in the arginine biosynthesis pathway of wild type yeast with four mutants with deletions of single genes in that pathway. The second experiment compared controls with cells treated with five different drugs targeting the arginine and ergosterol pathways. In the first experiment, the algorithm correctly identified all of the deleted genes. In the second experiment, the algorithm correctly identified the known drug-targeted genes. For the drugs whose targets have not been characterized, the results from the algorithm matched experts' judgements.



O4A-3

**IMPROVING PHOTOSYNTHESIS IN *ARABIDOPSIS THALIANA*: FUMARATE AS A POTENTIAL CARBON STORE**

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Increases in the efficiency of photosynthesis have the potential to increase the growth and yield of crop plants. However, the rate at which plants can assimilate fixed carbon is limited, and accumulation of intermediates can down-regulate photosynthesis. This is a potential explanation for the under-performance of crop plants grown at high CO<sub>2</sub> levels; carbon fixation increases are limited by the ability of plants to store fixed carbon as starch and/or sucrose. It has been suggested that the organic acid fumarate can act as an additional storage compound, potentially compensating for increases in carbon fixation. This would allow plants to increase their photosynthetic rate without being subject to down-regulatory processes.

We have used a systems biology approach in *Arabidopsis thaliana* to attempt to understand the mechanisms by which plants accumulate fumarate under different environmental conditions. Fourier Transform Infra Red spectrometry, Gas Chromatography Electron Impact Time of Flight Mass Spectrometry and enzymatic assays were used to examine metabolite levels in a range of plant lines under varying conditions. Under constant conditions, fumarate levels show a diurnal cycle of accumulation in the light and degradation overnight. Accumulation of fumarate forms a significant pool of fixed carbon under control conditions, and we have identified conditions (low temperature and long day) where this pool can be increased by over 200%. Under these conditions, photosynthetic rate is also significantly increased. Metabolomic analysis will allow us to identify metabolites and metabolite groups important in this accumulation process, leading to further physiological analysis.

**TARGETED AND NON-TARGETED LC-MS METABOLIC PROFILING IDENTIFIES SHIFTS IN AMINO ACID AND LIPID METABOLISM IN THE INFLAMMATORY SKIN DISEASE PSORIASIS**

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Psoriasis is a chronic immune-mediated skin disease. Pathology occurs when the immune system identifies healthy skin cells as a pathogen, triggering an over production of keratinocytes. Mechanisms of psoriasis are not understood, and the metabolic component is poorly studied. This study examined plasma from healthy controls (n=32), mild and (n=32), severe psoriasis (n=32), as well as Enbrel-treated severe psoriasis patients (n=16). Enbrel is a common therapeutic for treating psoriasis that inhibits tumour necrosis factor alpha (TNF $\alpha$ ) by fusing to the IgG1 antibody of the TNF receptor. Metabolomics utilised reversed phase and hydrophilic liquid interaction chromatography. Metabolite identification was performed by comparing accurate mass, retention time and fragmentation of peaks against a library of 434 standards. Targeted metabolic profiling was performed using LC multiple reaction monitoring of 34 sphingolipids, 87 eicosanoids and 14 lipid amides. Analysis of all data using orthogonal projection to latent structures (OPLS-DA) identified shifts in severe ( $R^2Y=0.947$   $Q^2 = 0.647$  CV-ANOVA  $p=1.6\times 10^{-4}$ ), and mild ( $R^2Y=0.579$   $Q^2=0.478$  CV-ANOVA  $p=3.4\times 10^{-4}$ ) psoriasis relative to controls. Observed shifts were due to increases in 1) amino acid metabolism with disease (specifically methionine, serine, arginine and the urea cycle), 2) sphingolipid levels, and 3) 12-LOX metabolism. Enbrel treatment resulted in significant shifts (OPLS-DA model:  $R^2Y=0.642$   $Q^2=0.496$  CV-ANOVA  $p=2.6\times 10^{-3}$ ). Treatment primarily reduced pro-inflammatory ceramides and urea cycle intermediates. Increases in the ceramides and their subsequent reduction with treatment suggest a mechanism by which Enbrel resolves the systemic inflammation associated with psoriasis. Relative 12/15-LOX activity affects skin disease pathology, as 12-LOX induces cell proliferation, whilst 15-LOX inhibits cell growth. Greater levels of 12-LOX activity increase cell proliferation, potentially explaining the overproduction of keratinocytes in psoriatic lesions. Higher levels of arginine potentially lead to greater levels of pro-inflammatory nitric oxide, produced from arginine by nitric oxide synthase (NOS). NOS can be induced by TNF $\alpha$ , hinting at a pathway for Enbrel to affect arginine metabolism via the urea cycle. This study demonstrated how non-targeted and targeted approaches can be combined to identify pathways in disease pathogenesis and treatment. Enbrel treatment modulated metabolism, reducing levels of urea cycle intermediates, potentially reducing nitric oxide production, and the pro-inflammatory ceramides, suggesting a mechanisms by which the systemic inflammatory component is mediated.

O4A-5

**AN AUTOMATED WORKFLOW TO REDUCE LC-MS DATA TO BIOLOGICALLY RELEVANT FEATURES ONLY, WITH SUBSEQUENT ANNOTATION, AND ITS APPLICATION TO *C.elegans* LONGEVITY MUTANT PROFILING**

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Model organisms produce a plethora of small molecules. Many of them are still completely unknown and not catalogued in any public databases. Metabolomic approaches can potentially compensate for this, by being completely untargeted. However, this also results in major drawbacks. Untargeted LC-MS profiling experiments, conducted on the latest high-resolution instrumentation, may end up with well over a hundred thousand peaks for a single file. In a typical profiling experiment, with several tens or even hundreds of samples, this results in barely analysable amounts of data. This hinders comprehensive molecule assignment, impeding the ultimate goal – (systems) biological interpretation.

As some studies recently showed, stable isotopes can be used to discern biological peaks from other chemical signal and help annotate these, by measuring the mass shift, which occurs during label incorporation. Many model organisms, such as bacteria, yeast, worms, flies and even cells (in culture) can readily be isotope enriched.

We have developed a fully automated workflow, that analyses stable isotope labelled samples, acquired by LC-MS, to reduce the total amount of data to non-redundant, biologically relevant information only, which is then annotated with a molecular formula.

We enriched *E. coli* and *C. elegans* extracts with three stable isotopes (C, N, S), and could cut down information by over 99.9 % and annotate the molecular formulae for the genuine biological molecules – many of them not yet found in databases. In a second step, we analysed a set of 30 long-lived *C. elegans* mutant strains. We present the data for relationships between longevity and metabolism, using only relevant and annotated compounds.

We believe that our new software tools will provide a valuable resource for the model organism small molecule community, to find and annotate new molecules and focus on biologically relevant compounds.

## O4A-6

### DISCOVERY OF PLANT DUAL COX AND LOX INHIBITORS AND MODELS TO PREDICTION THROUGH METABOLOMIC STUDIES AND ARTIFICIAL INTELLIGENCE

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Cyclooxygenase (COX) and lipoxygenase (LOX) pathways are of utmost importance in inflammatory processes and therefore dual inhibitors on COX-1 and 5-LOX should be anti-inflammatory (AI) medicines with high efficacy and low side effects<sup>1</sup>. Species from Asteraceae is well-known as AI plants, we screened 55 leaf extracts (EtOH-H<sub>2</sub>O 7:3, v/v) against COX-1 and 5-LOX, from three groups of plants: food, cerrado and known AI history. Among the tested extracts, 13 of them (26.6%, IC<sub>50</sub> range from 0.03-36 µg/mL) displayed the desired inhibition. One from the food group: *Cichorium intybus*; three from the group of previous AI evidence (through any mechanism of action): *Aldama robusta*, *Tithonia diversifolia* and *Vernonia polyathes*; and the others from cerrado biome: *Aldama trichophylla*, *Minasia scapigera*, *Piptolepis monticola*, *Prestelia eriopus*, *Vernonia herbaceae*, *V. platensis* and *V. rubriramea*. Each extract was further analysed by HPLC-HRFTMS. The data of all samples were processed employing a differential expression analysis software (MZmine 2.10) coupled to the Dictionary of Natural Products<sup>®</sup> and Aster DB for dereplication studies. The biomarkers of the dual inhibition property were determined from 6,052 peaks using genetic search followed by decision tree J48 in Weka 3 software. The same biomarkers were determined through OPLS<sup>™</sup> algorithm in the SIMCA-P 13.0.2.0<sup>®</sup> software. OPLS model established was also validated (cross and external validation) to prediction of new dual inhibitors just from the HPLC-HRFTMS data, without previous AI assay. A model using artificial intelligence was also established in Weka using multilayer perceptron (MP), in the same way that for OPLS model: 70% of active and non-active samples comprised the training group and 30% the test group for external validation. In summary, the models for prediction of the bioactivity of natural compounds, resulting in high percentage of correct predictions (OPLS: 75%; MP: 90%), high precision (100% in both models) for dual inhibition, and low error values (mean absolute error = 0.2 in both models) as also shown in the validation test. Thus, the biomarkers of the plant extracts were statistically correlated with their AI activities and therefore can be useful to predict new AI extracts as well as their AI compounds. Acknowledgements: CNPq, FAPESP. <sup>1</sup>Fiorucci S et al. 2001 Biochem. Pharmacol. 62:1433.

O4B-1

## EXPLORATIVE NMR METABOLOMICS IN THE METABOLIC CHARACTERISATION OF TBM IN CSF

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Background: The hematogenous spread of *Mycobacterium tuberculosis* (Mtb) to the brain can lead to the establishment of small loculated tuberculous lesions that can rupture, discharging cellular debris and Mtb into the subarachnoid space resulting in a hypersensitivity reaction that can lead to the extra-pulmonary form of tuberculosis, Tuberculous Meningitis (TBM). Traditional diagnosis of this chronic disease can be difficult due to vague clinical presentation and consequently treatment is often fatally delayed.

Experiment: The application of <sup>1</sup>H Nuclear Magnetic Resonance (NMR) spectroscopy as an untargeted global metabolomics explorative approach was used to characterise the metabolic profiles of TBM in South African infants and children (ages: 4 months to 12 years) and considered a first step to defining a possible TBM bio-signature in cerebrospinal fluid (CSF) for improved early diagnostic purposes. The three clinically well-defined experimental groups in this study were: 1) TBM cases, at various stages of the disease, 2) non-healthy, non-meningitis controls (i.e. sick individuals suspected of meningitis but later confirmed negative), and 3) healthy controls.

Results: The highly specific and unbiased nature of NMR yielded spectra whereby metabolites of significance were identified both qualitatively and quantitatively from these highly complex biological samples. Certain medications were also identified, based upon created pure compound spectra libraries; particularly TB-specific pyrazinamide which is well known for its high-penetration ability of the blood-brain barrier. In addition, some HIV co-infected individuals were identified as outliers, exhibiting unique NMR spectra.

**METABOLOMIC ANALYSIS OF OVARIAN CANCER: A MULTI-MODAL GC-MS, LC-MS AND NMR STUDY**

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Ovarian cancer is the deadliest cancer of the female reproductive system. The majority of ovarian cancers are detected at a late stage when the cancer is already metastatic and when the 5-year survival rate is about 30%. The etiology and tumorigenesis of ovarian cancer is poorly understood and the large number of distinct tumor subtypes further enhances the complexity of this disease. Despite recent advances, ovarian cancer incidence and mortality rates have largely remained unchanged in the last decades. We have applied a comprehensive multimodal metabolomics approach using untargeted gas chromatography mass spectrometry (GC-MS), targeted liquid chromatography mass spectrometry (LC-MS) and quantitative nuclear magnetic resonance spectroscopy (NMR) profiling in order to study the serum metabolome of ovarian cancer patients (N=31) and matched controls (N=31). Data on common risk factors such as the use of estrogen replacement and oral contraceptives, menopausal status and number of pregnancies was collected as well as histology reports. The metabolic profile of ovarian cancer patients was found to be distinct from the profile of the matched controls when using multivariate statistics tools in SIMCA-P+. The strongest separation was seen using the GC-MS data, generating an orthogonal partial least squares discriminant analysis (OPLS-DA) model with  $R^2=0.70$  and  $Q^2=0.58$  when applying a Variable Importance in Projection (VIP) cut-off of 1. The corresponding values for LC-MS and NMR were  $R^2=0.58$  and  $Q^2=0.33$  and  $R^2=0.50$  and  $Q^2=0.22$  respectively. All three metabolomics approaches detected a decrease in methionine while isoleucine and glutamate were found increased by both NMR and GC-MS (not present in the Biocrates LC-MS kit). The targeted LC-MS approach detected a distinct pattern in glycerophospholipids when comparing cancer patients and controls. In the future we plan to study the impact of tumor subtype and stage on the metabolome. In conclusion, we have detected a distinct difference in the metabolic profile between ovarian cancer patients and matched controls. This difference was in part due to differences in the amino acid profile as well as glycerophospholipid patterns. We expect that unravelling the biological mechanism underlying ovarian cancer will improve how such tumors are detected and treated and that metabolomics tools can provide such insight.

### O4B-3

#### **URINARY METABOLOMICS OF COLORECTAL CANCER – A PILOT STUDY SCREENING FOR CROSS-SECTIONAL MARKERS USED IN TRANSLATIONAL ONCOLOGY**

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Background: Colorectal cancer (CRC), the third most frequent cancer, is one of the top 10 causes of death in western countries. Metabolomics has the potential of becoming a valuable tool in cancer research as well as in clinical practice and translational oncology.

Methods: We investigated the metabolome of fasting spot urine from 115 CRC patients (stages I-IV) from the German part of a prospective patient cohort (ColoCare) as well as spot urine from 20 control subjects using an oximation/silylation GC-MS approach. Additionally, spot urine of patients was analyzed post-surgery (n=52 and n=32 at 6 and 12 month follow-up). Data were pre-processed with MZmine 2 and metabolites were identified based on Kovats retention indices (RI) and electron impact (EI) spectra compared to the Wiley/NIST 2011 database. Uni- and multivariate analyses were performed using Metaboanalyst 2.0. Receiver operator characteristic (ROC) curves were constructed with ROCcET.

Results and Discussion: A partial least square discriminant analysis (PLS-DA) model was able to differentiate CRC from controls with high accuracy (area under the ROC: 0.96; confidence interval (CI): 0.89-0.995). Moreover, colon cancer patients differed by stage (p<0.05 for 9 metabolites). In contrast, the metabolome of rectal cancer patients receiving neo-adjuvant chemotherapy across stages was more similar. Generally, metabolite levels in patients at 6/12 month post-surgery resembled the metabolome of control subjects. Overall, significantly altered metabolites included nucleotides and derivatives (uridine, pseudo-uridine, hypoxanthine and D-ribofuranose), dipeptides (gamma-glutamyl-valine, hydroxyproline dipeptide) and *p*-cresol-glucuronide, putatively derived from the gut microflora.

Conclusion and outlook: We were able to identify potential markers for CRC. These metabolites will now be validated within the multicentric ColoCare study (currently >500 CRC patients enrolled in Germany and the US) and assessed for their clinical use as prognostic or predictive markers.

#### O4B-4

### **METABOLIC PROFILING DURING PREGNANCY AND ASSOCIATIONS WITH MATERNAL HEALTH PARAMETERS AND BIRTH OUTCOMES**

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Our aim was to identify dynamic metabolic changes during pregnancy, and to determine their relationship with maternal health and birth outcomes. Metabolic profiling applications during gestation give an insight into the *in utero* environment of the foetus and can be associated with its future health outcomes. Urine samples were obtained from pregnant women at 12 and 34 gestational weeks in two different Spanish regions (Gipuzkoa, n=836 and Sabadell n=900) as part of a large mother-child population-based cohort study, the INMA cohort. An exploratory metabolic profiling approach was applied using <sup>1</sup>H nuclear magnetic resonance (NMR) spectroscopy.

Our key observations included dramatic metabolic changes between the 12<sup>th</sup> and 34<sup>th</sup> weeks of pregnancy characterised by <sup>1</sup>H NMR signals arising at 0.58(s) 0.63(s) and 0.92(s) ppm. These signals probably arising from steroid-by-products were characterised by <sup>1</sup>H-<sup>13</sup>C NMR heteronuclear spectroscopy and further experiments using LC-MS/MS. A series of statistical analyses were applied to determine the association between urinary metabolic profiles and maternal clinical parameters related to metabolic syndrome, together with their association with birth outcomes. Consistent findings across the two cohorts and the two periods of pregnancy underpinned complex relationships between maternal pre-pregnancy BMI, weight gain and lipid metabolism. In addition, environmental exposures such as smoking and alcohol consumption, but also diet-related exposures were included in the analysis.

This study demonstrates a role for metabolomics in pregnancy research and in identifying reliable biomarkers in a birth cohort study. Metabolic profiling during gestation potentially defines the impact of the early life environment on infant health.



O4B-5

## **METABOLOMIC ANALYSIS OF THE RESISTANCE RESPONSE IN SUNFLOWER ROOTS TO THE PARASITIC WEED *OROBANCHE CUMANA***

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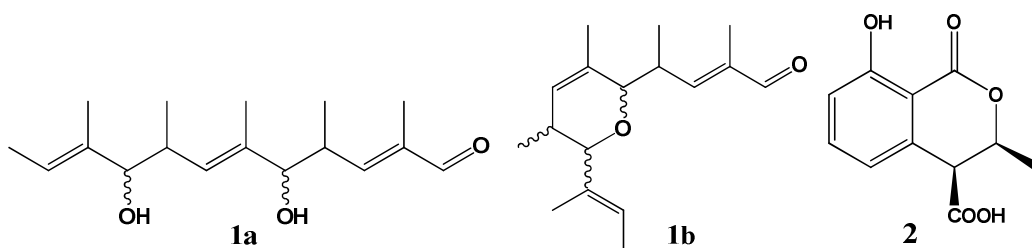
*Orobanche cumana* is a root parasitic angiosperm of sunflower that causes devastating losses in yield in Europe and Asia. This parasite is difficult to control and resistant cultivars play an important role in integrated control programmes. *O. cumana* seeds germinate in response to germination stimulants (e.g. dehydrocostus lactone) present in sunflower root exudates. The parasite radicle then differentiates (in response to a different suite of host derived signals) to form an attachment and penetration organ, known as the haustorium or tubercle. In a susceptible interaction, the parasite cells penetrate the host root cortex and endodermis and establish direct vascular connections to the phloem and the xylem of the host providing access to host nutrients. Resistance in sunflower cultivars can occur at different stages of parasite ingress into the root and microscopic studies have variously shown lignification of host cells, callose deposition, accumulation of phenolic compounds and cell death around the invading parasite. However, very little is known about the metabolic defence pathways underpinning different resistance reactions. Several studies have quantified specific defence metabolites but no global metabolomic analysis has been performed to identify the different biochemical pathways up or down regulated during a resistant interaction. In this study, we performed a non-targeted metabolomic analysis of the roots of a susceptible and resistant sunflower cultivar following inoculation with *O. cumana* using Ultra-high Performance Liquid Chromatography-high resolution Mass Spectrometry (UPLC-MS). We identified key defence pathways and metabolites involved in the resistance reaction. In addition we verified the identity and quantified key metabolites using standards and targeted UPLC-MS analysis of root samples. This study revealed that flavonoid and isoflavonoid biosynthetic pathways and the biosynthesis of coumarins, lignans and alkaloids (the latter derived from the shikimate pathway) were significantly upregulated during the defence response. For example, there was an accumulation of chlorogenic acid, ferulic acid, sinapic acid, caffeic acid glycoside and of the phytoalexin scopoletin. These data will be discussed in relation to our current knowledge of defence pathways in the sunflower-*O. cumana* interaction.

## METABOLOMICS AND DEREPLICATION STUDIES OF ENDOPHYTIC METABOLITES FROM SOME EGYPTIAN MEDICINAL PLANTS IN THE SEARCH FOR NEW POTENTIAL ANTI-CANCER DRUGS

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Endophytic fungi associated with medicinal plants represented a potential source of novel chemistry and biology. This study involved isolation of five endophytic fungal strains (*Aspergillus aculeatus*, *A. oryza*, *A. flocculosus*, *Curvularia sp.* and *Syncephalastrum racemosum*) from four different Egyptian medicinal plants (*Terminalia laxiflora*, *Dracaena deremensis*, *Tabebuia argentea*, *Markhamia platycalyx*). Identification of the strain has been achieved through molecular biological methods. Metabolomic profiling, using 2D-NMR and HR-ES/FTMS were done at different stages of the growth phase for both solid and liquid culture media. Dereplication studies were accomplished by utilizing the Mzmine software with the Antimarin database. The optimised method in terms of media, incubation time, and maximum production bioactive compounds were taken into account for the scale-up. The seven-day and thirty-day rice culture extracts of *Aspergillus aculeatus* and *A. flocculosus* respectively were fractionated using different high-throughput chromatographic techniques and subjected to selected bioactivity-guided isolation approaches. This led to the identification of compounds (1-12) from *A. flocculosus* and compounds (13-24) from *A. aculeatus*. Moreover, compounds 1a, 1b and 2 are novelties and possess moderate activity against NF $\kappa$ -b and *Trypanosoma brucei*.



## O4C-1

### IDENTIFICATION OF NOVEL BIOMARKERS OF DIETARY INTAKE

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Self-reported dietary intake assessment methods can be influenced by random and systematic errors. Advances in dietary intake assessment methods are therefore necessary to improve the classification of a person's dietary intake and also enhance our understanding of the link between diet, health and disease. The identification of novel biomarkers of dietary intake, through the application of metabolomics, offers the potential of a more objective measure of dietary intake.

This study aims to identify and quantify specific metabolites that reflect food group intake. Dietary intake data and  $^1\text{H}$  nuclear magnetic resonance ( $^1\text{H}$  NMR) urine spectra from 565 participants of the National Adult Nutrition Survey (NANS) were used for this analysis. Dietary intake data was obtained from 4-day food diaries and this was reduced into 34 food groups. Heat map analysis was performed to identify correlations between  $^1\text{H}$  NMR spectral regions and food group intakes. Positively correlated peaks within the spectral regions were then compared between low and high consumers of the specific food groups. Multivariate data analysis including principal components analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were also used to identify differences in  $^1\text{H}$  NMR spectra between high and low consumers of fruit and vegetable groups. Receiver operating characteristic (ROC) analysis was performed to assess sensitivity and specificity of biomarkers.

Heat map analysis identified high energy beverages as having strong correlations with a number of spectral regions. Spectral peaks which were positively correlated with high energy beverages and significantly increased in the high consumers were identified. The metabolites associated with high energy beverages were formate, citrulline, taurine and isocitrate. This panel of biomarkers had an area under the curve (AUC) of 0.9 for ROC analysis and a sensitivity and specificity of 80% and 90% respectively. Multivariate data analysis identified urinary metabolites associated with fruit and vegetable intake. Further validation of these markers is currently underway. Future work will ascertain how to translate these markers for use in nutrition epidemiology.

## O4C-2

### **ANTIDIABETIC EFFECT OF METFORMIN BY REDUCING CORTISOL LEVELS VIA THE AMPK/LXR $\alpha$ /POMC PATHWAY**

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**Background:** Metformin is used for the first-line drug in the treatment of diabetes mellitus type 2. Although the mechanism of action of metformin that activating AMP-activated protein kinase (AMPK) is generally considered, it is not fully understood about how activated AMPK roles in glucose regulation. The aim of this study is to identify the changes of urinary endogenous metabolites by metformin treatment and the molecular mechanism showing antidiabetic effect of metformin.

**Methods:** Fourteen healthy male subjects were orally administered metformin (1000 mg) once. 12h-urine samples taken before and after administration of metformin were analysed by following a holistic LC/Q-TOF MS based metabolomics combined with multivariate data analyses. Also, to investigate the molecular mechanism of antidiabetic effect of metformin, rat pituitary adenoma GH3 cells were used. After 8 hours of metformin treated incubation, the protein immunoblot assay was performed.

**Results:** Multivariate statistical data analyses satisfactorily classified samples between control and metformin group. Urinary metabolites affected by metformin treatment were as follows; while cortisol, hydroxycortisol and betaine were decreased, retinyl  $\beta$ -glucuronide and cholic acid glucuronide were increased. Given that cortisol stimulates gluconeogenesis, antidiabetic effect of metformin is to be attributed to reduced cortisol levels, which could be involved in the AMPK/LXR $\alpha$ /POMC pathway.

**Conclusion:** In this study, we identified metabolites affected by metformin treatment in healthy subjects through LC/Q-TOF MS analysis. Of importance, this study also demonstrated that metformin suppressed the cortisol levels via the AMPK/LXR $\alpha$ /POMC pathway.

O4C-3

**DEREPLICATION AND CHARACTERIZATION OF NOVEL 17-HYDROXYGERANYLLINALOOL DITERPENE GLYCOSIDES (HGL-DTGs) IN 24 SOLANACEOUS SPECIES BY U(H)PLC/ESI-TOF-MS AND MS/MS**

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The discovery of new bioactive metabolites in plants depends on efficient methods to distinguish known from unknown structures. However, *de-novo* structure elucidation and confirmation of known compounds (dereplication) are major bottlenecks in plant metabolomics, and few systematic dereplication strategies have been developed so far. Despite the increasing availability of mass spectrometers capable of accurate mass measurement, no methods that rely exclusively on this technology have been developed for entire compound classes so far. One reason for this is the complexity of most plant metabolic profiles which renders the characterization of compound classes across multiple plant species a challenging task.

17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) are abundant secondary metabolites whose biosynthetic steps of malonylation and glycosylation are regulated by the phytohormone jasmonic acid in *Nicotiana attenuata*. Due to the presence of multiple labile glycosidic bonds, HGL-DTGs exhibit extensive in-source fragmentation (IS-CID) which provides valuable information for structural elucidation. To reconstruct these IS-CID clusters from profiling data and identify precursor ions, we applied a deconvolution algorithm, performed a correlation analysis among the different fragments and created an MS/MS library in positive ionization mode from 16 different purified and semi-purified HGL-DTGs. With these MS/MS spectra, we annotated 251 non-redundant fragments (75% of the ions in deconvoluted clusters) and established a set of rules to characterize extracts of leaf, flower and fruit tissues from 24 solanaceous species. Using this strategy, 29 novel HGL-DTGs were predicted in plant species of the genus *Nicotiana*, *Capsicum* and *Lycium*.

Using this approach, we demonstrated that malonylation is a highly conserved biosynthetic step in HGL-DTG biosynthesis that is present in all HGL-DTG containing species. We were also able to identify conserved and tissue-specific variations in the response of these molecules to methyl jasmonate, a phytohormone that induces defensive responses in plants. In particular, we detected that malonylation is differentially regulated in different *Nicotiana* species. Finally, the established mass-spectrometry based workflow accelerates metabolite re-identification from new biological matrices and is readily applicable to many additional compound classes with known fragmentation patterns.

**DEVELOPMENT OF STRATEGIES FOR INTEGRATED FULL-SCAN PROFILING AND DATA DEPENDENT MS/MS AND MS<sup>n</sup> APPLYING CID AND HCD ON HYBRID ORBITRAP MASS SPECTROMETERS**

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The identification and characterisation of metabolites is a current bottleneck in untargeted metabolomics. Although accurate measurement of mass-to-charge ratios is applied routinely in UHPLC-MS applications for putative metabolite annotation, acquisition of MS/MS or MS<sup>n</sup> data is required for chemical search-space reduction and greater confidence in metabolite identity attribution [1].

We have assessed, and now provide strategies, for integrated full scan metabolic profiling using MS/MS and MS<sup>n</sup> data acquisition (CID and HCD) on LTQ-Orbitrap hybrid platforms. This research has shown that (i) the integration of MS/MS or MS<sup>n</sup> data applying CID and HCD can provide complementary information as shown for a diverse range of endogenous metabolites, (ii) different activation energies are required for acquisition of appropriate MS/MS and MS<sup>n</sup> spectra to enable identification of metabolites with diverse structural and physicochemical properties, and, (iii) applying narrow precursor ion *m/z* ranges (e.g. 100-200, 200-300) provide a larger number of informative MS/MS spectra compared to applying a large precursor *m/z* range (e.g. 100-1000). The strategies described provide for a suitable data acquisition first pass to improve metabolite identification capabilities. Further targeted identification studies will potentially be required in any given study to focus on identification of specific metabolites.

[1] Dunn, W.B. et al. Mass appeal: metabolite identification in mass spectrometry-focused untargeted metabolomics. *Metabolomics*, 2013, 9 (Issue 1 Supplement), 44-66.

## O4C-5

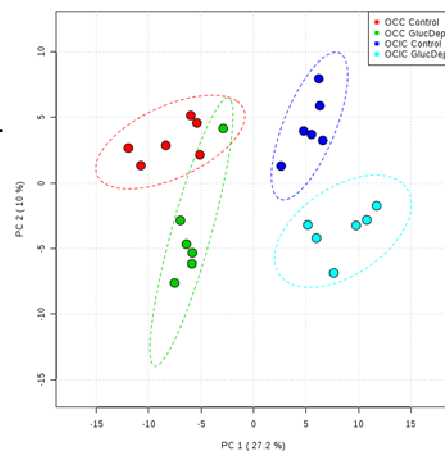
### OVARIAN CANCER METABOLOMIC PROFILES DIFFER IN OVARIAN CANCER INITIATING CELLS

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A key emerging area in cancer research is the study of stem-like “cancer initiating cells” (CICs) in tumor populations. CICs are a major concern for effective cancer treatment, as they can self-renew, can differentiate into cancer cells, and exhibit chemoresistance; they are thus suspected as a primary cause of cancer recurrence. Very little is known about the molecular workings of these cells, and we must start to understand them in order to effectively treat CICs. Metabolism is widely known to be dysfunctional in cancer, most famously via the Warburg effect, but there is limited knowledge about CIC metabolism. A systems-level understanding of the metabolic dynamics of CICs could profoundly affect our understanding, or even treatment, of cancer and CICs.

Here we present the results of an *in vitro* glucose deprivation experiment to model *in vivo* tumor conditions in ovarian cancer cells (OCCs) and ovarian CICs (OCICs). Extracellular and intracellular time-course metabolite samples were analyzed using GCxGC-MS. To our knowledge, this is the first metabolic profiling of OCIC metabolism. Distinct metabolic profiles were found for the two cell types and between the control and glucose-deprived cells (Fig 1). Interestingly, independent of the differences imposed on the cells by glucose deprivation, there is a characteristic set of intracellular metabolites that only appear in OCCs, but not OCICs. These metabolites are spread across multiple pathways, suggesting system-wide metabolic alteration of OCICs from OCCs and potentially serving as indicators of CIC transition or even as therapeutic targets.



**Figure 1: Separation between OCCs and OCICs is evident in the first principal component (PC), along with separation between glucose-deprived and control groups in the second PC.**

O4C-6

**NOVEL MASS SPECTROMETRY BASED LIPIDOMIC METHODS FOR INVESTIGATING GANGLIOSIDES IN MOUSE MODELS OF GUILLAIN-BARRÉ SYNDROME**

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Gangliosides have long been implicated as targets in autoimmune diseases such as Guillain-Barré Syndrome (GBS) and Multifocal Motor Neuropathy. In GBS, auto-antibodies bind native membrane gangliosides signalling immune-mediated breakdown of nerves causing acute flaccid paralysis. While the fundamental pathology is understood, differences in clinical presentation, and preference for motor over sensory nerves, have yet to be explained. To investigate, mouse models have been developed in which essential enzymes in ganglioside biosynthesis are knocked out and rescued in different tissues. To profile the recovered repertoire of gangliosides we have developed two mass spectrometry methods which could be expanded to a range of lipids from tissues and cells.

Gangliosides from brains of wild type, GalNACT knock-out, and rescue mice were extracted and separated by HPTLC, detected using direct, label-free MALDI mass spectrometry imaging and identified by mass and position of corresponding primulin stained bands. Brain extracts were also analysed by normal and reverse phase liquid chromatography (LC) with common solvent gradients and online MS detection with CID MSMS. An optimal method was then used to analyse extracts from single peripheral nerves.

Combining traditional TLC with MALDI MSI enabled detection and identification of almost all lipid bands visualised using primulin. The spatial resolution of MSI also meant that separation of fatty acid chains was observable within bands. The profiles in wild type and knock-out mice agreed with previously published studies. Both rescue models showed some rescued synthesis of complex gangliosides in brain tissue.

An optimal HPLC method for ganglioside separation using a simple IPA gradient on a reverse phase Acclaim C30 column was developed. Gangliosides were distinguishable by glycan head group and fatty acid tail structure using this method. MS detection enabled sensitive, accurate detection of low abundance species such as GQ1b and GM3. Characteristic fragment ions and neutral losses helped confirm putative identifications.



O5A-1

## DAILY RHYTHMS IN THE HUMAN METABOLOME AND THE EFFECT OF SLEEP AND SLEEP DEPRIVATION

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Determining metabolites that vary significantly with time of day and are influenced by exogenous factors such as sleep may impact greatly on the interpretation of results from metabolomics studies. Here, we have used liquid chromatography–mass spectrometry (LC-MS) to examine the effect of sleep and sleep deprivation on the human metabolome. Healthy male subjects ( $n = 12$ , aged  $23 \pm 5$  years (mean  $\pm$  SD)) maintained a regular sleep/wake schedule (23:00 – 07:00 h) for one week prior to an in-laboratory session. Blood samples were taken over a 48 h period beginning at 12:00 h and comprising a 24 h period which incorporated a sleep opportunity (23:00 – 07:00 h; day 1), followed by 24 h during which subjects remained continually awake (day 2). Subjects remained in highly controlled conditions with respect to environmental light/dark, sleep, meals and posture throughout the in-laboratory session. Two-hourly plasma samples were prepared using a methanol/ethanol liquid phase extraction prior to analysis using reverse phase UPLC-QTOF-MS. Untargeted analysis allowed the extraction of 368 features using XCMS. Principal component analysis showed a clear time-of-day effect in the metabolome. To assess this, time points 14:00 – 18:00 h inclusive (day 1) were compared with time points 02:00 – 06:00 h inclusive (day 1) in an OPLS-DA model in which the Y variable separated the two time periods (validated by permutation testing). For 11% of metabolites  $|p(\text{corr})| > 0.5$ , for 22%  $|p(\text{corr})| > 0.4$ , and for 35%  $|p(\text{corr})| > 0.3$ . To assess the effect of sleep and sleep deprivation, time points from 00:00 to 06:00 h during day 1 (sleep, fasting) and from 00:00 to 6:00 h during day 2 (no sleep, fasting) were analysed using OPLS-DA, so that features associated with sleep status (as validated by permutation analysis) could be determined. Only 2% of features had  $|p(\text{corr})| > 0.5$ ; 4% had  $|p(\text{corr})| > 0.4$ ; 8% had  $|p(\text{corr})| > 0.3$ . Time of day thus appears to have a greater effect on the metabolome than does sleep and sleep deprivation. Determining the full impact of exogenous factors (sleep, light/dark, meals/fasting) will be crucial for the future identification of biomarkers of disease and drug effects.

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## O5A-2

### LIPIDOMIC ANALYSIS OF BRAIN TISSUES AND PLASMA IN A MOUSE MODEL EXPRESSING MUTATED HUMAN AMYLOID PRECURSOR PROTEIN/TAU FOR ALZHEIMER'S DISEASE

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[Purpose] Alzheimer's disease (AD), the most common cause of dementia among neurodegenerative diseases, afflicts millions of elderly people worldwide. Although lipid dysregulation is suggested to participate in AD pathogenesis, its role in AD disease progression remains unclear. This study aimed to evaluate alterations in individual lipid species in brain tissues and plasma obtained from mice model for AD at various disease stages. [Methods] We performed a lipidomic analysis using brain tissues and plasma obtained from mice expressing mutated human amyloid precursor protein (APP) and tau protein (Tg2576×JNPL3) (APP/tau mice) at 4 (pre-symptomatic phase), 10 (early symptomatic) and 15 months (late symptomatic). [Results] Levels of docosahexaenoyl (22:6) cholesterol ester (ChE) were markedly increased in brains from APP/tau mice compared to controls at all stages examined. Several species of ethanolamine plasmalogens (pPEs) and sphingomyelins (SMs) showed different levels between brains from APP/tau and control mice at various stages of AD. In addition, 19,20-dihydroxy-docosapentaenoic acid (19,20-diHDoPE) and 17,18-dihydroxy-eicosatetraenoic acid (17,18-diHETE) were significantly increased in APP/tau brains during the pre-symptomatic phase, and concomitant changes occurred in plasma. Significant decreases in phosphatidylcholines and PEs with polyunsaturated fatty acids were also detected in brains at the late symptomatic phase, indicating a perturbation of membrane properties. [Conclusion] Although the exact mechanisms leading to the consistent increases in 19,20-diHDoPE and 17,18-diHETE in brain and plasma remain unknown, they might be candidate biomarkers for presymptomatic diagnosis of AD. Present results provide fundamental information on lipid dysregulation during various stages of human AD.

### O5A-3

#### **METABOLOMICS IN CEREBROSPINAL FLUID OF PATIENTS WITH AMYOTROPHIC LATERAL SCLEROSIS: AN UNTARGETED APPROACH USING HIGH-RESOLUTION MASS SPECTROMETRY**

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**Background:** the diagnosis of Amyotrophic Lateral Sclerosis (ALS) is clinical and, due to the absence of reliable diagnostic biomarkers, is often delayed a year or more from when symptoms begin. Patient-oriented research is inefficient because it depends on clinical outcome measures with high variance. Biological markers are needed to aid diagnosis, measure clinical progression and examine of disease physiology. Metabolomics, a promising approach to the identification of disease markers, provides metabolic profiles from biological fluids. Among the available high-throughput analytical platforms, liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) may be the most powerful. LC-HRMS has not yet been used to study metabolomics in cerebrospinal fluid (CSF) in ALS.

**Objectives:** to (i) devise an untargeted metabolomics methodology that reliably compares CSF from ALS patients and controls using LC-HRMS; (ii) ascertain a metabolic signature of ALS using the LC-HRMS platform and (iii) identify metabolites for use as diagnostic or pathophysiological markers.

**Methods:** we developed a method to analyze CSF components by UPLC (Ultra Performance Liquid Chromatography) coupled with a Q-Exactive mass spectrometer that uses electrospray ionization. Metabolomic profiles were created from the CSF obtained at diagnosis from ALS patients and patients with other neurological conditions. Multivariate analyses (OPLS-DA) used  $R^2$  and  $Q^2$  values to assess model quality. Univariate analyses assessed the contribution of individual metabolites as well as compounds identified in other studies.

**Results:** sixty-six CSF samples from ALS patients and 128 from control participants were analyzed. Metabolome analysis by LC-HRMS, correctly predicted the diagnosis of ALS in 80% of cases. OPLS-DA identified five features that discriminated diagnostic group ( $p < 0.004$ ). These compounds were characterized by  $MS^2$  and are currently being examined for identification.

**Conclusions:** untargeted metabolomics using LC-HRMS is a newly-developed and robust procedure to analyse a large cohort of CSF. This emerging technology generated a specific metabolic profile for ALS from CSF, and could be an important aid to the development of biomarkers for the disease.

O5A-4

## **METABOLOMIC MULTI-PLATFORM BASED ON DIRECT INFUSION MASS SPECTROMETRY FOR ALZHEIMER'S DISEASE DIAGNOSIS**

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The main challenge in metabolomics is to obtain comprehensive and unbiased metabolomic fingerprints due to the huge complexity, heterogeneity and dynamism of metabolome. In this sense, mass spectrometry represents a very interesting analytical platform, since complexity of metabolome may be overcome through the use of complementary atmospheric pressure ionization sources. Furthermore, direct infusion mass spectrometry (DIMS) has been postulated as an alternative in metabolomics, complementing hyphenated approaches. Thereby, DIMS exhibits several advantages such as the ability for high-throughput screening, fast analysis and more comprehensive metabolomic coverage, since there is not exclusion of compounds due to the separation device (liquid/gas chromatography, capillary electrophoresis).

Therefore, DIMS approaches provide broad information about the biological systems, very valuable for diseases diagnosis and study of pathogenesis. Alzheimer's disease (AD) is the most common neurodegenerative disorder, but its etiology is still unknown, although it is likely to be a conglomeration of different pathological entities. There is currently no cure for Alzheimer's disease, but early diagnosis could help to monitor disease progression, so identification of reliable biomarkers is becoming increasingly important.

The present work explores the application of a multi-platform based on direct infusion mass spectrometry for the comprehensive metabolomic analysis of blood serum samples from Alzheimer's disease patients. The approach supposes a multistage extraction procedure for releasing a broad range of metabolites, combined with MS analysis in positive and negative ionization modes and the complementary use of electrospray (ESI) and atmospheric pressure photoionization (APPI). The proposed method provided a global vision of Alzheimer's disease, confirming the involvement of multiple pathological processes, such as membrane destabilization, oxidative stress, hypometabolism or neurotransmission alterations, among others.

O5B-1

**COMPREHENSIVE METABOLOMICS ANALYSIS OF A YELLOWSTONE NATIONAL PARK HOT SPRING PHOTOTROPHIC MICROBIAL MAT OVER A DIEL CYCLE REVEALS A HIGH POTENTIAL FOR METABOLIC COUPLING AMONG COMMUNITY MEMBERS**

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Phototrophic microbial mat communities growing in geothermal springs are ecologically relevant to understanding microbial interactions among various producers and consumers of organic carbon. Several microbial species have been identified and physiologically characterized from the Mushroom Spring phototrophic microbial mat community in Yellowstone National Park (YNP); however, the extent of the metabolic interactions among community members is still not sufficiently understood. Several hypotheses on metabolite production, consumption, and exchange have been postulated. To evaluate these hypotheses, we performed targeted and untargeted metabolomics analyses of microbial mat cores collected from Mushroom Spring during a diel cycle. Metabolites and other molecules were segregated into four different chemical classes – volatile organic acids, polar metabolites, wax esters, and polyhydroxyalkanoates (PHAs) – and analyzed using separate gas chromatography-mass spectrometry methods, resulting in a total of 104 metabolites and molecules identified, with 72 quantified. Glycolate and acetate, predicted to have peak abundances during the day and night, respectively, showed the expected patterns of accumulation. Wax esters and PHAs showed higher abundances during the night, consistent with previously published transcriptomics data from a similar study that showed diel cycles among transcripts corresponding to the enzymes for synthesizing wax ester and PHAs. Approximately 60 polar metabolites were detected from the mat samples, with almost half of them showing certain fluctuating patterns during the diel cycle. Based on our knowledge, this is the first metabolomics study on phototrophic microbial mats in a YNP hot spring during a diel cycle.

**METABOLOMIC AND TRANSCRIPTOMIC ANALYSIS REVEALS ENDOCRINE DISRUPTION IN SKEENA RIVER (BRITISH COLUMBIA) SOCKEYE SALMON DURING THE 2008 SPAWNING MIGRATION**

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The health of British Columbia wild Sockeye salmon (*Onchorhynchus nerka*) populations has been the subject of increasing concern due to extreme variability in the number of fish returning to spawn annually. A 2008 study examining the health of 2 major Sockeye salmon runs (Fraser and Skeena Rivers) revealed marked differences in sex-specific hepatic gene expression profiles in fish collected at the spawning grounds of the Skeena relative to the spawning grounds of the Fraser. This finding was particularly surprising considering the Skeena passes through a relatively pristine environment while entry to the Fraser watershed bisects downtown Vancouver. To shed further light on potential sources of exposure, along with alterations in biochemical pathways which may affect the health of these fish, we examined the hepatic metabolome of the 2008 Skeena animals using targeted, quantitative metabolomics. Adult salmon were collected at the mouth of the Skeena River (n=76 fish) and at the spawning grounds in Fulton River (a tributary of the Skeena; n=43 fish). Individual salmon were inspected visually for the presence of milt or roe to establish gametic sex, scales were collected for DNA fingerprint-based stock assessment, and livers were collected for determination of genetic sex, status of hepatic mRNA transcripts, and metabolomic analysis. Quantification of 186 metabolites, including acylcarnitines (40), amino acids (21), glycerophospholipids (90),  $\Sigma$ hexose, sphingolipids (15), and biogenic amines (19) was accomplished using a kit-based approach with analysis by flow injection- or liquid chromatography-tandem mass spectrometry (FI-MS/MS or LC-MS/MS, respectively). Reference material (human plasma; spiked and unspiked) and blanks were included to assess method performance. Statistical and pathway analyses were carried out using MetaboAnalyst 2.0. Among the metabolites significantly associated with gender were  $\Sigma$ hexose, asparagine, phospholipids and sphingomyelins. Carnitine was the major metabolite associated with migration in both sexes. A number of sphingomyelins, phospholipids and amino acids were potentially associated with aberrant gender-specific hepatic mRNA profiles. Future work will investigate the role of significant metabolites in estrogen signalling (or other) pathways and how disruption of these pathways relates to adverse health outcomes in migratory fish populations.

**APPLICATION OF BOTH TARGETED AND UNTARGETED METABOLOMICS APPROACHES TO ASSESS POTENTIAL BIOLOGICAL EFFECTS OF SIMULATED SONAR SIGNALS ON BOTTLENOSE DOLPHINS**

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The effects of underwater noise associated with naval activities have retained the attention of the scientific community throughout the last decade. Multiple studies have reported the impact of such sounds on marine mammals. In a search for a deeper mechanistic understanding of the effects of underwater noise on marine mammals, and to explore potential biomarkers of the stress response, we have employed a metabolomics approach to characterise the stress response of bottlenose dolphins induced by simulated sonar signals.

Two approaches were used. A targeted approach focused on a single class of metabolites often reported to be good indicators of stress in mammals. An untargeted approach investigated new biomarkers of stress in dolphin serum. During the first part of the study, we developed a highly sensitive UHPLC-QQQ method to quantify six steroids of interest (i.e. cortisone, cortisol, corticosterone, 11-deoxycortisol, testosterone and progesterone). Each compound was identified using both retention time and multiple reactions monitoring (MRM). The untargeted approach was run on a UHPLC system coupled to a FT-ICR mass spectrometer in order to acquire very high resolution mass spectra.

While the targeted approach did not yield significant results, the untargeted approach led to more interesting findings. PLS-DA analysis of the different class of samples pre, test and post (i.e. before the sound exposure, immediately following the sound exposure, and one week after the sound exposure) yielded a robust model to discriminate dolphin samples before and after exposure to the simulated sonar signal. Univariate statistical analyses of the data suggested that several tens of compounds were involved in the stress response.

Identification of these biomarkers will improve our ability to identify and understand biologically significant effects of sound exposure on marine organisms and especially marine mammals. This should improve the effectiveness and efficiency of efforts to minimize the risks of anthropogenic noise.

## O5B-4

### **METABOLIC PROFILING OF A NON-HODGKIN'S LYMPHOMA AND BREAST CANCER CASE-CONTROL STUDIES TO IDENTIFY BIOMARKERS OF EXPOSURE AND DISEASE: RESULTS FROM THE ENVIROGENOMARKERS STUDY**

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The discovery of novel biomarkers of exposure to environmental pollutants can improve understanding of the biological mechanisms of diseases with an environmental aetiology. The EnviroGenoMarkers Project is a nested matched case-control prospective study within two pre-existing cohorts (NSHDS/Sweden and EPIC/Italy) aimed at the discovery of biomarkers to aid the study of the role of environmental agents in Breast Cancer (BC) and Non-Hodgkin Lymphoma (NHL). EnviroGenoMarkers applies the “*meet in the middle*” approach to identify -omics biomarkers associated with both exposure and disease.

Metabolic profiling was carried out to plasma samples by UPLC-QToF profiling, and data analysis by XCMS package. Initial analyses conducted on the NSHDS cohort; 194 B-cell NHL case-control pairs and 224 BC case-control pairs matched on sex, age (+/- 5 years) and centre. Findings were validated in the EPIC-/Italy cohort: 27 matched pairs for NHL and 242 for BC.

Three potential ‘meet-in-the-middle’ candidates were identified by classical and supervised statistical analyses for the test NHL dataset and were also associated with at least one exposure however they were not identified in the validation dataset. One metabolite feature was in the top ten p-value rankings of the associations for both the test and validation NHL cohorts.

Multiple features were associated with disease status in the two BC cohorts, however only one was significant in both datasets. Multiple features were associated with blood concentrations of the POPs and heavy metals in both cohorts. Moreover, there was high correlation between the features associated with various exposures, particularly for the PCBs. These results will significantly contribute to the characterisation of human *exposome*, which is the total of human environmental exposures from conception onwards, and the effect of these exposures to human health.



## O6A-1

### UTILISATION OF METABOLOMICS TO STUDY THE PRODUCTION OF SECONDARY METABOLITES IN BACTERIAL ENDOSYMBIONTS ISOLATED FROM MARINE SPONGES

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SeaBioTech is an EU-FP7 project designed and driven by SMEs to create innovative marine biodiscovery pipelines as a means to convert the potential of marine biotechnology into novel industrial products for the pharmaceutical (human and aquaculture), cosmetic, functional food and industrial chemistry sectors. Sponges are a rich source of novel metabolites that are of medicinal interest particularly as anticancer compounds. Sponge-associated endosymbiotic bacteria produce a plethora of novel secondary metabolites which may be structurally unique with interesting pharmacological properties. They provide chemical defence against environmental stresses such as predators and overgrowth of fouling organisms. Some of these secondary metabolites can be produced in large quantities on a biotechnological scale without the need to harvest the sponge and are therefore an economically viable source of commercial quantities of metabolites of interest.

As a case study for SeaBioTech, the metabolomic methods of dereplication and metabolic profiling were used to identify pharmacologically relevant secondary metabolites from sponge-associated endosymbiotic bacteria using LC-FTMS and NMR spectroscopy to analyse microbial extracts from solid cultures and liquid broths. Following taxonomic identification and bioassay screening, multivariate statistical analysis methods were employed for bioactive compound discovery. Several species of *Vibrionaceae* were identified that showed either strong or moderate anti-mycobacterial activity against *Mycobacterium marinum*. Differences in the mass spectrometry and NMR spectra from active and inactive bacterial extracts were probed using multivariate analysis in an attempt to uncover metabolites responsible for the bioactivity during the dereplication process. This study illustrates how metabolomics can be utilised to accelerate the selection of endosymbionts to take forward to scale-up, potentially fast-tracking the drug discovery process.

## O6A-2

### **CAN METABOLOMICS HELP TO ELUCIDATE THE BIOACTIVE COMPOUNDS CONTRIBUTING TO THE HEALTH BENEFICIAL PROPERTIES OF POMEGRANATES?**

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The traditional importance of the pomegranate as a medicinal plant is now supported by data obtained from modern science showing that the fruit has health beneficial activities. These activities are attributed to the pomegranate's high content of total polyphenols and high antioxidative activity. In order to identify and define the chemical nature of the antioxidants and additional health beneficial phenols, we have screened 29 pomegranates accessions. Peels exhibited approximately 40-fold higher antioxidative activity than the aril juices. To define whether metabolic profiling can assist in the identification of the antioxidants in peels, metabolic profiling was performed to each of these accessions using HPLC. We have measured the area of the 70 peaks detected in the chromatogram for each of the 29 accessions. By using correlations tests between the antioxidant activities of the peel's homogenates to each of the 70 peaks from the 29 accessions, we could predict which of the peaks contribute to the antioxidant activity. To verify whether the predicted peaks have indeed antioxidative activity, each peak was collected from the HPLC column and its antioxidative activity measured. We have found that almost all of the predicated peaks have indeed antioxidative activity. By using HPLC Q-TOF the chemical nature of many of the compounds found in the metabolic profile were identified. Among the identified are punicalagin, punicaline, and punigluconin, unique compounds of pomegranates. The advantages of our method over the customary method (liquid-liquid extraction) are that: (a) it enables high-throughput screening of numerous different compounds since bioactive compounds are not lost during the process; and (b) once the data set of this metabolic profiling is organized, it enables the discovery of other compounds exhibiting health benefiting activities.

## O6A-3

### **METABOLOMICS AS A TOOL IN THE IDENTIFICATION AND PRODUCTION OF NEW MARINE-DERIVED ANTIBIOTICS FROM SPONGES AND ENDOSYMBIOTIC BACTERIA**

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Metabolomic methods can be utilised to screen diverse biological systems for potentially novel and sustainable sources of antibiotics and pharmacologically-active drugs. Marine sponges and their endosymbionts have proven to be abundant in bioactive compounds. HR-LCFTMS and NMR were used in the identification of compounds isolated from a bacteria and its host sponge, as well as in the dereplication and metabolic profiling of other sponge-associated bacteria.

24-methylenecholesterol and two novel steroids, both significantly active against *Trypanosoma brucei*, were isolated from the Irish Sea sponge *Haliclona simulans*. Extracts from *Streptomyces* sp. isolated from *H. simulans* demonstrate anti-bacterial and anti-fungal activities. HR-LCFTMS assisted in identifying antimycins as the anti-fungal compounds. NMR and HR-LCFTMS were applied to the dereplication of extracts from bacteria from Mediterranean sponges. EG4 was selected and its cultivation optimised from small scale to larger scale production with the aid of metabolomic methods to identify and trace biomarkers.

Metabolomics has become a powerful tool in systems biology which allows us to gain insights into the potential of natural marine isolates for synthesis of significant quantities of promising new agents, and allows us to manipulate the environment within fermentation systems in a rational manner to select a desired metabolome.

O6A-4

**DOES ANTIBIOTIC IONISATION STATUS AFFECT THE MICROBIAL METABOLOME: AN INVESTIGATION INTO *ESCHERICHIA COLI* K12 CHALLENGED WITH TRIMETHOPRIM AT VARYING pH**

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Antibiotics are one of the most repeatedly prescribed therapeutics group. They are chemical agents that can selectively kill or inhibit the growth of bacteria. Here we apply metabolomics-based approaches to understand the interaction of trimethoprim, a basic dihydrofolate reductase inhibitor that acts as an indirect inhibitor of nucleic acid synthesis, with *Escherichia coli* K12. Two pH levels (5 and 7) were selected to mimic the urine pH of a healthy person, so that a further understanding of the drug action and the changes in metabolic levels *in vivo* can be developed. The minimum inhibitory concentration (MIC) of trimethoprim at the optimum bacterial growth conditions were determined and used to challenge the bacteria along with two lower sub-MICs. Growth curves were produced using a Bioscreen and from these it was found that whilst the drug was fully ionised at pH 5, it still had a negative effect on the growth curve of the bacteria. Therefore an LC-MS experiment using UHPLC coupled to a Thermo LTQ-Orbitrap XL MS system was employed to detect trimethoprim molecules inside the cell. This proved that the drug was taken up by the cell and thus affected growth at pH 5, as shown by the Bioscreen results. In addition, GC-TOF/MS was used to produce a metabolic profile of each condition. As anticipated, it was found that the primary effect of the drug was on biosynthesis of nucleotides at pH 7. However, there was an unusual response from guanine at pH 5 (upper regulated) and there were found to be many amino acids following this response. In addition, osmoprotectants including trehalose were significantly increased when the drug was highly active causing a change in metabolites (i.e. sugars and TCA members), finally glutamate was observed to be upregulated with its products following the same trend.

O6A-5

**EVALUATION OF URINARY ENDOGENOUS METABOLITES AS MARKERS FOR CYP3A INHIBITION AND INDUCTION IN HUMAN USING LC-tofMS**

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Background: The endogenous markers for CYP3A activity are useful for early identification of CYP3A inhibitors or inducers. This study identified the urinary endogenous markers for CYP3A activity in healthy subjects.

Methods: An open-label, 1-sequence, 3-period, 3-treatment, crossover study was conducted in 24 healthy Korean male volunteers. Each subject received a 1 mg of midazolam in each of 3 study periods: midazolam alone (control phase); midazolam after pretreatment with 400 mg ketoconazole once daily for 4 days (CYP3A inhibited phase); and midazolam after pretreatment with 600 mg rifampicin once daily for 10 days (CYP3A induced phase). The 12-hours interval urine samples were collected for metabolomics analysis between 24 hours before and 24 hours after of midazolam administration in each study period. Urine metabolites were determined using LC-TofMS. The principal metabolites listed from the MS or MS/MS scanning and multivariate analysis.

Results: After statistical analysis, L-octanoylcarnitine, Isobutyryl-L-carnitine, 16-ketoestradiol, 5 $\alpha$ -dihydrotestosterone-sulfate, androsterone-sulfate, and etiocholanolone-sulfate were suggested as candidates for CYP3A inhibition. Moreover, tetrahydroaldosterone-3-glucuronide, argininosuccinic acid, N-succinyl-L,L-2,6-diaminopimelate, 3 $\beta$ , 16 $\alpha$ -dihydroxyandrostenedione sulfate, androsterone-sulfate, 5 $\alpha$ -dihydrotestosterone-sulfate, etiocholanolone-sulfate, sebacic acid, and glycochenodeoxycholate-3-sulfate were proposed as candidates for CYP3A induction markers.

Conclusion: Metabolomic approach using LC-TofMS could provide markers for CYP3A4 activity and insight about variability in CYP-mediated drug metabolism.

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O6A-6

**ULTRAFAST STATISTICAL PROFILING: FT-ICR BASED PROFILING OF MYXOBACTERIA NATURAL PRODUCTS FOR RAPID DETECTION AND IDENTIFICATION OF MARKER COMPOUNDS**

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Myxobacteria represent an important source of novel natural products exhibiting a wide range of biological activities. Some of these so-called secondary metabolites are investigated as potential leads for novel drugs. Traditional approaches to discovering natural products mainly employ bioassays and activity-guided isolation from different myxobacterial isolates, but genomics-based strategies become increasingly successful to reveal additional compounds. These "metabolome-mining" approaches hold great promise for uncovering novel secondary metabolites from myxobacterial strains, as the number of known compounds identified to date is often significantly lower than expected from genome sequence information.

Currently, successfully established methods are based on LC-QTOF-MS analysis requiring about 20 min analysis times per sample. Facing several thousand myxobacterial strain isolates as well as numerous genetic knock out mutant strains available to analyze for the presence of novel secondary metabolites, analysis time becomes a bottleneck.

In this proof of concept study we analyzed several metabolite extracts from genetic knock-out mutants by ultra-high resolution FT-ICR direct infusion measurements, requiring only about 1 min / sample to measure. Principal Component Analysis (PCA) of the acquired MS spectra showed a clustering of the samples according to the bacterial genetic background, i.e. wild type and mutants could be differentiated. PCA loadings pointed to compounds responsible for this differentiation. By measuring these regions of interest in continuous accumulation of selected ions (CASI) mode, the sensitivity and resolution of these mass ranges could be enhanced significantly. The instrument resolving power of  $R > 750.000$  provided isotopic fine structure information. This enabled to literally "read out" the correct elemental composition for the target compounds. We have demonstrated for myxoprincomide, a recently discovered myxobacterial secondary metabolite, that this approach enables an unequivocal molecular formula generation for a compound with MW >1000. The ultra high resolution of an FT-ICR instrument enables for the first time an elemental composition determination for a molecular weight range where mass accuracy of existing mass spectrometers would be not enough for an unequivocal decision.

O6B-1

## TOWARDS A GENERATIVE MODEL OF LC/MS DATA TO IMPROVE METABOLITE IDENTIFICATION

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The identification of molecules from mass spectra represents a significant bottleneck in the use of high-throughput mass spectrometry (MS) to routinely analyse the metabolic makeup of an organism. Typically, MS is preceded by a physical separation (e.g. by liquid chromatography) and data are collected as a series of spectra as molecules elute. It is well understood that molecules eluting at the same time (and with the same temporal peak shape) are likely to be related: e.g. they are different isotopomers of the same molecule or different adducts/fragments.

The presence of these related peaks can help in identifying molecules by, e.g., comparing the mass differences and intensity ratios with those theoretically computed for particular molecules and their isotopes and adducts. We have recently developed a Bayesian method that simultaneously groups related peaks (based on their elution time and/or shape) and assigns the groups to particular chemical formulas. However, formula identification is only one step towards metabolite identification due to the possibility of many isomers for some formulas.

In this work, we extend our model to incorporate freely available network data using the Bayesian approach introduced in [1] as well as predicted elution times using the models in [2]. By incorporating these additional forms of information, an extra level of identification is added to the model, that can distinguish metabolites with the same formula. We investigate the performance of this extended model for both synthetic and real LC/MS-derived metabolomics data.

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**GLOBAL METABOLOMICS OF STRESSED *CAENORHABDITIS ELEGANS* USING ISOTOPIC RATIO OUTLIER ANALYSIS**

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The nematode *Caenorhabditis elegans* is exposed throughout its life to a variety of stressful conditions which makes it an ideal host to study stress response. A primary interest is the study of the metabolomic responses of *C. elegans* under stressful conditions in order to understand how the animals respond to adverse conditions. We examined worms' metabolomic responses to heat shock using a new LC-MS based technique called Isotopic Ratio Outlier Analysis (IROA). IROA allows the discrimination between molecules of biological origin and non-biological artifacts in two-group studies. Worms are isotopically labeled with 95% or 5% <sup>13</sup>C for the control and experimental groups. The two groups are mixed together for uniform extraction, sample preparation, and LC-MS analysis. Because both samples are simultaneously analyzed, many of the errors created by normal sample-to-sample variance are controlled, most particularly ion suppression and losses due to sample preparation. The isotopomers that arise from the combined groups are readily distinguished and form a recognizable pattern that can be used to identify true biological metabolites as well as to quantify relative amounts of each compound, determine the number of carbons in the compound, and determine an accurate molecular formula. We present here a method for identifying, quantifying, and analyzing IROA peaks in LC-MS spectra. A possible source of error in this experiment stems from the differential labeling of the groups, and so we also present a method for correcting for isotope effects. Additionally, we present a correlation-based analysis approach for identifying co-regulated compounds using the fold-change information contained within the IROA peaks. Using our technique with heat-shocked *C. elegans*, we unambiguously identified, in at least 3 out of 4 replicates, the molecular formula for 486 compounds, 77 of which changed by 2-fold or more. We found significant changes in several ascarosides – pheromones which are involved in behavioral and developmental responses such as male attraction and dauer formation (an alternative, stress-resistant developmental stage). This approach allows for precise quantification of the fold-changes of hundreds of compounds in a global, untargeted manner and can provide detailed metabolic information in any organism that can be isotopically labeled.



## O6B-3

### **METABOLOMICS-BASED ASSOCIATION NETWORKS: A CRITICAL DISCUSSION**

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Association networks based on comprehensive metabolomics measurements have become very popular. This is due to their simplicity to calculate – only (partial) correlations have to be used – and their potential to communicate with biologists. The apparent simplicity is, however, hiding some of their drawbacks: to what extent is the network reliable and really revealing underlying biology?

The last years we have studied two fundamental aspects concerning the validity of correlation-based metabolic association networks: i) the influence of measurement error correlation and ii) the reproducibility of the network when generated similarly from a new sample set from the same study population.

Only recently, measurement error correlation has emerged as one of the fundamental problems in high-throughput metabolomics measurements. These errors can be so severe that they mask the real biological correlation, or induce false associations. This will hamper the validity of metabolomics-based association networks.

When sets of samples drawn from the same population are used to infer association networks from, it is expected that these networks are very similar. A measure for this similarity gives a quantitative means for establishing the reliability of the association network. Furthermore, this serves as a reference when networks from different populations or reflecting different conditions have to be compared.

We will give real-life and simulated examples of both aspects discussed above – measurement error correlation and network consistency – for a range of metabolomics-based association networks. We will also discuss diagnostics for studying the reliability of the association networks and how to validate these networks in practice. The real-life examples concern both dynamic and static human lipidomics studies and illustrate that validated biochemical (pathway) information can be obtained from plasma samples using a network inference based approach.

## O6B-4

### METABOLOMICS APPLICATIONS OF THE BioCyc DATABASES AND PATHWAY TOOLS SOFTWARE

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The BioCyc.org database collection provides metabolic reconstructions for 3,000 organisms with sequenced genomes [1]. These Pathway/Genome Databases (PGDBs) integrate information on metabolic pathways, reactions, metabolites, genes, and proteins, providing a whole-organism context for interpreting metabolomics datasets. Highly curated BioCyc PGDBs for organisms such as *E. coli*, *S. cerevisiae*, Arabidopsis, mouse, and humans include information gathered from tens of thousands of publications. For example, the *E. coli* database alone has been curated from 24,000 publications. The MetaCyc database is a multi-organism metabolic pathway database describing 2,000 experimentally elucidated pathways and their components from thousands of organisms. MetaCyc has been curated from 35,000 publications.

BioCyc databases are accessible via the BioCyc.org web site, and in conjunction with the locally installable Pathway Tools software [2]. Metabolomics analysis tools available in BioCyc include genome-scale monoisotopic mass search, over-representation analysis on metabolite sets, and visualization tools allowing metabolite sets to be painted onto diagrams of individual pathways, and onto organism-specific metabolic map diagrams, to aid in determining the pathway distribution of metabolomics data.

The MetaFlux component of Pathway Tools is used to create and execute steady-state flux balance analysis (FBA) models from PGDB [3]. Our group has created FBA models for *E. coli* and for humans. Atom-mapping data is another recent addition to BioCyc. Pathway Tools can now compute the path of each atom in a reactant compound to its terminus in the product compound of a reaction [4]. Atom mappings for 9,200 reactions in BioCyc and MetaCyc were computed. A metabolic route-finding program is now available in Pathway Tools web mode (Tools->Metabolic Route Search). It computes optimal paths, based on atom mappings, from a source metabolite to a target metabolite in the metabolic network, with the option to insert reactions from MetaCyc for metabolic engineering problems. BioCyc data are available as downloadable files in multiple formats, and via web services.

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O6B-5

## APPROACHES FOR THE RAPID PROCESSING AND ANNOTATION OF MASS SPECTROMETRY DATA

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Compound identification constitutes one of the major bottlenecks in metabolomics. It is therefore valuable and time-saving to use databases and automated tools to aid identification and limit the amount of manual *de novo* structure elucidation. However, querying general compound databases only using accurate mass leads to thousands of compound candidates and there is a need to rationally filter and rank compound candidates from such a query.

We have created a data processing and metabolite identification pipeline which leads to a rapid and semi-automated ranking of compound candidates. Data from metabolite fingerprinting using LC-ESI-QTOF/MS were processed with several open source software packages, including XCMS and CAMERA to detect features and group features into compound spectra.

Next, using the MetShot package, we automatically schedule tandem mass spectrometry acquisitions to acquire a large number of MS/MS spectra while minimizing the number of data acquisitions as much as possible. Subsequently, the MS/MS data is submitted to computer assisted annotation for identification using the R package, Rdisop, and the MetFusion application.

We also implement a simple retention time prediction model using only predicted lipophilicity  $\log D$ , which predicts retention times within 42 s (6 min gradient) for most compounds in our setup, which on average is sufficient to remove 35 % of candidate compounds. We putatively identified 44 common metabolites including several amino acids and phospholipids at Metabolomics Standards Initiative (MSI) levels two and three, and confirmed the majority of them by comparison with authentic standards at MSI level one.

**COMPUTER ASSISTED IDENTIFICATION OF URINE METABOLITES ORIGINATING FROM GREEN TEA**

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The breakdown and biotransformation of small molecules present in food can give rise to a large variety of potentially bioactive metabolites in the human body. However, the absence of reference data for many of these components limits their identification in complex biological samples, such as plasma and urine. We present an *in silico* workflow to enhance the extraction of chemical information from metabolic profiling experiments using liquid chromatography coupled to multistage accurate mass spectrometry (LC-MS<sup>n</sup>). Our workflow is based on automatic annotation of MS<sup>n</sup> spectral trees by matching *in silico* substructures from candidate molecules with observed fragment ions.<sup>1</sup> Candidate chemical structures are obtained from databases of known compounds and by using biotransformation rules for generating potentially novel human metabolites. We evaluated the workflow by applying it to entire LC-MS<sup>n</sup> datasets of green tea and of urine after its consumption. For 623 fragmented molecular ions in the green tea dataset, in total 116240 candidate structures were automatically retrieved from the PubChem database. 85 previously identified components were successfully prioritized, with a median rank of 3, among hundreds of alternative candidates on the basis a substructure matching score. Furthermore, 24 novel assignments were made. Subsequently, 109 green tea components were systematically converted by *in silico* biotransformation rules, defining possible modifications in the human gut and liver before their excretion in urine. The resulting virtual library of 6416 potential metabolites was used to automatically annotate the urine LC-MS<sup>n</sup> datasets. The results were evaluated based on >80 previously identified components<sup>2</sup> and lead to putative identification of 20 additional urinary metabolites originating from green tea consumption. 75% of the annotated metabolites were not present in the Pubchem database, indicating the importance of using *in silico* biotransformation for analysis of metabolites. We will discuss the potential of our approach to support annotation of unknown metabolites in LC-MS<sup>n</sup> data for metabolomics experiments.

<sup>1</sup> Ridder et al. (2012) Rapid Commun. Mass Spectrom. 26: 2461-2471

<sup>2</sup> Van der Hoof et al. (2012) Anal. Chem. 84: 7263-7271

## O7A-1

### **INTERPRETING MECHANISM FROM METABOLIC FOOTPRINTING DATA OF SINGLE-GENE *PSEUDOMONAS AERUGINOSA* KNOCKOUTS**

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*Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium, that can form opportunistic infections in a range of hosts, including man. As well as acute infections, it also causes chronic lung infections in cystic fibrosis (CF) patients, and a majority of CF patients ultimately die from the long-standing inflammation caused by these chronic infections.

A whole-genome transposon knockout library is available for *P. aeruginosa*. We have carried out a proof-of-principle metabolic footprinting study on around 80 mutants, chosen to include metabolic enzymes as well as regulatory proteins. We grew the strains in 96-well plates on a medium designed to mimic the nutritional content of CF sputum, and used 1D  $^1\text{H}$  NMR spectroscopy to gather information on the main metabolic changes during growth - both excretion and consumption of metabolites from the medium. We were able to obtain fitted concentration values for the major metabolites, using a freely-available Bayesian software tool for deconvolution of the spectra.

We could use clustering to validate metabolic responses of functionally-related mutants – for example, subunits of pyruvate dehydrogenase clustered together in metabolic space, and furthermore pyruvate accumulated in the medium. In addition, kinase/regulator pairs of two-component system mutants clustered together. However, one mutant - for the regulatory sigma-54 gene *rpoN* - had a completely unexpected phenotype: it excreted large amounts of gluconate.

We determined the mechanistic links between the gene deletion and metabolic phenotype: analysing a series of additional knockouts enabled us to determine that the link was via regulation by a noncoding RNA of carbon catabolite repression of 6-phosphogluconate dehydratase. This gluconate-excretion phenotype was also variable in a clinical isolate panel taken from CF patients, showing correlations with genotype, and also a relationship to antibiotic resistance for clinically relevant antibiotics. In summary, we have demonstrated a semi-automated NMR metabolomic pipeline, that can identify novel and clinically relevant phenotypes in *P. aeruginosa*.

O7A-2

## IMPROVED BIOPROCESS BY METABOLITE PROFILING AND MODELLING ON THE CELLULAR LEVEL

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Increasing yield and productivity of high-performance strains is of increasing importance.

Strains are used for the production of low molecular weight compounds such as amino acids or vitamins, as well as for the production of complex molecules such as monoclonal antibodies. To reach new yield and productivity levels, information on limitations in metabolic pathways is required. Identification and elimination of bottlenecks in the product pathway represent complex tasks because limitations are usually distributed among multiple reaction steps. The advancement in novel technologies and more specifically metabolomics, that is the global and parallel measurement of hundreds of metabolites open the possibility to accomplish those targets. Metabolomics in combination with predictive network models allows the quantitative analysis of the underlying cellular mechanisms and identification of over-expression and attenuation targets. Here, we will provide an example of this approach in recombinant protein production using *E. coli*. The example shows how combining dynamic network models and metabolite profiling provides new quantitative information on limitations in precursor supply. At the same time it demonstrates how this approach can be used for metabolic engineering of strains and cell lines.

## SEARCHING FOR THE NEW ZEALAND SAUVIGNON BLANC JUICE AND WINE *TERROIR* THROUGH METABOLOMICS

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Sauvignon blanc (SB) is the major wine variety produced in New Zealand (NZ). This grape variety is cultivated in most of the wine producing regions, but only wines from Marlborough is well recognised and famous for their fruity and tropical flavours. The exact cause of variation between the wines from different regions is not clearly understood. But it is known that the difference in *terroir* (soil, climate and winemaking practices) may cause the production of different qualities of grape juices and wines. Detailed chemical analysis of grape juices is becoming an important tool for winemakers to decide which juices should be used for making premium wines before starting fermentations in the winery. Metabolite profiling of grape juice and wine provide important information about grape chemical composition, and wine typicity and quality. Therefore, we aimed to search for "*terroir*" in New Zealand SB grape juices and wines using a global metabolomics approach. Initially, a total of 63 grape juices were collected from different regions of New Zealand over 5 years (2006-2010). These juices were fermented under controlled laboratory conditions using a commercial yeast strain (EC1118) at 15°C. Comprehensive metabolite profiling of these 63 juices and wines were performed by combining different analytical techniques, including both mass spectrometry and nuclear magnetic resonance. In addition, detailed oenological parameters of grape juices and wines were determined using Fourier Transform Infra-red Spectroscopy (FTIR) WineScan. The complete metabolite profiles of these SB grape juices showed almost no effect of seasonal and regional *terroir*, while the resulting wines clearly showed a presence of seasonal *terroir*. Specifically, 2010 Marlborough wines were completely different than all other seasons. To confirm this observation, we analysed over 200 grape juices and wines from 2011 and 2012 harvest using GC-MS. The results confirmed that seasonal difference was more prominent than the regional difference in both grape juices and wines. We also identified the group of metabolites that play the vital roles behind the variation in grape juices and wines.

O7B-1

**DIFFERENTIAL FATTY ACID PROFILES IN LPS-TREATED MICE LIVER USING STABLE ISOTOPE LABELLING AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY WITH SINGLE SAMPLE ANALYSIS**

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Fatty acids (FAs) are involved in a variety of important biological processes, including inflammation, neural disorder, metabolic disorder and tumorigenesis. This study proposed a new method to rapidly screen for differential FAs in biological samples with single sample analysis. FAs in experimental and control groups were separately derivatized using  $d_0$  and  $d_3$  methanol to form fatty acid methyl esters (FAME). To quantitatively compare the FAs between the two groups, calibration curves of 24 FAs were generated using mixtures of  $d_0/d_3$ -labelled FA standards at ratio of 1:9, 1:3, 1:1, 3:1, and 9:1. The precision and accuracy for the relative quantification of FAs were between 5.09% to 11% and 85.64% to 95.13%, respectively. We used lipopolysaccharide (LPS) treated mice as our tested model. Liver samples obtained from seven LPS treated B6 mice and eight control B6 mice were analyzed by the proposed stable isotope labelling (SIL) and gas chromatography-mass spectrometry (GC-MS) method. Pooled livers of control group and LPS treated group were labelled with  $d_0$  and  $d_3$ -methyl esters, respectively. Equal volumes of derivatized samples were mixed prior to GC-MS analysis. A total of 6 saturated FAs and 10 polyunsaturated FA (PUFA) were identified in mice liver. Relative quantitative comparison revealed LPS induced higher expression of short chain FAs but lower expression of long chain FAs. The results were compared with the data obtained using traditional metabolomic approach. We observed that pooled samples using SIL and GC-MS analysis gave similar results, but dramatically reduce analytical time. The method was demonstrated as an effective and rapid strategy to identify potential FA markers in biological samples.



**A NOVEL LIPIDOMIC STRATEGY TO INVESTIGATE THE DYNAMICS OF INTRACELLULAR LIPID PATTERN IN SKELETAL MUSCLE CELLS UNDER LIPOTOXIC CONDITIONS**

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Increased plasma free fatty acid concentrations (FFA) are a hallmark of obesity and one major risk factor leading to skeletal muscle insulin resistance and the development of type 2 diabetes. Palmitate, the dominating FFA in plasma, lead to insulin resistance and apoptosis in cultured skeletal muscle cells. However, the exact mechanism, in particular the dynamics of the palmitate-mediated intracellular lipotoxic changes of the lipid pattern, are still less understood. We developed a novel strategy for the in-depth analysis of the dynamics of lipid metabolism performing stable isotope-labelling experiments of human skeletal muscle cells from muscle biopsy material, UHPLC-Orbitrap-MS lipidomics and data processing by a new software tool for global isotopomer filtering and matching. Muscle cells were cultured for 4, 12 and 24 h with 250  $\mu$ M [U-<sup>13</sup>C]-palmitate. 692 isotopomers were detected in the myocellular lipid extracts, assigned to 203 labeled lipid species spanning 12 lipid (sub-)classes. Our approach revealed increased production of several lysophosphatidylcholine (LPC)- and lysophosphatidylethanolamines (LPE)-species and <sup>13</sup>C-palmitate-incorporation. Less pronounced increases of diacylglycerides, ceramides, dihydroxy ceramides as well as phosphatidylinositols were detected. Incorporation of <sup>13</sup>C-palmitate in several triglycerides and PCs indicated high turnover rates of these lipid classes but no changes in the lipid levels. We detected elongation and degradation of FAs in lipid species. Of note, pharmacological inhibition of LPC and LPE production blocked lipotoxicity. The novel lipidomics strategy presented here allows to follow the synthesis, transformation and degradation of individual lipid species and has the potential to open new detailed insights into the dynamics of lipid metabolism that may lead to a better understanding of lipotoxic effects thereby elucidating new targets for therapeutic intervention.

**FROM BLIND FINGERPRINTING TO FUNCTIONAL PROFILING: CERAMIDE AND RELATED COMPOUNDS IN EXPERIMENTAL MODELS OF OXYGEN SENSING**

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Hypoxic pulmonary vasoconstriction (HPV) response represents a physiological adaptive mechanism that responds to oxygen variation. Sphingolipids are a heterogeneous class of lipids involved in several physiological mechanisms. The synthesis of ceramides involves the activity of sphingomyelinase (SMase) on membrane sphingomyelin (SM), and its levels could play a crucial role as mediator. In addition, ceramidase breaks ceramides (Cer) to obtain sphingosine (SP) which in turn can be phosphorylated by SP kinase to generate SP-1 phosphate (SPP). These enzymes have been associated to hypoxia in different studies. In order to investigate the response to different conditions, embryonary arteries from rats or chickens were used in different experiments. Arteries were dissected free of surrounding tissues and cut into rings of around 2.5 mg average weight. Vessels were then exposed to normoxia, hypoxia, or to cycles hypoxia/normoxia. Moreover, selected conditions such as incubation with high concentration of potassium or with SMase were tested as methodological controls. After treatment, each sample was fingerprinted by reverse-phase Ultra High Performance Liquid Chromatography (UHPLC), coupled with a QTOF mass analyzer (QTOF-MS). High sensitivity and mass accuracy of the analytical platform was a requirement as the main challenge of this study dealt with the fingerprinting in very small biological samples. Compounds were eluted with a linear gradient, using ammonium formate and methanol, in less than 12 min per sample. After data processing around 4000 molecular entities were thus found, and the list of extracted masses was compared against public databases. Features for sphingolipids and other compounds of interest were identified, with error below 5ppm ( $\pm 0.0003$  amu approx.), and selected for further profiling. More than 40 different SM and Cer could be identified, and their proportion evaluated. Moreover, the ratios of SP and SPP could be evaluated too. The ratios showed that under hypoxia all the ceramides increased in PA, and returned to initial values after re-exposure to normoxia, and this fact was associated to changes in other related compounds. High-throughput profiling from UHPLC-QTOF-MS fingerprints has proven to be an effective tool for rapid, quantitative and precise evaluation in biological samples, providing new elucidations of the complex mechanisms involved in acute oxygen sensing.

## O8A-1

### **DIFFERENT METABOLIC RESPONSES OF CAFFEINATED AND DECAFFEINATED GREEN TEA EXTRACT DURING REST AND MODERATE INTENSITY EXERCISE**

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Both acute and chronic supplementation of green tea extract (GTE) has been suggested to increase energy expenditure and fat oxidation under resting and exercise conditions. These effects have been mainly attributed to catechins, although caffeine alone has also been shown to promote thermogenesis and fat oxidation.

This study compared the effects of caffeinated (cGTE) and decaffeinated (dcGTE) GTE supplementation on numerous plasma metabolites at rest and during moderate intensity exercise.

In two similar, but separate, placebo-controlled double blind, randomized studies, healthy male subjects consumed (i) cGTE (1200mg catechins, 240mg caffeine / day) for 7 days or (ii) dcGTE (1200mg catechins, / day) for 1, 7 and 28 days. On each experimental day, subjects completed a moderate intensity cycling exercise after the consumption of a single bolus of c/dcGTE or placebo (PLA) followed by a 2-hour resting period. Blood samples were collected on each experimental day at baseline, after the resting period and throughout exercise. Plasma was analyzed using untargeted GC-MS- and LC-MS-based metabolite profiling as well as targeted profiling of catecholamines. The data were analyzed using multivariate statistics and ANOVA mixed-effects models.

At rest, cGTE led to changes in metabolites reflecting increased fat oxidation ( $\beta$ -hydroxybutyrate), increased lipolysis (glycerol), reduced Cori-cycle (alanine) and enhanced TCA cycle (citrate) activity when compared to PLA. In contrast, 7-days dcGTE only induced changes reflecting increased fat oxidation, which was also slightly enhanced after 28 days under resting conditions. During exercise, cGTE caused changes indicative of reduced fat oxidation, increased glycolysis (lactate, pyruvate), and enhanced Cori-cycle activity, while no significant effects of dcGTE were observed after 7-day and 28-day of dcGTE. In addition, neither dcGTE nor cGTE supplementation appeared to alter adrenergic stimulation (adrenaline and noradrenaline) during rest or exercise.

In conclusion, our comprehensive metabolite profiling approach broadened our understanding on the potential modes of action of dc/cGTE and highlighted the significant contribution of caffeine to the metabolic effects of GTE.

## **METABOTYPING IN CARDIOVASCULAR RISK SUBJECTS REVEALS DIFFERENCES IN THEIR URINARY PROFILES AFTER WINE INTAKE**

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Metabotypes promises to be a tool in nutritional interventional studies. Diet composition has been shown to influence metabolism and to impact on the risk of cardiovascular disease. The aim was to classify the subjects in different phenotyping groups attending to their anthropometric and clinical characteristics and subsequently, to evaluate the changes on urinary metabotypes by 1H-NMR metabolomics approach in response to red wine, dealcoholized red wine and gin intake.

In a prospective, randomized, crossover and controlled trial, 24h-urine samples of fifty-seven male volunteers with high cardiovascular risk factors were collected at baseline and after followed three dietary interventions (28 days): dealcoholized –red-wine (D) (272ml/day, polyphenol-control), red-wine (W) (272ml/day) and gin (G) (100ml/day, alcohol-control). Anthropometric and clinical parameters at baseline were analyzed by k-means to obtain the phenotypes. Phenotype differences at baseline and after dietary interventions were assayed by 1H-NMR-based metabolomic.

The analysis produced 4 clusters. Metabolomic revealed particular differences between a cluster with high cardiovascular risk diabetic (HCD-C) and a cluster with low cardiovascular risk cluster (LCV-C). These differences were associated to significant higher levels in LCV-C phenotype of BCAA (valine, isoleucine, leucine), TCA intermediate metabolites and acetate. The glucose was statistically higher in HCD-C. After W and G interventions, metabolomics differences were maintained between these two clusters. After D intervention, BCAA levels for both cluster groups achieved levels which were not significantly different from LCV-C and HCD-C after red wine and gin interventions.

The results suggested that individuals with different clinical phenotype show different response to the beverages intakes, translated in a different metabolic profile. The results showed new insights that could open the way to go towards a personalized nutrition.

**NEW BIOMARKERS OF COFFEE CONSUMPTION IDENTIFIED BY NON-TARGETED METABOLOMIC PROFILING IN COHORT STUDY SUBJECTS**

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Coffee is one of the most widely consumed beverages in the world and contains various bioactives implicated with health. Controversies still exist regarding the risks and benefits of consumption. More epidemiological and clinical studies are needed in which coffee intake can be accurately assessed. To identify sensitive biomarkers of intake, metabolomic profiling of urines from 20 high coffee consumers (median 290 mL/day) and 19 non-consumers from the SU.VI.MAX2 cohort was performed using UPLC-ESI-QTOF-MS. Of the 1111 ions detected (932 in ESI+; 179 in ESI-), all but one of 132 statistically discriminant ions (ANOVA-BH p-value < 0.05) had a higher intensity in the group of high consumers than in that of the low consumers, suggesting an exogenous origin. In parallel, a Partial Least Squares Discriminant Analysis (PLS-DA) after Orthogonal Signal Correction filtration (OSC) clearly distinguished urine samples from low and high coffee consumers, with an excellent validation of the model (Q2(cum)=0.85, permutation test plot). The ANOVA BH p-value and the OSC-PLS-DA VIP value gave very similar ion rankings. Many interesting ions were related to caffeine metabolism and were easily identified by comparison with authentic standards. However, caffeine metabolites are not specific for coffee intake. Of the other highly discriminating ions, three clusters were related to non-caffeine derived compounds and may be more specific to coffee consumption, with excellent performance in the Receiver Operating Characteristics (ROC) curve test. Using LTQ-Orbitrap (Thermo Scientific) for ultra-high resolution and MSn capabilities, in combination with prediction of MS fragmentation (Mass Frontier™, Thermo Scientific) and metabolism prediction (Meteor, Lhasa Ltd), the parents of these discriminant clusters have been tentatively identified as Atractyligenin glucuronide, Cyclo(leucyl-prolyl) and Trigonelline. Further investigations must be carried out to confirm these compounds as specific biomarkers of coffee consumption usable in any population, but analytical data and information on their dietary sources and metabolism make them very promising new biomarkers for nutritional epidemiology.

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**DISCOVERY BIOMARKERS OF BREAD INTAKE IN CARDIOVASCULAR HIGH-RISK PARTICIPANTS. A MASS SPECTROMETRY-BASED METABOLOMICS APPROACH**

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Bread is a key component of the Mediterranean Diet which provides important amounts of complex carbohydrates, fiber and B vitamins, among other bioactive compounds, especially whole-grain bread (WG-B). Nutrimetabolomics explore the complex relationship between the consumption of dietary compounds and the maintenance of health or disease development with the aim to discover new biomarkers of intake and effect, respectively. On this regard, the study of food metabolome measure diet exposition to food consumption.

In this study, a metabolomics strategy designed to discover new biomarkers of food consumption was applied by classifying participants of the Predimed study according their reported bread consumption (either white bread (WH-B) or WG-B).

Bread intake was defined according to FFQ which was previously completed by the free-living elderly Mediterranean population at high cardiovascular risk. Subjects of the Predimed study were stratified at baseline by their consumption of bread: 2 groups of Regular-bread consumers ( $\geq 1$  portion of bread/day either WH-B or WG-B) or non-bread consumers (NBC) ( $\leq 3$  portions of bread/month). Urine samples of subjects were analyzed by HPLC-Q-TOF-MS followed by multivariate data analysis (OSC-PLSDA and HCA).

Urinary metabolome showed differences between both regular-bread consumers and NBC. The metabolite furosine was tentatively identified showing no differences between both types of bread consumption. This metabolite has been related to roasting bread processing. Furthermore, regular-consumers of WG-B showed significantly higher levels of 2,8-dihydroxyquinoline- $\beta$ -D-glucuronide than NBC and WH-B. This metabolite has been associated with PPAR-alpha activity.

The results reinforce the capacity of metabolomics to explore the metabolism impact of dietary components and the ability to obtain new biomarkers of intake and effect combining epidemiological nutritional data and metabolomics.

O8B-1

## FROM METABOLITE PROFILING TO BIOMARKERS, GLYOXYLATE AND TYPE 2 DIABETES

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Background and aims: Timely detection of and intervention in type 2 diabetes reduces the risk of developing complications. Metabolic changes can be detected several years prior to a clinical diabetes phenotype, but their relevance in pathophysiology is poorly understood and their systematic exploration for a diabetes risk remains to be explored. We discovered metabolites associating with the development of diabetes through broad profiling. In addition metabolite profiling in different animal model and tissues allowed for the generation of mechanistic hypothesis regarding their function. These metabolites guide development of biomarkers for diabetes comorbidity risk assessment.

Materials and methods: Applying metabolite profiling in blood plasma to several large clinical studies we observed changes that associated with diabetes development several years prior to a clinical diabetes phenotype. Interpretation of differential profiling data from clinical studies (>300 metabolites quantified) showed new metabolites, such as glyoxylate a small reactive aldehyde, involved in the generation of diabetes associated advanced glycation end products. Glyoxylate concentrations were affected by metformin in vivo and associated with diabetes complications suggesting glyoxylate as a central morbidity associated metabolite.

Results and Conclusion: Our quest for new diagnostic diabetes markers led to the discovery of metabolites with prominent physiological significance. One of them, glyoxylate, significantly associated with diabetes diagnosis and diabetes risk. The small and reactive aldehyde serves as detoxification product of advanced glycation endproducts (AGE), is the single precursor of oxalate, an essential molecule in diabetic nephrolithiasis, and is generated during peptide hormone activation including GLP1. In the context of generating validated diagnostic biomarkers we discuss individual metabolites and how their role in diabetes pathology may lead to new therapeutic strategies.

**A METABOLOMIC EVALUATION OF SHORT AND LONG TERM EFFECTS OF DIFFERENT MACRONUTRIENT INTAKE IN OVERWEIGHT AND OBESE POSTMENOPAUSAL WOMEN**

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With menopause there is an increased risk of central obesity associated with insulin resistance and cardiovascular disease, for partly unclear reasons. Thus, there is a need for intervention studies, with concomitant biochemical analyses, to develop effective treatment of postmenopausal overweight and obesity. We performed a global evaluation of metabolic responses, i.e. metabolomics, using gas chromatography-time of flight/mass spectrometry (GC-TOF/MS) in plasma samples from 66 obese and overweight postmenopausal women consuming a Paleolithic-type diet (PD) or a diet according to the Nordic Nutrition Recommendations (NNR diet) for 24 months. The PD diet was composed of 30 E% protein, 30 E% carbohydrate and 40 E% fat, mainly monounsaturated fatty acids (MUFA), while the NNR diet consisted of 15 E% protein, 55 E% carbohydrates and 30 E% fats, balanced according to MUFA and saturated fatty acids (SFA). In addition, the PD diet was evaluated in a short-term study over five weeks in ten obese and overweight postmenopausal women. The different diets resulted in significant weight loss. Differences in dietary metabolic response were extracted by orthogonal partial least squares-discriminant analysis (OPLS-DA). We found differences in metabolites and metabolite patterns when comparing a PD diet to NNR. Furthermore, we found similarities and differences between the short- and long-term interventions of a PD diet. Notably, by utilizing the metabolomics and multivariate bioinformatics approach, we found dietary effects that would not have been detected if only interpreting p-values, such as seasonal and individual variation to dietary weight loss as well as robust dietary metabolite patterns. The PD diet resulted in a cohesive increase in *myo*-inositol and docosahexaenoic acid (DHA) as well as a decrease in the SFA lauric acid (12:0) in both the short- and long-term intervention. Ethanolamine and cholesterol, which can be associated with increased cardiovascular disease risk, were decreased in the PD diet while increased in the NNR diet. Differential metabolic responses are seen after diet interventions with different macronutrient composition in postmenopausal women. This may be of importance to reduce risk for future metabolic and cardiovascular morbidity.



### O8B-3

#### DISCOVERY OF NOVEL BIOMARKERS FOR FABRY DISEASE USING A MASS SPECTROMETRY METABOLOMIC APPROACH

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Fabry disease is an X-linked lysosomal disorder characterized by the accumulation of glycosphingolipids, mainly globotriaosylceramide (Gb<sub>3</sub>) and globotriaosylsphingosine (lyso-Gb<sub>3</sub>) in biological fluids, and different organs. Enzyme replacement therapy (ERT) is provided to both males and females depending on the severity of symptoms.

Objectives: 1) To discover novel urinary and plasma biomarkers related to lyso-Gb<sub>3</sub> which reflect disease severity and progression using a metabolomic approach; 2) To elucidate the chemical structure of the targeted biomarkers; 3) To quantify the biomarkers of interest by tandem mass spectrometry (LC-MS/MS).

Materials and Methods: Urine and blood samples from 63 untreated Fabry patients and 59 healthy controls were collected. The analyses were done on a Synapt ESI-QTOF-MS system coupled to an Acquity UPLC (Waters Corporation, Milford, MA). The mass spectrometry results generated by the metabolomic approach were processed by MarkerLynx XS (Waters Corp.), and EZInfo (Umetrics, Umeå, Sweden) for multivariate analysis.

Results: Apart from lyso-Gb<sub>3</sub> (mass/charge ( $m/z$ ) = 786), seven novel biomarkers were specifically detected in urine and plasma with the following  $m/z$  ratios: 758, 774, 784, 800, 802, 820, and 836. All seven biomarkers are analogues of lyso-Gb<sub>3</sub> with modifications on the sphingosine moieties. A lyso-Gb<sub>3</sub>-glycine internal standard was synthesized for relative quantification of all biomarkers using LC-MS/MS. Fabry patient urine specimens presented analogues which were excreted in larger quantities than the lyso-Gb<sub>3</sub> biomarker itself. No significant quantities of lyso-Gb<sub>3</sub> analogues were found in normal healthy controls. Statistical studies showed that the urinary excretion levels of the analogues correlate with patient gender and enzyme replacement therapy (ERT) treatment in men. One male Fabry patient with a cardiac variant mutation (N215S) had a normal urinary Gb<sub>3</sub>/creatinine level, but he presented abnormal levels of urinary and plasma lyso-Gb<sub>3</sub>, as well as urinary lyso-Gb<sub>3</sub> analogues.

Conclusion: To our knowledge, this metabolomic study using a time-of-flight mass spectrometry approach is the first to demonstrate the presence of lyso-Gb<sub>3</sub> analogues. Relative quantification of these promising urinary biomarkers revealed that they are excreted in variable quantities in patients, suggesting complex biochemical and physiological profiles pertinent to each patient.

**EXAMINING RESPONSE TO ASPIRIN THERAPY USING A PHARMACOMETABOLOMICS-INFORMED-PHARMACOGENOMICS APPROACH**

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Aspirin is a well established anti-platelet agent, but the mechanisms responsible for variation in response to aspirin therapy are poorly understood. In the present study, we utilized information from metabolomic profiling to conduct focused genetic association analyses using a *pharmacometabolomic-informed-pharmacogenomics* approach. We assessed healthy subjects from the Heredity and Phenotype Intervention (HAPI) Heart Study using a GCTOF metabolomics platform. Mass spectrometry (MS)-based metabolomic profiling was performed on serum samples collected before and after low-dose aspirin therapy to characterize the metabolomic signature of aspirin exposure and identify important pathways affected. We also investigated differences in metabolomic profiles between good and poor responders to aspirin therapy. Many metabolites, including known aspirin catabolites, changed upon exposure to aspirin and pathway enrichment analysis identified purine metabolism as significantly affected by drug exposure. Further, several metabolites from the purine pathway were associated with aspirin response. Post-aspirin levels of inosine and adenosine were significantly higher in the poor compared to good responders ( $p$ 's<0.01). Using our established "pharmacometabolomics-informs-pharmacogenomics" approach we identified genetic variants in *adenosine kinase (ADK)* associated with aspirin response. The most strongly associated SNP was rs16931294. The G allele was associated with higher platelet aggregation post-aspirin than the more common A allele ( $\beta$ =0.8;  $p$ <.003). The G allele of rs16931294 was also significantly associated with higher post-aspirin levels of inosine ( $p$ =0.007). Combining metabolomics and genomics allowed for more comprehensive interrogation of mechanisms of variation in aspirin response - an important step toward personalized treatment approaches for cardiovascular disease.

O9A-1

**VITAMIN B-6 RESTRICTION IN HEALTHY MEN AND WOMEN AFFECTS METABOLITE PROFILES REFLECTING ALTERED ONE-CARBON METABOLISM AND TRYPTOPHAN CATABOLISM**

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Vitamin B6 is important in many facets of human metabolism. Pyridoxal phosphate (PLP), the coenzyme form of vitamin B6, is involved in the interconversion and catabolism of amino acids, several phases of one-carbon (1C) metabolism, the formation of key organic acids, heme synthesis, gluconeogenesis and glycogenolysis. Low vitamin B6 status is associated with greater risk of several forms of vascular disease and cancer. In this study, we evaluated 23 health men and women after a 2-day controlled diet (basal) and following a 4-wk low vitamin B6 diet ( $0.37 \pm 0.04$  mg/d). We obtained fasting and postprandial plasma samples before and after B6 restriction. Recent findings from this study included that vitamin B6 restriction alters global metabolomic patterns and amino acid profiles (Gregory et al., PLOS One, 2013) as well as fatty acid profiles (Zhao et al, J Nutr 2012). Here we report further analysis of this study by using LC-MS/MS metabolite profiling of major constituents and biomarkers of 1C metabolism and the tryptophan-kynurenine catabolic pathway.

Statistical methods accounting for multiple testing with paired data showed that B6 restriction yielded significant overall changes in both 1C and tryptophan catabolic profiles in both pre- and postprandial states. Of the 1C metabolites, vitamin B-6 restriction yielded greater plasma concentration of cystathionine ( $P < 0.0001$ ) and serine ( $P < 0.05$ ), and lower concentration of creatine ( $P < 0.0001$ ), creatinine ( $P < 0.05$ ), and dimethylglycine ( $P < 0.05$ ), relative to their initial levels in the vitamin B-6 adequate state. In the tryptophan pathway, vitamin B-6 restriction yielded increased 3-hydroxykynurenine ( $P < 0.01$ ) and decreased kynurenic acid ( $P < 0.01$ ). Multivariate analysis of combined datasets (1C plus tryptophan metabolites) using multilevel PLS-DA that accounts for the paired structure clearly discriminated between vitamin B-6 adequate and restricted states. Ratios of biomarkers also changed markedly. These findings illustrate how metabolic profile analysis provides insight into the impact of nutritional inadequacy and identifies useful biomarkers indicative of functional effects of nutrient deficiency.

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## O9A-2

### **AN EXPLORATION OF THE URINARY METABOLOME IN THE EUROPEAN PROSPECTIVE INVESTIGATION ON CANCER AND NUTRITION (EPIC) COHORT TO IDENTIFY NOVEL DIETARY BIOMARKERS**

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Dietary biomarkers are increasingly used for validation of dietary measurements and as a more objective assessment of methods based on questionnaires to study associations between diet and disease risk. This biomarker approach utilizes the huge diversity of constituents commonly found in foods and after digestive transformation in human biofluids. Metabolomics of urine samples within the European Prospective Investigation into Cancer and nutrition cohort (EPIC) should allow identification of novel dietary markers.

A cross-sectional study nested within EPIC was conducted on 481 subjects for which detailed 24-hr dietary recalls (24HDRs) and 24-hr urine samples were available for analysis. Urine samples were first normalised by dilution to the same specific gravity. Data were acquired using a high-resolution UPLC-QToF-MS. An optimised data processing workflow was developed in the R language which included peak-picking, QC based signal drift correction/filtration (feature RSD <30%), automated multivariate analysis, data-dependent MS/MS accurate mass matching and the monoisotopic mass identification through the screening of on-line and customized databases (Phenol-Explorer, FooDB, HMDB, MassBank, Metlin). Additionally the workflow allowed automated outlier removal and application of iterative regression and/or discriminant analysis methods to each Y-variable (i.e. dietary category) returning putative biomarkers above user-defined thresholds. Integrated hierarchical clustering of metabolomic, food intake and food constituent data was then used to examine metabolite-metabolite and metabolite-diet associations.

The efficiency of this fully automated approach for data analysis allowed the rapid identification of over 300 unique mass spectral features corresponding to dietary polyphenols, their phase II and gut microbial metabolites. These biomarkers matched confidence criteria for correlation to the consumption of a variety of specific foods rich in polyphenols. They included phenolic compounds originating from coffee, chocolate, citrus, wine and tea consumption like caffeoylquinic acids, feruloylquinic acids and phenolic compounds derived from coffee roasting. Furthermore, 671 MS features correlated with coffee intake ( $r > 0.3$ ,  $n = 371$ ,  $p < 0.01$ , Bonferroni corrected) were identified corresponding to approximately 100 metabolites, putative novel biomarkers for coffee intake. Identification of these biomarkers is on-going in our laboratory.

This unique approach will be extended to the rest of the food metabolome (>27,000 known food constituents in the FooDB database) to identify a variety of biomarkers for different dietary exposures. This biomarker identification approach will be implemented in future metabolome-wide association studies in the EPIC or similar cohorts.

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**ANALYTICAL STRATEGIES TO IDENTIFY LOW ABUNDANT METABOLITES IN COMPLEX SAMPLE MATRICES: BIOAVAILABILITY OF POLYPHENOLS AS SHOWCASE**

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There is an increasing interest in the absorption, disposition, metabolism, and excretion of phytochemicals present in the human diet. In particular, polyphenols have recently received much attention in view of their potential health benefits to human. The complete structural elucidation of polyphenols in food and especially their biotransformation products in human body fluids is still a tedious task, due to their variable abundance and many possible isomeric forms. Recent technological advances in mass spectrometry (MS) and nuclear magnetic resonance (NMR) and a systematic analytical approach enable the more precise detection of small molecules (Van Der Hooft et al. 2013). We show how a combination of high resolution MS multistage fragmentation (MS<sup>n</sup>) and one-dimensional -proton (<sup>1</sup>H)-NMR of metabolites semi-purified by liquid chromatography coupled to solid phase extraction (LC-SPE) can circumvent the need for isolating extensive amounts of the compound of interest to elucidate its structure (van der Hooft et al. 2012). During this presentation, examples will be provided of the identification of metabolites in crude food extracts, as well as their colonic catabolites found in human body fluids as a result of bioconversions by the gut microflora. Moreover, these semi-purified phenolic conjugates could be quantified using NMR. This approach resulted in quantitative cumulative excretion profiles of, for instance, valerolactone conjugates in urine. Also, the automation of metabolite annotation and identification based on MS<sup>n</sup> and NMR will be discussed. The efficient identification approach for metabolites down to micromolar concentrations opens up new perspectives for in depth studying the role of dietary polyphenols and their specific break-down products in the human body.

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O9A-4

## USING LIPIDOMICS TO STUDY THE METABOLISM OF DIETARY LIPIDS AND THE EFFECT ON CANDIDATE MARKERS FOR CARDIO METABOLIC DISEASES

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Background: The risk for Insulin resistance has been associated with specific plasma phosphatidylcholines and triglycerides, using open lipid profiling and lipidomics approaches<sup>1,2</sup>. These studies were unable to show that dietary factors contributed or otherwise affected the concentrations of these candidate markers. In addition to the profiling the intact lipids it is also possible to determine the fatty acid profile after hydrolysis of the lipids. We used a combination of fatty acid profiling and intact lipid profiling to study the effect of specific lipid-based dietary interventions on the lipid profile of healthy volunteers. The aim of this work is to understand the relationship between the two methods and to identify candidate markers for increased risk of cardio-metabolic disease that can be modified by diet.

Materials and Methods: Plasma sample sets from different specific dietary supplementation (fish oil)<sup>3</sup> and other intervention studies in volunteers were selected. Following derivatisation, fatty acid profiling was performed by GC. Intact lipid profiles were obtained by direct infusion using chip based nanospray coupled to a benchtop Orbitrap.

Results & Discussion: Supplementation with long chain n-3 PUFA such as found in oily fish led to significant increases in the concentrations of these fatty acids in the phospholipid fraction. The same effect could be observed in the intact lipid profile, where polyunsaturated fatty acid containing lipids strongly where significantly changes by the fish oil supplementation and correlated with fatty acid data obtained by GC, Pearson correlation coefficient between fatty acid DHA (FA(22:6n-3) and intact lipid PC(40:6) was 0.9 ( $P < 0.1E-06$ ). Many of the lipids that are associated with the risk for type 2 diabetes could be significantly changed through fish oil supplementation. Lipids that other studies<sup>2</sup> showed to be associated with increased risk for type 2 diabetes like PC(38:3) were significantly reduced by fish oil supplementation, while lipids associated with a decreased risk like (PC-38:6) increased after fish oil supplementation. This shows that dietary interventions can significantly change intact lipids that are associated with the risk for insulin resistance.

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3. Browning et al. Am. J. Clin. Nutr. 2012;96:748-58.

**EFFECTS OF MARGINAL SELENIUM DEFICIENCY IN MICE ON LIVER METABOLISM**

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Selenium (Se) is an essential trace element in mammals, which mainly administrates its functions after being incorporated into selenoproteins. In addition, different selenocompounds can directly affect cellular metabolism. In Europe, the majority of the population does not reach the recommended amount of Se supply, thus suboptimal Se status prevails. As the liver plays a crucial role in Se metabolism we were interested in the liver metabolome of mice experiencing marginal Se supply. Male C57BL6/J mice were fed either a Se adequate (0.15 mg Se/kg) diet or Se deficient (0.086 mg Se/kg) diet starting directly after weaning for 7 weeks. The main Se form in the diet was selenomethionine. Changes in the Se status were confirmed by decreased activity of total glutathione peroxidase in liver homogenates, which resulted in elevated levels of 4-hydroxynonenal, a marker for oxidative stress. Alkaline phosphatase (ALP) activity as clinical marker for liver distortion was increased in plasma samples but decreased in liver tissue of Se deficient animals. For GC-MS analysis, metabolites were extracted from hepatic tissue using a methanol:chloroform mixture. After phase separation, samples were derivatized using MSTFA. Metabolite detection and quantification was performed using TargetSearch. In addition, amino acids and their derivatives were quantified following the iTRAQ-labeling method using the AA45/32™ Kit in combination with LC-MS/MS. Spectra were processed using the Analyst® 1.5 Software. Carbon-one metabolites such as betaine, dimethylglycine (DMG) and choline were analysed by LC-MS/MS, using d<sub>11</sub>-betaine as internal standard. Se deficient liver was characterized by significantly elevated levels of hypotaurine, taurine, homocysteine (Hyc) and glutamic acid, while methionine levels were not altered. In addition, betaine/choline and DMG/choline ratios tended to be down-regulated in selenium deficiency, while betaine/DMG ratios were unaffected. Expression analysis of enzymes involved in Hyc metabolism showed a marked decrease in cystathione-β-lyase levels under Se deficient conditions. In summary, a low Se status can be linked to higher Hyc levels in the liver which appears to be caused by an impaired transsulfuration pathway indicated by lower CBS expression. Whether the observed oxidative conditions in Se deficiency are crucial for the metabolic changes needs to be further investigated.

O9B-1

## DEVELOPMENT OF A GENERAL METHOD FOR THE HPLC/MS-BASED ANALYSIS OF COENZYME A DERIVATIVES AND COFACTORS FROM CELL EXTRACTS

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Every nutrient that enters a cell is converted to at least one intermediate bound to CoA during metabolism. CoA intermediates also play a crucial role in the degradation of more complex substrates. Unfortunately, due to their size, polarity and diversity, they cannot be measured properly with the common analytical methods like gas chromatography (GC), classical reversed phase liquid chromatography (C18-RP-HPLC) or hydrophilic interaction liquid chromatography (HILIC).

The here described method applies RP-HPLC on a pentafluorophenyl column to exploit additional selectivity while circumventing the use of ion pairing reagents. Detection on a high resolution TOF mass spectrometer enables MS<sup>2</sup> and accurate mass measurement. The optimized solid phase extraction (SPE) sample preparation protocol is facile and universally applicable.

More than 16 different CoA derivatives could be detected in a single analysis of several model organisms, covering intermediates from whole catabolic pathways (6-8 subsequent CoAs). Additionally several CoA derivatives, for which no pure standards were available, were identified using MS<sup>2</sup>, isotopic patterns and sum formula calculation from accurate masses. The identification procedure will be verified using new CoA standards when available. As a bonus, the method also allows parallel detection of several important metabolic cofactors like adenosine phosphates and nicotinamide derivatives which are important for the cell's energy metabolism.

Our method was successfully applied to Gram negative and Gram positive bacteria, as well as to archaea and yeast, including for example pathogen *P. aeruginosa* as well as the industrially utilized bacterium *C. glutamicum*.

All in all, our method shows a great potential to be applied in concert with GC-MS in metabolome analysis and related research to provide valuable information towards full scale metabolomics. More application oriented goals could lie in the investigation of biodegradation pathways.



**DISSECTION OF THE CARBON AND ENERGY METABOLISM OF APICOMPLEXAN PARASITES IMPORTANT FOR VIRULENCE USING <sup>13</sup>C-STABLE ISOTOPE RESOLVED METABOLOMICS**

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The Apicomplexa protozoan parasites, *Plasmodium falciparum* and *Toxoplasma gondii*, are responsible for the most severe form of malaria and the potentially devastating disease, toxoplasmosis, respectively. While *P. falciparum* proliferates within a limited number of cell types in the mammalian host (red blood cells, hepatocytes), *T. gondii* can invade and grow within any nucleated cell. These parasites lack a mitochondrial isoform of pyruvate dehydrogenase (PDH) and were thought to have a highly streamlined carbon metabolism in which most of their cellular ATP was generated from aerobic glycolysis of host glucose. We have recently reassessed the carbon metabolism of the major intracellular stages of *P. falciparum* and *T. gondii* using <sup>13</sup>C-stable isotope resolved metabolomics. Unexpectedly, we find that all developmental stages of these parasites exhibit an active mitochondrial metabolism in which both host glucose and glutamine are variably catabolized in a canonical TCA cycle, indicating the presence of an alternative mitochondrial dehydrogenase that substitutes for PDH. Both parasites also have a partial (*P. falciparum*) or complete (*T. gondii*)  $\alpha$ -aminobutyric acid (GABA) shunt that plays a key role in regulating glutamine flux into the TCA cycle. Following genetic and chemical inhibition of these pathways, we find that a functional TCA cycle is essential for intracellular growth and survival of *T. gondii* parasites, while inhibition of the GABA shunt leads to attenuated virulence and a severe motility defect. Interestingly, gluconeogenesis was not detected in intracellular *T. gondii* stages, but the gluconeogenic enzyme, fructose-1,6-bisphosphatase, is essential for growth, possibly reflecting a role for this enzyme in regulating glycolytic flux. In contrast to *T. gondii*, the TCA cycle is not essential for intracellular growth of asexual red blood cells stages of *P. falciparum* which primarily utilize the TCA cycle to catabolize glutamine. However, the differentiation of these stages to transmission competent gametocytes is associated with a marked change in TCA cycle fluxes and mature gametocyte stages become sensitive to TCA cycle inhibitors. These studies highlight unanticipated complexity and stage-specific changes in the metabolism of these parasites that may underlie parasite-specific differences in host cell range.

O9B-3

**PLASMODIUM FALCIPARUM PHOSPHOENOLPYRUVATE CARBOXYLASE IDENTIFIED AS A KEY ENZYME IN REDOX MAINTENANCE AND ENERGY GENERATION**

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Phosphoenolpyruvate carboxylase (PEPC), catalyses the carboxylation of phosphoenolpyruvate to oxaloacetate. The enzyme is absent from humans but encoded in the *Plasmodium falciparum* genome, suggesting that PEPC has a parasite-specific function. To investigate the role of the enzyme and its importance in erythrocytic stages of the parasite, we generated a mutant lacking the *pepc* gene (D10<sup>Δpepc</sup>). This was only possible when the growth medium was supplemented with malate, a reduction product of oxaloacetate. Withdrawing malate from D10<sup>Δpepc</sup> cultures resulted in severely restricted parasite growth, suggesting that *pepc* is effectively an essential gene. This growth defect was reversed by addition of either malate or fumarate, but not by citrate, glutamine, succinate, aspartate or glycerol. To analyse the contribution of PEPC to intermediary metabolism, a targeted metabolomics analysis using high resolution liquid chromatography-mass spectrometry and stable isotopes of glucose, glutamine and bicarbonate was conducted. This revealed that the major conversion of glycolytically-derived phosphoenolpyruvate into malate (via oxaloacetate), fumarate and aspartate was abolished in D10<sup>Δpepc</sup> with concomitant increased generation of pentose phosphate pathway metabolites and glycerol-3-phosphate. Metabolism of glutamine and pyrimidine biosynthesis appeared largely unchanged. The mutant parasites were also more sensitive to the aminotransferase inhibitor L-cycloserine, which was reversed with malate. The data overall confirm the activity of PEPC in *P. falciparum* and suggest that it catalyses a key step in carbon metabolism providing metabolites for pathways involved in redox maintenance and energy generation. They also provide some general insights into the pathways of intermediary carbon and amino acid metabolism operating in erythrocytic stages of *P. falciparum*.

## ABSOLUTE QUANTIFICATION OF METABOLITES FOR FLUX ANALYSIS IN TRYPANOSOMES USING ORBITRAP-BASED LC-MS

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Human African Trypanosomiasis (HAT) is a parasitic disease in sub-Saharan Africa caused by the protozoan parasite *Trypanosoma brucei* (*T. brucei*) and transmitted by tsetse flies. It is fatal if untreated, so effective drug treatment is necessary. In the face of emerging resistance and unacceptable side effects of existing treatments, new drugs are urgently required and the discovery of new targets for chemotherapy is of great importance.

The metabolism of this extracellular parasite has some unique features when compared to other eukaryotes: for example, for its energy supply the parasite in the human bloodstream relies exclusively on glycolysis, which is partly compartmentalised in specific organelles. A computational model of glycolysis in *T. brucei* has previously been demonstrated of use in understanding the metabolism of this system, with the aim of developing optimised anti-parasite drugs. However, a full understanding of cellular responses requires both qualitative and quantitative information, including absolute metabolite concentrations across the metabolic network beyond glycolysis. In order to obtain absolute concentrations of individual metabolites in trypanosomes, uniformly (U)-<sup>13</sup>C-labelled *E. coli* metabolite extracts were used as an internal standard; absolute intra- and extracellular concentrations of metabolites were then estimated by adding known amounts of U-<sup>13</sup>C-labelled extract to the trypanosome sample prior to the extraction procedure.

Here we present the first global quantitative analysis of metabolite concentrations and metabolic fluxes in trypanosomes. Based on the fate of (U)-<sup>13</sup>C-labelled glucose during a short-term (up to 2 hours) and a long-term (48 hours) we were able to infer alternative metabolic network topology of the glucose-based metabolism in *T. brucei* beyond glycolysis. In a second step, using the LC-MS-based absolute quantification of intra- and extracellular metabolites, we were then able to compute metabolic fluxes throughout the system. This will now allow us to identify the most plausible metabolic network topology and drive the extension of the computational models to a more comprehensive representation of the metabolic capacity of the parasite.

O9B-5

## **METABOLOMICS-GUIDED ADVANCES IN CELL CULTURE AND DRUG DISCOVERY FOR TRYPANOSOMIASIS AND MALARIA**

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New drugs are urgently needed to treat parasitic tropical diseases including malaria and human African trypanosomiasis. Metabolomics technology enables simultaneous detection of many small molecule metabolites in a biological system, and facilitates new approaches for the discovery of novel anti-parasitic drug candidates.

Metabolomic analyses of in vitro cultures of *Trypanosoma brucei* were performed with a HILIC-Orbitrap LC-MS metabolomics platform. The IDEOM software was developed to achieve both targeted quantitative, and untargeted semi-quantitative, data analysis with automated putative identification of metabolites based on a combination of metabolite databases and authentic metabolite standards.

The intra- and extra- cellular *T. brucei* metabolomes provided a detailed understanding of nutritional requirements, and allowed the rational development of a simplified culture medium. 35 components in standard culture medium were found to be present at non-physiological and unnecessarily high concentrations. Removal of these components provided an optimal medium with metabolite levels that more closely mimic the in vivo environment of the parasite. The simplified medium showed significantly increased sensitivity in drug screening assays for trypanocidal compounds that have metabolic modes of action or uptake, as demonstrated by the 400-fold decreased IC<sub>50</sub> for the anti-folate drug methotrexate.

Furthermore, metabolomic analysis of *T. brucei* and *Plasmodium falciparum* following incubation with several antiprotozoal compounds revealed compound-specific metabolic responses to drug treatment. This approach allowed unbiased identification of the mode of action for compounds that inhibit metabolic enzymes and transporters, providing direct evidence for drug target validation in a cell-based system. These findings demonstrate the application of metabolomics to the advancement of discovery programs for new anti-parasitic drug candidates and drug targets.

O9B-6

## **TRICHOMONAS VAGINALIS PRODUCES S-methyl-L-cysteine USING CYSTEINE SYNTHASE**

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*Trichomonas vaginalis* infects the human urogenital tract causing trichomoniasis, an exceptionally common sexually transmitted disease recently identified as a co-factor in the transmission of HIV. The parasite lacks mitochondria and has a fermentative metabolism based on specialised organelles called hydrogenosomes. *Trichomonas vaginalis* does not produce glutathione but contains high levels of cysteine, which is the main intracellular thiol and redox buffer. The PLP-dependent enzyme cysteine synthase produces cysteine by *de novo* synthesis from phosphoserine and hydrogen sulphide.

Using metabolomic profiling with high resolution LC-MS we show that *Trichomonas vaginalis* contains S-methyl-L-cysteine (SMC), a thioether analog of methionine. SMC is found in several leguminous plants and the amitochondriate parasite *Entamoeba histolytica* but has not been previously detected in trichomonads. The identity of SMC was confirmed by fragmentation analysis and by comparison with an SMC standard. The intracellular concentration of SMC was determined using a standard curve and published values for cell volume. Very low levels of SMC were found in uninfected growth medium but this was increased 10-fold in the supernatant obtained after the cells were removed from cultures by centrifugation. The concentration of SMC was reduced 25-fold when the parasite was grown under conditions previously found to down-regulate cysteine synthase activity. Recombinant cysteine synthase was shown to produce SMC from phosphoserine and methanethiol *in vitro* using a coupled enzymic assay. Kinetic parameters of the bi-functional cysteine synthase in the synthesis of SMC and the synthesis cysteine were compared in the same assay. A possible role of SMC in antioxidant defence is discussed.

Overview: LC/MS analysis revealed that *Trichomonas vaginalis* cell extracts contain S-methyl-cysteine (SMC), identified by fragmentation analysis and comparison with a standard. The intracellular concentration of SMC was determined using a standard curve and published values for cell volume. SMC was exported into the medium during growth of the culture and levels were greatly reduced when cells were grown under conditions previously shown to down-regulate cysteine synthase activity. Recombinant cysteine synthase produced SMC from phosphoserine and methanethiol. synthase produced SMC from phosphoserine and methanethiol. previously shown to down-regulate cysteine synthase activity. Recombinant cysteine synthase produced SMC from phosphoserine and methanethiol.

LC/MS analysis revealed that *Trichomonas vaginalis* cell extracts contain S-methyl-cysteine (SMC), identified by fragmentation analysis and comparison with a standard. The intracellular concentration of SMC was determined using a standard curve and published values for cell volume. SMC was exported into the medium during growth of the culture and levels were greatly reduced when cells were grown under conditions previously shown to down-regulate cysteine synthase activity. Recombinant cysteine synthase produced SMC from phosphoserine and methanethiol.

**NEW METABOLITE MARKERS IMPLICATING ADAPTIONS OF THE HUMAN HOST TO MYCOBACTERIUM TUBERCULOSIS AND VISA VERSA**

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To date, the majority of the tuberculosis (TB) research has been done using genomics and proteomics, and limited to investigating various aspects of the microbe only. Until only recently, due to metabolomics, the interaction of the host and bacteria, and the adaptations that each makes in response to each other, can be investigated. In this study, a GCxGC-TOFMS metabolomics research approach was used to identify new metabolite markers from TB infected patient sputum, to better characterize TB and the response of the host to the disease and visa versa. Due to the large variation in the individual patient sputum metabolite profiles detected, an innovative approach, using various multivariate and univariate statistical methods was used to identify those metabolites best describing the differentiation seen between the TB-positive and TB-negative patient sputum sample groups. These metabolite markers were subsequently classified as: 1) those associated with the *M. tuberculosis* cell wall; 2) *M. tuberculosis* markers related to altered *in vivo* growth in response to the host and; 3) metabolite markers suggesting alterations to the human host metabolome in response to the active TB disease state. The interpretation of these new metabolite markers led to a number of hypotheses, including: 1) support of the previously proposed citramalate cycle in *M. tuberculosis*; 2) the interaction of this cycle with an up-regulated glyoxylate cycle during pulmonary *M. tuberculosis* infection; 3) the increased utilisation of fatty acids and glutamate as alternative carbon sources in *M. tuberculosis* during pulmonary infection; 4) an alternative mechanism by which the host produces hydrogen peroxide via glucose oxidation, in order to eliminate the bacterial infection; 5) inhibition of the host respiratory chain due to pronounced oxidative stress during an active TB disease state, resulting in increased concentrations of various neurotransmitters and other metabolites previously associated with an inborn error of metabolism (MADD / GA type II); and 6) elevated concentrations of neurotransmitters associated with a number of previously described symptoms of TB.

**METABOLIC RESPONSE OF *CANDIDA ALBICANS* TO PHENYLETHYL ALCOHOL UNDER HYPHAE-INDUCING CONDITIONS AND ITS ROLE ON MORPHOGENESIS**

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Phenylethyl alcohol was one of the first quorum sensing molecules (QSMs) identified in *C. albicans*. This extracellular signalling molecule inhibits the hyphal formation of *C. albicans* at high cell density. Little is known, however, about the underlying mechanisms by which this QSM regulates the morphological switches of *C. albicans*. Therefore, we have applied metabolomics and isotope labelling experiments to investigate the metabolic changes that occur in *C. albicans* in response to phenylethyl alcohol under defined hyphae-inducing conditions. Our results showed a global upregulation of central carbon metabolism when hyphal development was suppressed by phenylethyl alcohol. By comparing the metabolic changes in response to phenylethyl alcohol to our previous metabolomic studies, we were able to short-list 7 metabolic pathways from central carbon metabolism that appear to be associated with *C. albicans* morphogenesis. Furthermore, isotope-labelling data showed that phenylethyl alcohol is indeed taken up and catabolised by yeast cells. Isotope-labelled carbon atoms were found in the majority of amino acids as well as in lactate and glyoxylate. However, isotope-labelled carbon atoms from phenylethyl alcohol accumulated mainly in the pyridine ring of NAD<sup>+</sup>/NADH and NADP<sup>-</sup>/NADPH molecules, showing that these nucleotides were the main products of phenylethyl alcohol catabolism. Interestingly, two metabolic pathways where these nucleotides play an important role, nitrogen metabolism and nicotinate/nicotinamide metabolism, were also short-listed through our previous metabolomics works as metabolic pathways likely to be closely associated with *C. albicans* morphogenesis. Further studies we are carrying out involve the use of knockout mutants of *C. albicans* with glutamate dehydrogenase (GDH2 or GDH3) deleted, which we expect to affect the redox balance of the fungal cells. So far we have found that the mutants exhibited altered morphogenetic abilities when cells were cultured in arginine and proline as the sole carbon and nitrogen sources, but not when grown under other hyphae-inducing conditions. This indicates that certain amino acids such as arginine and proline induce morphogenesis in *C. albicans* through nitrogen metabolism, most likely altering the redox balance of the cell. However, more studies are being carried out in order to fully characterise the effect of GDH2 and GDH3 deletions on *C. albicans* metabolism.

# POSTER ABSTRACTS

P1-1

## **METABOLOMICS UNVEILS THE MOLECULAR PHENOTYPE OF “OMEGA-3” TRANSGENIC MICE**

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Essential fats, such as omega-3 and omega-6 fatty acids, must be obtained through the diet and cannot be synthesized de novo in mammals. In 2004, the fat-1 transgenic mouse model was developed, enabling the mouse to endogenously convert omega-6 to omega-3 fatty acids. Research has demonstrated that the fat-1 mouse is protected against a wide variety of diseases and conditions related to inflammation including colitis, pancreatitis, asthma, hepatitis, liver disease, atherosclerosis, insulin resistance, and several types of cancer (breast, colon, pancreatic, liver).

In this study, we used a state-of-the-art, high-throughput assays for the analysis of metabolites in tissue samples from fat-1 and wild-type mice, providing new clues to the pathways and mechanisms that may be involved in the health benefits associated with alterations of the omega-6/omega-3 fatty acids ratio.



## THE PSEUDOXANTHOMA ELASTICUM MOUSE MODEL: UNTARGETED METABOLOMICS TO SEARCH FOR THE *Abcc6* SUBSTRATE

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Pseudoxanthoma elasticum (PXE), caused by mutations in the gene encoding the ATP-binding cassette (ABC) transport protein ABCC6, is a serious genetic disorder with ectopic tissue mineralization affecting skin, eye and the cardiovascular system. Interestingly, ABCC6 protein is virtually absent in affected organs, whereas a high expression is seen in hepatocytes. It is expected that PXE is a metabolic liver disease where circulatory changes affect the peripheral mineralization process, and it has been proposed that the disease is caused by impaired ABCC6-mediated export of an anti-mineralization compound from the liver. Alternatively, the ectopic mineralization in PXE could be due to hepatic accumulation of ABCC6 substrate(s) causing altered secretion of known anti-mineralization factors. At present, the identity of substances transported by ABCC6 remains unknown. However, this search has high priority because it may shed light on the PXE pathomechanism.

The present study employs *Abcc6* knockout mice and an untargeted metabolomics-based screening approach to identify *Abcc6* substrates. The *Abcc6* knockout mouse lacks functional *Abcc6* protein and develops a PXE-like mineralization phenotype. It therefore represents a useful animal model to study PXE. Due to its non-functional *Abcc6* protein, the *Abcc6* knockout mouse is expected to accumulate *Abcc6* substrates in the liver. Accordingly, liver tissue extracts from *Abcc6* knockout mice were analyzed and compared to extracts from wildtype mice that harbor functional transporter protein. Samples were analyzed by ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-qTOF-MS), and data were processed with the XCMS software package. Data evaluation by principal component analysis (PCA) demonstrated that the hepatic metabolic profile of knockout and wildtype mice did indeed differ. We are currently in the process of identifying the dysregulated metabolites accounting for the observed difference.

Our preliminary data justify untargeted metabolomics as a tool to characterize the PXE mouse model. Identification of *Abcc6* substrate(s) can be a major breakthrough in the understanding of PXE pathogenesis and may ultimately facilitate the development of a new therapy for PXE patients.

**METABOLITE PROFILING OF *MYCOBACTERIUM SPP.*: BIOCHEMICAL DIVERSITY AND ADAPTION**

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*Mycobacterium tuberculosis* (Mtb) is the causative agent of tuberculosis. Studied intensively since its discovery in 1882 by Robert Koch, its genome sequence revealed considerable metabolic plasticity. However, its metabolism is still poorly understood. Over the last few decades the increasing occurrence of drug-resistant Mtb strains has resulted in renewed interest in Mtb and related *Mycobacterium* species. In order to develop modern “omic” approaches for the study of Mtb, *M. smegmatis*, a fast-growing *Mycobacterium*, and *Bacillus Calmette-Guérin* (BCG), the tuberculosis vaccine, are employed as model organisms.

Previously published research of Mtb has mainly focused on genomic, transcriptomic and proteomic approaches. In recent years the lack of knowledge of metabolic networks underlying phenotypes in *Mycobacterium* has led to a need for metabolite profiling platforms in these organisms. For instance, variations in central carbon metabolism have been revealed as virulence determinants. Therefore the development of a metabolomic platform for *Mycobacterium* is invaluable for future multi-level “omics” characterisation.

The metabolite profiling approaches developed include quenching and extraction procedures for GC-MS analysis. The most effective treatments were an isotonic methanol quenching followed by a methanol/ chloroform extraction including saponification. Analysis of derivatised extracts by GC-MS have identified around 250 metabolites in one chromatographic separation of which around 160 have been confirmed by authentic standards. The protocol has been applied to monitor metabolic changes of both model organisms during three different growth stages.

P1-4

**METABOLOMIC SCREENING FOR IDENTIFYING THE OXIDATIVE STRESS VULNERABLE ENZYMES THAT INVOLVE IN CELLULAR METABOLISM**

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Oxidative dysfunction in the metabolism has long been implicated in diverse biological disorders, including aging. There is a substantial number of metabolic enzymes whose activities are sensitively regulated by oxidative stress. However, identifying the targets of these enzymes remains difficult due to a lack of comprehensive observations of the metabolism acting through the stress response. We herein developed a non-targeted metabolomics strategy using integrative liquid chromatography-mass spectrometry (LC-MS) and observing rapid metabolome changes in response to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced oxidative stress in human cells. Unexpectedly, one pair of the most characteristic metabolites uniquely indicated carnitine palmitoyltransferase 1 (CPT1), the critical enzyme for mitochondrial beta-oxidation of long-chain fatty acids, to be a target for oxidative inactivation. Subsequent analyses using isolated mitochondria demonstrated the enzymatic activity of CPT1 to significantly decline, although reversibly, by H<sub>2</sub>O<sub>2</sub>, as well as other reagents that generate reactive oxygen species (ROS) or the intermediates. Thus, our results provide important insights into the mechanisms present in human cells for oxidative dysfunction in mitochondrial metabolism via the regulation of CPT1 activity.

**UNTARGETED AND TARGETED APPROACHES FOR METABOLOMICS BASED ON ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY**

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Metabolomics, as one of the key components for systems biology, primarily concerns small molecular metabolites. The two common approaches are untargeted and targeted metabolomics. The former was aimed to gain global metabolite profiling of complex samples, such as biological fluids or tissue extracts. Ultra high performance liquid chromatography–mass spectrometry (UHPLC-MS) with high resolution and rich information was a pillar for untargeted metabolomics. As for targeted metabolomics, multiple reaction monitoring (MRM) MS/MS with more specificity and sensitivity was typically applied for specific compounds. Systematic and accuracy analysis could be achieved via the combination of untargeted and targeted metabolomics approach. Herein, an untargeted approach based on UHPLC-Q-TOF MS and a targeted approach for free amino acid after 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivation based on the dynamic MRM MS/MS were developed and validated. Sample pretreatment and chromatography conditions were optimized by total responses and number of the metabolites in untargeted metabolomics approach. While, fragmentor voltage and collision energy were investigated for targeted free amino acids detected. The both methods were validated prior to the analysis of samples including intra- and inter-day precision, method repeatability and post-preparative stability for untargeted metabolomics approach and intra- and inter-day precision, method repeatability and linear range for targeted metabolomics approach. Stable relative standard deviation of overwhelming majority metabolites were achieved in QC samples. The methods have been successfully used to haemolymph analysis of silkworm, a typical complete metamorphosis insect of Lepidoptera which is well-known for its industrial importance in sericulture. About 70 metabolites, covering diacylglycerol, phosphatidylcholine, phosphatidyl ethanolamine, sphingomyelin, lysophosphatidyl choline, lysophosphatidyl ethanolamine, were identified in global metabolic profiling. And 34 kinds of free amino acid were detected accurately via the dynamic MRM. This method has a major significance for distinguishing different varieties of silkworm and uncovering further biological mechanism.

## COMBINED NMR-BASED METABOLOMIC ANALYSES IN TROUT NUTRITION STUDIES

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In a context of limited marine resources and continuous development of world aquaculture, developing aquafeeds with a reduced content of fish-meal and fish-oil is a real challenge. Alternative protein and lipid sources must meet the fish nutrition features, namely high protein requirements for growth, preferential use of amino acids for energy production, low efficiency of carbohydrate utilization, particularities in lipid metabolism (biosynthesis and retention of polyunsaturated fatty acids). Rainbow trout was used as a fish model to assess the metabolic effects of the changes in dietary ingredients. Fish were fed, from the first feeding stage for one year, using two experimental diets: a classical one made of marine-resources and a new one made of plant products. NMR-based metabolomic profiling of aquafeed polar extracts were analysed to characterize each diet. Their effect on rainbow trout metabolism was assessed through plasma analysis. Plasma <sup>1</sup>H-NMR spectra analysed by principal components analysis (PCA) revealed that rainbow trouts fed on the new diet were metabolically different from rainbow trouts fed on the classical diet. These preliminary results show that NMR-based metabolomic analysis is a useful integrative tool in fish nutrition studies providing information for the development of new feeding strategies in aquaculture. They will be complemented with <sup>1</sup>H-NMR profiling of liver and muscle.

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P1-7

**METABOTYPING OF THE C. ELEGANS *SIR-2.1* MUTANT USING IN VIVO LABELING AND <sup>13</sup>C-HETERONUCLEAR MULTIDIMENSIONAL NMR METABOLOMICS**

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The roles of *sir-2.1* in *C. elegans* lifespan extension have been subjects of recent public and academic debates. We applied an efficient workflow for in vivo <sup>13</sup>C-labeling of *C. elegans* and <sup>13</sup>C-heteronuclear NMR metabolomics to characterizing the metabolic phenotypes of the *sir-2.1* mutant. Our method delivered two orders of magnitude higher sensitivity than unlabeled approach, enabling 2D and 3D NMR experiments. Multivariate analysis of the NMR data showed distinct metabolic profiles of the mutant, represented by the increases in glycolysis, nitrogen catabolism and initial lipolysis. The metabolomic analysis defined the *sir-2.1* mutant metabotype as the decoupling between enhanced catabolic pathways and ATP generation. We also suggested the relationship between the metabotypes, especially the branched chain amino acids, and the roles of *sir-2.1* in the worm lifespan. Our results should contribute to solidifying the roles of *sir-2.1*, and the described workflow can be applied to studying many other proteins in metabolic perspectives.

**COMPREHENSIVE METABOLOMIC ANALYSIS REVEALS MAJOR DIFFERENCES IN THE MACROPHAGE INFLAMMATORY RESPONSE BETWEEN YOUNG AND AGED MICE**

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Inflammation is an evolutionarily conserved response to foreign pathogenic infections and tissue injury. Bacterial lipopolysaccharide (LPS) is a potent inducer of inflammation. After repeated or prolonged exposure to LPS, macrophages enter a transient hyporesponsive state termed "LPS tolerance". Aging is often reflected by an impaired immune response that leads to elevated susceptibility to infection and systemic inflammation. Genomic studies have shown that peritoneal macrophages obtained from young mice were able to develop LPS tolerance much more efficiently than aged mice when repeatedly exposed to LPS. Ceramides, phosphatidylinositols and other lipid mediators have been linked to regulate immune responses in macrophage. Our study is the first comprehensive metabolic analysis of the cellular response of murine macrophages to LPS induction and LPS tolerance, and that examines how macrophage responses to these stimuli change with age.

We examined the metabolic profiles of mouse bone marrow-derived macrophages using HILIC-LC-TOF-MS. Bone marrow progenitors from old (18-22 months) and young (6-8 months) mice were harvested and allowed to differentiate for 7 days. The differentiated macrophages were treated with 100 ng/mL LPS for 16 hours, following 100 ng/mL LPS for 4 hours with 2 hours rest in between. Macrophages were collected at 16 and 22 hours for the metabolomic analysis. Approximately  $3 \times 10^5$  cells were lysed and extracted using MeOH/EtOH/H<sub>2</sub>O (2:2:1) then centrifuged to afford an extract that allowed simultaneous analysis of both polar and lipid metabolites using HILIC-LC-TOF-MS. Metabolite features were extracted using XCMS and CAMERA, then analyzed using multivariate analyses such as OPLS-DA, HCA and Student's t test. The metabolomic profiles resulted from LPS tolerance can be distinguished from untreated macrophages as well as macrophages that were treated with LPS only once without experiencing LPS tolerance. The macrophages from older mice were hyposensitive to LPS and LPS tolerance compared to those from younger mice. Changes in levels of pantothenic acid (vitamin B<sub>5</sub>) and a number of phospholipids were found during LPS induced and LPS tolerance study; the behaviour of these metabolites was also found to be age-associated.

**METABOLOMIC PROFILING OF MALE AND FEMALE DROSOPHILA MELANOGASTER BY DIFFERENT CHROMATOGRAPHIC METHODS IN COMBINATION WITH HIGH RESOLUTION MS**

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The genes that are present in the single male X chromosome have to be compensated so that these genes can attain similar levels of expression to those present in females who have two X chromosomes. Thus it was of interest to see how one versus two X chromosomes could affect global metabolite expression. Metabolic profiling was carried out on male and female *Drosophila melanogaster* using three different column selectivities coupled to Orbitrap Exactive detection. Quite a number of differences were present. An important gene which is present on the X chromosome is the White (*w*) gene. This gene codes for an ABCG transporter gene which among other things is responsible for transport of cGMP and tryptophan into tissues and this impacts on the synthesis of pterin and xanthomatin eye pigments respectively and flies where this gene is deficient lack these pigments. Thus if the single X chromosome in males is undercompensated for then pigment levels would be lower in males. All the pterin pigments were significantly higher in males and, although formylkynurenine and kynurenine were lower in males, the levels of xanthomatin pigments did not differ between males and females. Thus the single X chromosome in males appears to be overcompensated for with respect to the *w* gene. One mechanism for compensation is gene silencing via epigenetic modifications such as methylation. The methylated nucleobases methylcytosine, methyladenosine, methylguanidine, methylxanthine, dimethylguanidine and the methylated RNA nucleosides methyladenosine and methylguanosine were all elevated in males by about x2 suggesting that methylation may have some regulatory role with regard to gene compensation in males although possibly mediated via RNA rather than DNA. The methylating metabolite S-adenosylmethionine (SAM) was at a similar level in males and females but in males there was a previously undescribed metabolite which is decarboxy SAM which was about 20 times higher in males and present at about twice the levels of SAM. In addition, the reversed phase chromatographic method revealed a series of juvenile hormones which were higher in females than males, these hormones are important in both male and female flies for promoting the maturation of ovaries and male accessory gland.



P1-10

**PROFILING MULTIPLE AUXOTROPHIC YEAST STRAINS BY UNTARGETED METABOLOMICS WORKFLOW BY HIGH RESOLUTION LC-MS/MS**

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We have developed an untargeted metabolomics approach for investigating a series of auxotrophic yeast strains. Previous analysis of the auxotrophic mutant used in most chemical genomics studies showed perturbations in the degradation pathway of tryptophan in comparison to its prototrophic counterpart. In order to better study the involvement of the auxotrophic mutations, a series of new mutants were analysed to pinpoint which implicated the disruption of tryptophan metabolism. Our approach is untargeted in order to not only monitor tryptophan metabolites but identify other metabolites that are modulated in the series of eight mutants investigated in this work.

Eight yeast strains were cultured under similar conditions. Triplicate aliquots were sampled from three separate cultures of each strain (n=9 samples per group). Metabolites were extracted with 50% methanol using ultrasonication with a mix of isotope-labeled internal standards was added to yeast pellets prior to extraction for data normalization. Extracts were separated by two complementary chromatographic methods, using reverse-phase and aqueous normal phase columns. Analysis was performed on a high resolution high speed 5600 TripleTOF™ (AB Sciex) using IDA-MS/MS with dynamic background subtraction in both positive and negative ion modes. Data were processed using MarkerView™ software for statistical analysis. Metabolites of interest were tentatively identified using ChemSpider and Metlin using accurate mass measurements in MS and MS/MS mode. From this data, it should be possible to pinpoint which mutation is causing the disruption of tryptophan degradation and also shed light on why these seemingly unrelated pathways are intertwined in yeast metabolism.

P1-11

**OXIDATIVE STRESS-INDUCED ADAPTIVE CHANGES IN METABOLISM IN G6PD-DEFICIENT ERYTHROCYTES**

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G6PD deficiency is associated with increased susceptibility of erythrocytes to reactive oxygen species, which can cause hemolytic anemia. In this study, we studied the changes in metabolic responses of erythrocytes to oxidative stress. Diamide induced disturbances in glutathione homeostasis, nucleotide pool, and accompanied AMPK activation in G6PD-deficient erythrocytes. These findings suggest that anomalous redox homeostasis in G6PD-deficient erythrocytes after diamide treatment, and AMPK is activated as an attempt of cells to compensate for energy deficit.

## P1-12

### **METABOLITE ALTERATIONS IN ENU MUTAGENESIS DERIVED *ASGR1*<sup>MHDABAP005</sup> MICE AS A MODEL FOR HUMAN IDEOPATHIC HYPERPHOSPHATASEMIA**

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The Metabolomic Platform of the Genome Analysis Center at HMGU is designed to mediate progress in science through development of new metabolomic methods and provision of analytical measurements applicable to man, animal models, plants, environmental samples, and *ex vivo* systems. We already established several LC-MS/MS based targeted and non-targeted metabolomics methods.

We describe the quantification of 163 or 186 metabolites (lipids, amino acids, acylcarnitines, carbohydrates) out of 10 µL plasma using the Biocrates Absolute<sup>™</sup>IQ kits p150 and p180, respectively. The measurements perform very well with high reproducibility and have been applied to studies in human cohorts and in animal models.

Here we present the metabolomic measurement results of the phenotypic characterization of a C3HeB/FeJ mouse line that was generated within a genome-wide *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen. The isolated mouse line revealed significantly elevated levels of alkaline phosphatase activity carrying a dominant biallelic missense mutation in the *Asgr1* (*asialoglycoprotein receptor 1*) gene (c.815A>G, p.Tyr272Cys). At 36 weeks of age mutant male mice showed significantly decreased glucose levels in plasma. By targeted metabolomic analysis we could demonstrate that lipid-transport was not affected in homozygous *Asgr1*<sup>Mhdabap005</sup> mice, but interestingly glycerophospholipids were decreased in plasma. In addition, several amino acids were significantly increased and some acylcarnitines decreased in these animals, demonstrating the potential of targeted metabolomics to specify the physiological consequences of loss-of-function mutations in the mouse.

**FILLING IN THE 'GAPS' OF *DROSOPHILA MELANOGASTER* METABOLIC MAP USING METABOLOMICS APPROACHES**

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A computer-annotated metabolic map of *Drosophila* has been predicted by Kyoto Encyclopedia of Genes and Genomes (KEGG). However, 'gaps' still remain with no identified *Drosophila* orthologues for metabolic enzymes. We previously generated a computational model of core metabolism in the fruit fly, which identified 145 'gaps' in the metabolic map. One of these gaps is *Drosophila* gene *CG30016*. Based on sequence homology it is hypothesised to be a homologue of 5-hydroxyisourate hydrolase (5-HIUH) involved in purine metabolism. Enzyme defects within purine metabolism in humans result in IEMs including hyperuricemia. An animal model of hyperuricemia is required for the identification and screening of new treatments. Here, we propose a method for identifying metabolic 'gaps' using metabolomics approaches. Liquid chromatography-mass spectrometry (LC-MS) was performed for *CG30016* knockout fly strain and a control. This was carried out for fly Malpighian tubules, where *CG30016* is exclusively expressed. The comparison confirmed that *CG30016* is involved in purine metabolism and specifically urate degradation. Interestingly, our results suggested that *CG30016* is either catalysing a reaction downstream from 5-HIUH (OHCU decarboxylation), or is a bifunctional enzyme, playing a role as both 5-HIUH and OHCU decarboxylase. Moreover, an apparent tubule phenotype was observed in *CG30016* knockout line. Flies from this line were observed to have an inflated ureter and evidence for luminal occlusion. This is consistent with a blockade of purine metabolism and might reflect what is observed in hyperuricemia and subsequent kidney damage. Filling in the 'gaps' in *Drosophila* metabolic map is essential for completion of the core metabolome of the fruit fly and its validation as an in vivo model for human metabolism and metabolic disorders. It will also provide a rapid and cheap modelling system of inborn errors of metabolism and kidney damage related to metabolic imbalances.

**METABOLOMICS PROFILING OF MULTIFUNCTIONAL PROTEIN 2 (MFP-2) KNOCKOUT MOUSE**

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Multifunctional protein-2 (MFP-2) plays an important role in peroxisomal beta-oxidation. Deficiency of this enzyme in human causes severe developmental syndrome with multiple abnormalities, particularly in the brain. Most of affected individuals die within the first year of life. Accumulation of branched-long-chain fatty acids and very-long-chain fatty acids and a disturbed synthesis of bile acids were documented in these patients. However, there is no report on metabolite profile nor what is the pathophysiology and etiology of malfunctions in several organs. To understand the effected pathways of this disease, we analyzed metabolomics of MFP-2 knockout mouse (MFP2 KO) which partly phenocopies the human disease. Low survival of the MFP-2 KO mice into adulthood shows the importance of this enzyme for the development of organs. Plasma metabolic concentrations of 4 and 10 weeks old mice were relatively quantified with a sensitive and robust UPLC-MS/MS method. This method applies an Acquity UPLC (Waters) coupled to Linear Ion Trap LTQ XL MS (Thermo Scientific) with a total instrument analysis time of 24 min per sample in positive and negative ion modes. The resulting MS/MS2 data are matched to Metabolon's database library that include retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as their associated MS/MS spectra for all molecules in the library. This study shows for the first time difference metabolomic profile between MFP-2 KO and wild type mice that enable the investigation of metabolic pathway affected by deficiency of MFP-2 enzyme.

**THE EFFECTS OF HYDROGEN PEROXIDE ON CELLULAR METABOLISM OF HEPATOMA CELLS USING UPLC/Q-TOF MS**

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Metabolomics provides a powerful platform for discovering the changes of the cellular metabolites involved in redox homeostasis with H<sub>2</sub>O<sub>2</sub> treatment. In this study, the metabolic profiles of HepG2 cells showed in a dose-dependent change by PCA distribution. The major alterations in the metabolome included glutathione and purine metabolism based on the VIP analysis. Our findings suggest that glutathione acts as a H<sub>2</sub>O<sub>2</sub> scavenger, and purine metabolism provides a compensatory mechanism to protect H<sub>2</sub>O<sub>2</sub>-induced oxidative damage.

**MS-BASED METABOLITE PROFILING REVEALS CIS-UROCANIC ACID AND CHOLESTEROL AS TIME-DEPENDENT SKIN BIOMARKERS IN UVB-RADIATED MICE**

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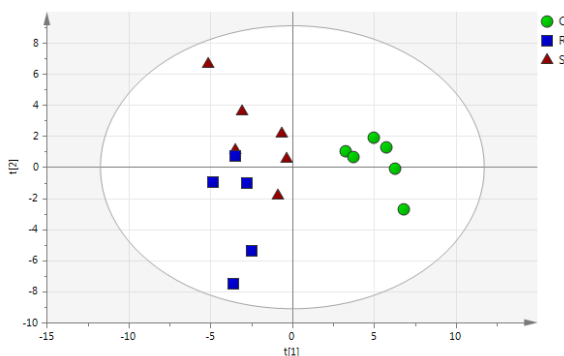
Metabolite profiling related to ultraviolet (UV) radiation in skin has not yet been performed despite many physiological evidence-based molecular mechanism studies on the relation between the skin and UV radiation. Earlier, we observed the clinical and histological changes including the increased TEWL, epidermal thickness and inflammatory cells and the changes of collagen fibre in chronic exposed mice skin to UVB radiation for 12 week. In this study, ultra-performance liquid chromatography (UPLC)-quadrupole time-of-flight (Q-TOF), gas chromatography (GC)-TOF, and Nanomate tandem-mass spectrometry (MS)-based metabolite profiling of the mice skin following UVB irradiation for 6 and 12 weeks revealed amino acids, organic compounds, fatty acids, lipids, nucleosides, carbohydrates, lysophosphatidylcholines (lysoPCs), lysophosphatidylethanolamines (lysoPEs), urocanic acids (UCAs) and ceramides (CERs) as candidate biomarkers of histological changes. Especially, *cis*-UCA and cholesterol showed a dramatic increase and decrease in 6 and 12 wks, respectively. In addition, both of the changes in primary and secondary metabolite by UVB exposure were generally higher in 12 than 6 week. These results from primary, secondary metabolite and CER profiles proposed the prolonged chronic exposure to UVB light may have a great influence on skin by altering more metabolites, and comprehensive MS-based metabolomic approach for determining regulatory metabolites in UV-induced skin will lead to a better understanding relationship between skin and UV.

**NMR-BASED METABOLOMICS TO INVESTIGATE NAPHTHALENE TOXICITY USING THE URINE OF A NAPHTHALENE TOLERANT MODEL**Hao-Jan Liang, [Ching-Yu Lin](#)*National Taiwan University, Taipei, Taiwan*

Bioactivated naphthalene (NA), one of the most common polycyclic aromatic hydrocarbons, exists widely in the environment. Human are exposed to NA easily from workplace or the environment (i.e. cigarette smoking and gasoline combustion). Previous studies demonstrated that NA caused species and site selective acute toxicity. Acute injury of Clara cell, a bronchiolar epithelial cell type, was observed after mice exposed to NA. However, repeated NA exposure resulted in Clara cell tolerant to further injury. In this study, we intend to understand NA toxicity in mice by using the mouse tolerant model. Tolerant mouse model was conducted by seven repeatedly daily injections of NA (200 mg/kg, ip.) following the mice challenged with 300 mg/kg NA. The urine from the repeated dose (tolerant), single dose (injury), and the control mice were collected at 12 different time points; then, analyzed by 600MHz  $^1\text{H-NMR}$  followed by multivariate analysis. PLS-DA model demonstrated a clear separation between urine samples from the tolerant, injury, and the control groups, 24h after challenge dose. The clustering is contributed from both NA metabolites and endogenous metabolites. We conclude that the urine recorded NA metabolism and NA-induced metabolic turbulences which can be monitored and associated with cell injury.

**Figure 1.** The PLS-DA model from the NMR spectra of mouse urine, 24 hr after a challenge dose of naphthalene.  $R^2X=0.521$ ,  $R^2Y=0.637$ ,  $Q^2=0.443$ .

C: Control; R: Repeated dose (tolerant);  
S: Single dose (injury).

**Acknowledgements:**

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**WHEN INFORMATION FROM URINE AND BLOOD ISN'T ENOUGH - SYSTEMIC AND ORGAN SPECIFIC METABOLOMICS OF MTKO MICE**

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An untargeted metabolomics approach was used to study metabolic differences between wildtype (WT) mice and mice lacking expression of metallothioneins 1 + 2 (MT), which are ubiquitously expressed small metal-binding proteins believed to be involved in energy metabolism. Three groups of WT and MT knockout mice were used to study their metabolic differences in unchallenged conditions and after challenged with exercise and a high-fat diet. As the expression and importance of MTs vary between organs, it was decided to screen the metabolome content of certain organs in addition to the commonly preferred exometabolome media (urine and blood). Liver, gastrocnemius, brain, urine and blood samples were prepared and analysed with GC-MS and LC-MS. Two-way ANOVA and ASCA were used to analyse the data while PCA was used merely for visualization of the data in a multi-block manner. Little metabolic variation was found after comparing the metabolite levels of the urine, blood and gastrocnemius samples from WT and MT knockout mice. Only a few metabolites differed markedly between these mice even after the metabolism was challenged. However, moderate to marked differences were found in the liver and brain samples. The latter were particularly different between the mouse strains and more so when the metabolism was challenged. Hence, the importance of metallothioneins in the brain's metabolism is obvious and needs further investigation. This key finding would have been overlooked if only blood or urine were studied since the metabolic variation found in the brain was not evident from the exometabolome.

## **METABONOMICS OF THOROUGHBRED RACEHORSES: A PILOT STUDY**

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Horses are hindgut fermenters and therefore heavily dependent upon colonic bacteria for digestion of their cellulose-rich diets. Some types of intestinal disease in the horse are strongly associated with equine oral stereotypy (EOS), a behavioural phenotype which has interesting similarities with human autism spectrum disorder (ASD). Altered gut microbial populations have been demonstrated in ASD patients compared to controls, and they have phenotypic metabolic differences that suggest altered bacterial metabolism. Metabonomics provides an indirect method of characterising global changes in the microbiota using a combination of high-resolution spectroscopic techniques and mathematical modelling; we propose that these techniques could usefully be applied to the investigation of equine intestinal microbial co-metabolism.

The pilot study reported here was designed to determine the degree of inter- and intra-horse metabolic variation in a homogenous population of healthy Thoroughbred racehorses. Horses ( $n=32$ ) from four race yards were sampled (urine, plasma) weekly for 10 weeks. Bio-fluids were analysed using high resolution <sup>1</sup>H NMR spectroscopy with multivariate statistical analysis of the data. Statistically robust OPLS-DA models indicated a high degree of variability in gut microbial co-metabolites (hippurate, phenylacetyl glycine, *p*-cresyl glucuronide and 3-indoxyl sulphate). Samples were observed to cluster according to yard (husbandry factors) and age. The effects of interruption in training and antibiotic treatment were also explored with this dataset. Metabolic profiles of a small number of horses demonstrating oral stereotypic behaviour revealed gut microbial co-metabolite differences compared to neurotypical horses. Discriminant analysis revealed differences in quinic acid, glutamine and 3-aminoisobutyrate, the latter two metabolites known to be associated with neurological function. This study demonstrates the utility and sensitivity of <sup>1</sup>H NMR metabonomic approaches to investigating equine intestinal microbiota. It provides essential data on normal variation in the equine metabome and suggests differential production of microbial metabolites in horses that demonstrate oral stereotypic behaviour.

## CHARACTERIZING A NOVEL *E. COLI* AMINO ACID N-ACETYLTRANSFERASE USING METABOLOMICS

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A large number of enzymes remain uncharacterized even in *E. coli* K-12 whose genome has been known for over 15 years. Metabolomics can be useful to study such enzymes and particularly to identify their endogenous substrates. Here, we combined classical enzyme assays and metabolite profiling to characterize the activity of YhhY, a putative metal-inducible acetyltransferase. Screening using CoA assays revealed that the enzyme can transfer acetyl groups to the N-terminal of amino acids, preferably methionine, phenylalanine and histidine. The enzyme was found to be inhibited by thiol-reactive agents and preliminary kinetics data shows reaction rates differ among each amino acids.

To confirm the *in vitro* results and rule out the possibility of activity resulting from a contaminating enzyme, we monitored metabolite changes, by capillary electrophoresis-mass spectrometry, in *E. coli* cells that overexpress YhhY. We observed elevated levels of methionine, phenylalanine and histidine in the overexpressing cells, confirming that the amino acid acetylating activity is connected with YhhY. Finally, in order to better understand the physiological activity of YhhY, we are profiling metabolites in *E. coli* cells in which the YhhY gene has been disrupted. Following treatment with cobalt, preliminary data shows broad metabolite changes between wild-type and knock-out strain. The most significant changes were in nucleotide, pentose phosphate, and glutathione pathway intermediates. Overall, our results demonstrate that YhhY is an acetyltransferase displaying activity on several amino acids. While further details are necessary to delineate its exact biological role, its activity appears important during the metabolic response to metal and/or oxidative stress.

P1-21

**A POOLED SAMPLE APPROACH TO DIFFERENTIAL METABOLOMICS USING GCXGC TOFMS - STANDARDIZED METHODS AND A REFERENCE FEATURE FOR BIOMARKER SCREENING IN ETHANOL FED MICE**

John Heim<sup>1</sup>, Lorne Fell<sup>1</sup>, Lorraine Kay<sup>2</sup>, Juergen Wendt<sup>2</sup>, Joe Binkley<sup>1</sup>, Jeffrey Patrick<sup>1</sup>

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Metabolomic studies produce a substantial quantity of information which creates significant challenges for data analysis. This research presents a standardized approach for metabolomic GCxGC-TOFMS analysis consisting of a recommended uniform sample preparation procedure, chromatographic parameters, and mass spectrometric instrumental conditions to generate consistent and reliable results. The method was developed using amino acid and fatty acid standards as well as NIST certified human plasma. Equal aliquots from 5 ethanol fed and 5 normal control mouse liver extracts were pooled and dried prior to derivatization. The dried mouse liver extracts were derivatized using methoximation and silylation with BSTFA plus 1% TMCS. This research utilizes GCxGC-TOFMS analysis of pooled samples and data processing by means of a software driven "Reference" feature to discover significant metabolite variation between healthy normal control and unhealthy populations. The data analysis approach utilized a "Reference" developed from the pooled control sample results. The "Reference" method determines the differences between the results from the normal control and the ethanol fed mouse liver extract sample pools by comparison of user defined tolerance limits for retention time, peak area, and spectral match. Peak table results list whether the reference standard components were a match, not found, unknown, or out of tolerance with the peak area percentage from reference compounds. This study presents a standardized analytical approach from sample preparation to GCxGC-TOFMS analysis with software driven data interpretation "Reference" features that can quickly screen potential metabolite biomarkers contrasting normal control versus unhealthy sample pools.

**CAENORHABDITIS ELEGANS, AN EXCELLENT MODEL ORGANISM FOR METABOLOMICS**

Ramadan Ajredini<sup>1</sup>, Chaevien S. Clendinen<sup>1</sup>, Gregory Stupp<sup>1</sup>, Rafael Freire<sup>2</sup>, Arthur S. Edison<sup>1</sup>

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The nematode *Caenorhabditis elegans* is a free-living animal that lives in soil and composting fruit. It has been an excellent model organism for decades, due to, among other things, its fast development time (fertilized egg to adult in 3.5 days). *C. elegans* and related nematodes release signaling molecules called ascarosides that regulate dauer formation, mating, aggregation, and dispersal. Although *C. elegans* is one of the best-studied animals genetically, less attention has been given to its metabolism. This poster will describe the methods that we have developed to generate high-quality synchronized samples of both the exo- and endo-metabolomes as a function of developmental stage in *C. elegans* and related species. *C. elegans* is safe and easy to grow in the laboratory, using *E. coli* strains OP50, HB101 and MG1655 as its primary food source. Thousand of mutant strains can be obtained from Caenorhabditis Genetic Center (CGC), which are important for genetic and metabolomic studies. The worm develops through several larval stages: L1, L2, L3, L4, young adult and adult with eggs, and the timing of each stage is precisely known. Large quantity of synchronous worms can be grown in liquid culture to any of the developmental stages. We have developed a worm water (WW) protocol to collect worm exometabolome at any developmental stage, and this protocol allows for us to expose the worms to a variety of environmental or physiological conditions. The worms can be separated from bacteria using sucrose flotation, washed with buffer, and then placed in any buffer or water to collect pure exometabolome that is free from bacterial contaminants. The worm pellet can be homogenized and used for studying changes in metabolic pathways within the worms. We have applied our methods to successfully label the worms with stable <sup>13</sup>C isotope. These methods provide the material for IROA and NMR studies that are described in other posters in this meeting.

**P1-23**

**THE APPLICATION OF METABOLOMICS TO INVESTIGATE THE BURDEN OF RECOMBINANT PROTEIN PRODUCTION ON YEAST CELLS**

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Recombinant proteins are important industrial products, with a vast number of existing and potential applications. The synthesis of recombinant proteins requires complex systems that can only be found in living cells. The complexity of these "cell factories" is far beyond that of man-made production systems and far less understood. As a consequence, protein production is unpredictable. The optimisation of host cells in recombinant protein production is often performed using a trial and error approach. To improve the predictability of protein production it is necessary to understand the effect of heterologous protein production on the host cell metabolism (the metabolic burden).

In this project we are applying a systems biology approach integrating experimental and mathematical approaches to predict protein production and increase product yields. *Saccharomyces cerevisiae* was used as the host cell for production of heterologous protein. The microorganism was cultured at the maximum growth rate in permissive cultures. Control experiments were performed without the expression of recombinant protein. Mass spectrometry based analysis of the intra- and extra-cellular metabolome was performed. Metabolic profiles were compared between the experimental conditions to understand the burden of heterologous protein production on the host cell. This will facilitate the development of alternative feeding strategies to improve protein yields and improve the predictability of protein production.

**POTENTIAL BIOMARKERS OF DISEASE IN THE SERUM AND URINE OF MICE INFECTED WITH SCRAPIE AT PRECLINICAL STAGE**

Seyed Ali Goldansaz<sup>1</sup>, Dagnachew Hailamariam<sup>1</sup>, Trent Bjorndahl<sup>2</sup>, Rupasri Mandal<sup>2</sup>, David Wishart<sup>2</sup>, Burim Ametaj<sup>1</sup>

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The objective of this study was to search for potential metabolites in the serum and urine of prion infected mice. Two groups of 15 FVB/N wild type strain female mice each were inoculated subcutaneously (sc) either with RML (Rocky Mountain Lab scrapie) or RML+ lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4. A one time injection of RML ( $10^7$  ID 50 units) was given to the animals with LPS (0.1  $\mu\text{g/g}$  of body weight) being infused sc for 6wk using ALZET® osmotic mini pumps. Uninfected control mice (n=15) were infused for 6wk with saline only. Five animals from each group were euthanized at 11wk post inoculation (pi) with no clinical signs of disease, and the rest were left to develop clinical signs of prion disease. All remaining mice treated with RML+LPS and 80% of the RML-treated mice developed clinical signs of disease at  $203\pm 24$  and  $213\pm 12$  days pi, respectively, while all controls remained healthy. Serum samples were taken prior to euthanasia from the heart at two time points, 11wk pi and at the terminal stage with developed clinical signs (D). Urine samples were collected on 11wk, two months before showing clinical signs (D-2), D-1, and D. Due to limiting sample size, equal amounts of sample from different mice at each time point were pooled. Nuclear Magnetic Resonance (NMR) spectroscopy was conducted and the spectra were analyzed using Chenomx NMR Suite 7.1. Comparisons were made in a longitudinal manner using the different time points to study the metabolite trend over time from 11wk to D, versus the controls with  $P < 0.05$  as cut off value. Results from serum samples showed similarity between the altered metabolites of the two treatment groups with the exception of carnithine and malonate, and acetate and glutamate that uniquely increased in the RML and RML+LPS groups, respectively. Serum glucose, isobutyrate, isoleucine, sn-glycero-3-phosphocholine, and 3-methylhistidine in the RML group, and citrate, glycine, hypoxanthine, and leucine in the RML+LPS group were also uniquely decreased in concentration only in their corresponding groups. Furthermore, serum creatine increased in the RML+LPS group and decreased in the RML-treated mice. Urine analyses showed increased acetoacetate and 3-methyl-2-oxovalerate in the RML and RML+LPS groups, respectively. Moreover, 3-indoxylsulfate, arginine, carnithine, dimethylamine, ethanol, and lactate in the RML group, and betaine, o-acetylcarnithine, and scyllitol in the RML+LPS treatment decreased in time. All metabolites listed above were significantly different from the controls in one or more time points. In conclusion, several changes were observed in the serum and urine metabolites of mice over the period of 11wk pi to development of clinical signs of disease.

P1-25

## HYALURONIC ACID-DEPENDENT PROTECTION AGAINST UVB-DAMAGED HUMAN CORNEAL CELLS

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UVB is the most energetic and DNA-damaging of the Ultraviolet (UV) irradiation that reaches the Earth surface. Many previous reports suggested that exposure to the UVB irradiation causes corneal pathologies due to the direct damage on DNA molecules and the generation of reactive oxygen species (ROS) which interfere downstream signaling cascades via post-transcription modifications of cysteine-residues in target proteins resulting in losing their biological functions. However, the detailed molecular and biochemical mechanisms that lead to corneal pathologies by UVB are yet to be clarified. Hyaluronan, a high-molecular-weight glycosaminoglycan, serves as an important extracellular matrix component and is known to promote corneal epithelial wound healing. The aim of this study is to *in vitro* investigate if hyaluronan is able to decrease UVB-induced toxicity and promote cell repair system. Accordingly, human corneal epithelial cell lines were irradiated with UVB and treated with two different molecular weights hyaluronan (hyaluronan 100 kDa and hyaluronan 1000 kDa, 0.05% and 0.3%, w/v) to monitor the role of hyaluronan on cell physiology. Proteomic analysis was also performed to investigate hyaluronan modulated cell responses. Our data demonstrated that hyaluronan do protect corneal epithelial cells from UVB-irradiation and hyaluronan molecular weight is a crucial factor in cell protection. In addition, high molecular weight hyaluronan significantly increases cell migration via modulation of cytoskeleton we observed. 2D-DIGE combined MALDI-TOF/TOF analysis showed that high molecular weight hyaluronan might modulate cytoskeleton and signal transduction to protect UVB-damaged corneal epithelial cells from cell death. The current work will be extended into a metabolomics study of these systems. To our knowledge, we report for the first time the ability of high molecular weight hyaluronan (1000 kDa, 0.3% (w/v)) with maximally protecting human corneal epithelial cells against UVB-irradiation and providing cell mechanism to elucidate hyaluronan-induced corneal cell responses via proteomic analysis.



## P2-1

### MEASURING NITROGEN FLUX IN PRIMARY AND SECONDARY AMINE CONTAINING METABOLITES USING LABELLING WITH THE $^{15}\text{N}$ STABLE ISOTOPE

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This presentation will describe a new approach to flux measurements. Comparative and quantitative measurements of steady-state levels of metabolites in a typical metabolomics experiment, for example where control samples are compared to experimental groups, may not provide the researcher with the correct information on the role of individual metabolites. The concentration of a metabolite may be maintained even though the turnover rate is higher under a certain treatment. If only concentrations were compared no change would be detected in this situation. Typically fluxomics experiments utilise  $^{13}\text{C}$ -labelled substrates which are metabolised and therefore alter the relative abundances of  $^{12}\text{C}$  and  $^{13}\text{C}$  ratios. The  $^{13}\text{C}$  labelling pattern and the  $^{12}\text{C}/^{13}\text{C}$  ratios of the newly synthesised metabolites change depending on the type and activity of the synthetic pathway. This stable isotope labelling approach can also be applied to N-containing metabolites and has been used in proteomics but rarely in metabolomics. This approach can be applied to a range of biological systems. For example, in hydroponically grown plants the nitrogen containing salts can be completely substituted with  $^{15}\text{N}$  labelled salts.

In this paper, seedlings of *Noccaea caerulescens*, a metal hyperaccumulating plant, were grown in a normal hydroponic solution for two weeks. The seedlings were then transferred to solutions containing  $^{15}\text{N}$  labelled salts. Some plants were also treated with heavy metals to initiate a change in metabolism. Extraction and derivatisation of metabolites containing primary and secondary amines using an analytical protocol which targets these functional groups was used.<sup>1</sup> These derivatives were then analysed using LC-MS to determine quantitatively the incorporation of  $^{15}\text{N}$  into N-containing metabolites, in particular amino acids and biogenic amines. Clear changes in incorporation rates were observed. This presentation will describe the methodology including the challenges as well as the application of both low and high resolution mass spectrometry instrumentation.

1. B. A. Boughton & D. L. Callahan, et al. *Analytical Chemistry* 2011, 83. 7523-7530.

P2-2

## **IN VIVO <sup>13</sup>C STABLE ISOTOPE TRACING OF PRIMARY METABOLISM DURING LEAF DEVELOPMENT**

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The GC-MS based profiling of primary metabolism in plant tissues is an efficient and routine tool for metabolic phenotyping. But often the changes of metabolite pool sizes are difficult to interpret, not least because metabolite pool sizes may stay constant while the fluxes are altered. Hence, following the path of labelled carbon through metabolite pools may enable a deeper understanding of the metabolic phenotype. We developed an *in vivo* plant feeding method where labelled precursors, such as U-<sup>13</sup>C-sucrose, are fed into the plant using a reverse petiole assay (Lin *et al.* 2011). This assay is suitable for plants grown on soil in a phytotron, but could equally be applied to plants growing in a greenhouse or even in the field. Single leaf analysis of *Arabidopsis thaliana* rosettes grown on soil was performed capturing different developmental stages in their transition from a sink to a source tissue. The combination of metabolite profile data and stable isotope tracing leads to a more detailed metabolic phenotyping and improved understanding of primary metabolism.

## USE OF BIOINFORMATICS TOOLS FOR FLUXOMICS ANALYSIS OF THE METABOLIC CHANGES DURING ADIPOCYTE DIFFERENTIATION

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**Background and Aims:** White adipose tissue mass is a critical factor determinant for obesity and associated health risks, and the formation of new adipocytes from the differentiation of pre-adipocytes is accompanied with important metabolic changes. Stable isotope tracer data is used to reveal the metabolic flux profile in cells under studied conditions, and thus to provide an insight into the cell phenotype. The *de novo* adipocyte differentiation and the associated changes of metabolic flux profile were studied on the 3T3–L1 cell line incubated with stable isotope tracers.

**Materials and Methods:** 3T3–L1 preadipocytes were cultured and differentiated. Biochemical determinations of glucose, lactate, glutamate and glutamine were performed from day 0 to day 9. Incubations with [1,2-<sup>13</sup>C<sub>2</sub>]-D-glucose were performed at day 0 (pre-adipocytes), day 4 (immature adipocytes) and day 8 (mature adipocytes). The biochemical concentrations and isotopologic patterns of several metabolites were measured at the beginning and at the end of the incubations, the rates of production and consumption were calculated, and Mass Isotopomer Distribution Analysis and flux estimation with our software Isodyn was performed.

**Results:** The biochemical measurements indicated three distinct periods according to the state of differentiation. Using the isotopologue data obtained in our experiments and our custom metabolic software Isodyn we quantified the metabolic flux distribution in the glucose metabolic network. The changes in the label distribution in Krebs cycle intermediates, amino acids, and several intracellular and extracellular metabolites were taken into account.

**Conclusion:** The analysis of the isotopic distribution allowed us to evaluate the dynamics of metabolic fluxes in the central carbon metabolism. Further analysis with Isodyn allowed us to define the whole set of metabolic fluxes inside the cell, defining the changes in the metabolic phenotype during the differentiation process.

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**ACUTE EFFECT OF CIGARETTE SMOKE ON HUMAN BRONCHIAL EPITHELIAL CELL METABOLOME IS REVERSIBLE BY UPF1**

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We assessed protective capacity of glutathione analogue UPF1 (4-methoxy-L-tyrosinyl-γ-L-glutamyl-L-cysteinyl-glycine) against cigarette smoke condensate (CSC)-induced alterations in metabolic profile of human bronchial epithelial cells (HBEC).

HBEC were exposed to 10 µg/mL CSC for 1 h, followed by treatment with 0-10 µM UPF1 or 2 mM N-acetylcysteine (NAC) for 1-12 h. Cell lysates were analysed on a Q-Trap 3200 mass spectrometer to obtain spectra between m/z ratios of 50-1700 Da. The data were subjected to principal component analysis, ANOVA, Pearson correlation analysis and hierarchical clustering.

CSC affected the highest number of signals at 2 h (245 signals, 13.4%). Signals affected by CSC with/without UPF1 or NAC formed 3 major clusters of metabolites that followed similar changing pattern within each group over time. In cluster 1, CSC caused a significant decrease of signals within 4 h, which was initially inverted by UPF1, before the signals returned to baseline. In contrast to UPF1, NAC had no redeeming effect. Among metabolites in cluster 1 species of phosphatidylcholines were identified using public databases. Signals of cluster 2 were decreased by CSC before returning to baseline after 4 h, followed by prolonged up-regulation by both UPF1 and NAC. Glutamine and glutamic acid were among the metabolites identified in cluster 2. Signals in cluster 3 were elevated by CSC and inverted by UPF1 and NAC showing a negative correlation with cluster 1 ( $r=-0.5$ ).

UPF1 effectively inverts the immediate effect of CSC on certain metabolites and may thus provide basis for designing drugs for protecting HBEC to slow down the development of COPD.

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**TIME-DEPENDENT METABOLIC VARIANCE IN MOUSE KIDNEY UPON CISPLATIN-INDUCED ACUTE KIDNEY INJURY**

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Cisplatin (cis-diammine-dichloroplatinum (II), CDDP) is an antineoplastic drug in the treatment of many solid-organ cancers, including those of the head, neck, lung, testis, ovary, and breast. While toxicities include ototoxicity, myelosuppression, and allergic reactions, the main dose-limiting side effect of cisplatin is nephrotoxicity. Previous researches reported that CDDP generates reactive oxygen species, which in turn cause oxidative damage in the kidney. However, the exact mechanism of cisplatin-induced acute kidney injury (AKI) still remains unclear. Many current studies have suggested that comprehensive analysis of endogenous metabolites as compound-level-phenotype of genomic information can elucidate biological phenomena and develop diagnostic and/or mechanistic biomarkers. In the present study, we applied a metabolomic technique to investigate the time-dependent renal metabolic dynamics upon CDDP-induced AKI.

To investigate the CDDP-induced metabolic variance, we used male mouse kidneys at different time interval after single intraperitoneal injection of saline or 30 mg/kg of CDDP (3, 6, 24, and 72 h). As a general method for biochemical evaluation of renal function, serum levels of blood urea nitrogen (BUN) were measured for evaluating the effect of CDDP on renal function using absorption spectrometer. Endogenous metabolites were extracted from whole kidney, and then analyzed by liquid chromatography-mass spectrometry. The levels of BUN were stable during after 6 h, but the significant increase was observed at 72 h. On the other hand, several of metabolites showed a significant change not only at late phase (24 and 72 h) but also at early phase (3 and 6 h). In addition to glycolysis and TCA cycle metabolites, amounts of ATP and 5-Amino-4-imidazolecarboxamide ribonucleotide (AICAR), the precursor of AMP, were markedly elevated at earlier phase. It is known that cellular energy state is positively regulated by 5'-AMP-activated protein kinase (AMPK), and AICAR is able to activate AMPK. At early phase of AKI, the AMPK activation due to the elevation of AICAR may be involved in the activation of glycolysis and TCA cycle, thus leading to increase of ATP level. We are currently underway to further investigate about the potential relationship between pathological mechanism(s) of AKI and above-mentioned metabolic variance.

**PATHWAY-WIDE METABOLITE ANALYSIS OF CENTRAL CARBON METABOLISM USING THREE COMPLEMENTARY LC-ESI-MS METHODS AND ITS APPLICATION**

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Central carbon metabolism (CCM) is a complex enzyme-mediated energy metabolism network. Several dozens of known metabolic precursors, intermediates and end products are involved in CCM. Qualitative and quantitative measurements of these metabolites are necessary for metabolic studies, for example, for isotope-resolved metabolic flux analysis. However, this is often challenging in practice and multiple analytical platforms are usually needed. In addition, some analytical techniques have low analytical sensitivity or limited ability of resolving structural isomers of some metabolites. In this work, we developed three new or improved UPLC-ESI-MS-based methods for comprehensive and complementary pathway-wide analysis of the CCM metabolites in various biological samples.

An ion-pairing UPLC-(-)ESI-MS method using tributylamine (TBA) as the positively-charged paired ion was improved for precise determination of approximately forty phosphate-containing metabolites, with the capacity of resolving multiple stereoisomers, *e.g.*, glucose-1,6-bisP vs. fructose-1,6-bisP, and glycerate-2P vs. glycerate-3P. A new UPLC-(+)ESI-MS method that combined reductive amination using 3-amino-9-ethylcarbazole and polar PFP reversed-phase LC was developed for selective detection and quantitation of more than ten free and phosphorylated reducing sugars. Another chemical derivatization UPLC-(-)ESI-MS method was proposed and optimized for sensitive and accurate quantitation of ten key CCM carboxylates, including all the Krebs cycle intermediates, as their 3-nitrophenylhydrazones.

Precise and accurate quantitation was conducted by UPLC-multiple reaction monitoring- MS on a triple quadrupole instrument, with assay accuracy of 85% to 114% for the two chemical derivatization methods. Isotope-resolved metabolic profiling was conducted by UPLC-full-scan MS on a high resolution instrument. With the three developed LC-MS methods, full-pathway analyses of >55 CCM metabolites in mouse liver, lung, heart and goldfish white muscle tissues, mouse red blood and several bacterial cells were achieved, with the analytical sensitivity ranging from femtomoles to low picomoles. These methods were successfully applied to isotope-resolved metabolic flux analysis in <sup>13</sup>C-glucose-labeling wild-type and knockout mice related to an ischemic myocardial metabolism study.

P2-7

## **IMPROVING BIOPROCESS FERMENTATIONS BY STABLE ISOTOPE LABELING AND FLUX ANALYSIS**

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Using metabolomics the metabolite inventory of a cell can be analysed and quantified. Thus, biochemical pathways can be suggested that are present in the cell, tissue or organism. Furthermore, metabolic changes can be calculated between different physiological states. The addition of stable isotope labeled metabolites allows the chase through the metabolism of the target organism. Thus, biochemical bottlenecks or highways can be identified. In industrial bioprocesses organisms are exploited in order to synthesise high amounts of product. The organisms are often engineered to allow product synthesis and achieve high yield. However, little is known about the physiological conditions and activities of these production hosts. The analysis of the metabolome and of isotopically metabolites enables the detailed understanding of the biochemistry. This allows the identification of biochemical bottlenecks or highways. Even the identification of flux into by-products will be accurately quantified. Thus, targets for genetic engineering can be identified that lead to higher product levels or enhance the cellular physiology. Feed media and fermentation parameters can be adjusted to optimally aid the fermentation. In the end the process can be improved at different levels due to stable isotope labeling and metabolomics analysis. Here, we investigate the flux of a carbon source into a small molecule end product in *E. coli*.

P2-8

**THE COMPLETE TARGETED PROFILE OF THE ORGANIC ACID INTERMEDIATES OF THE CITRIC ACID CYCLE USING A SINGLE STABLE ISOTOPE DILUTION ANALYSIS, SODIUM BORODEUTERIDE REDUCTION AND SELECTED ION MONITORING GC/MS**

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The quantitative profiling of the organic acid intermediates of the citric acid cycle (CAC) presents a challenge due to the lack of commercially available internal standards for all of the organic acid intermediates. We developed an analytical method that enables the quantitation of all the organic acids in the CAC in a single stable isotope dilution GC/MS analysis with deuterium-labeled analogs used as internal standards. The unstable  $\alpha$ -keto acids are rapidly reduced with sodium borodeuteride to the corresponding stable  $\alpha$ -deutero- $\alpha$ -hydroxy acids and these, along with their unlabeled analogs and other CAC organic acid intermediates, are converted to their *tert*-butyldimethylsilyl derivatives. Selected ion monitoring is employed with electron ionization. We validated this method by treating an untransformed mouse mammary epithelial cell line (NMuMG) with well-known mitochondrial toxins affecting the electron transport chain and ATP synthase, which resulted in profound perturbations of the concentration of CAC intermediates.



**P3-1**

**URINARY METABOLOMIC ANALYSIS OF BLADDER CANCER BY UPLC-FTMS AND UPLC-ION TRAP MS<sup>N</sup>**

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124 bladder cancer and 65 hernia urine samples were analyzed using a metabolomic platform coupling UPLC-FTMS and UPLC-ion trap MS<sup>n</sup>. The platform improved retention time, mass accuracy and signal stability. The product spectra obtained from ion trap MS<sup>n</sup> were useful for elucidating the metabolite structures, especially when authentic standards were not available. Using this technology platform, two metabolite panels were used to differentiate BCa from hernia and to differentiate early stage BCa and hernia, respectively.

**FINGERPRINTING OF BREAST CANCER CELLS VS. NORMAL MAMMARY CELLS BY SECONDARY ELECTROSPRAY IONIZATION MASS SPECTROMETRIC ANALYSIS OF VOLATILE COMPOUNDS FROM THE CULTURE MEDIUM**

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**Background:** A number of reports suggest that dogs are able to identify cancerous versus healthy tissues simply by sniffing them out. This suggests that there exists a volatile metabolic signature of malignancy. The identification of such volatile metabolic compounds may provide potential biomarkers for early diagnosis of malignant diseases.

**Method:** We tested this hypothesis by analyzing the volatile metabolic signature of three types of human breast cancer cell lines (T47D, SKBR-3, and MDA-MB-231) versus normal human mammary cells (HMLE). Volatile compounds in the headspace of conditioned culture medium were directly fingerprinted by secondary electrospray ionization-mass spectrometry. The mass spectra were subsequently treated statistically to identify discriminating features between normal vs. cancerous cell types.

**Results:** We were able to classify the different samples by using feature selection followed by principal component analysis (PCA). In addition, high resolution mass spectrometry and fragmentation of the most discriminant molecules allowed us to hypothesize their chemical structure.

**Conclusions:** Our study supports the hypothesis that cancerous cells release a characteristic odorous signature that may be used as disease markers. As opposed to dogs, mass spectrometry has the potential to identify the chemical nature of these.

## EXPOSURE TO CARCINOGENIC POLYCYCLIC AROMATIC HYDROCARBONS THROUGH TOBACCO SMOKE

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Background: People are exposed to carcinogenic polycyclic aromatic hydrocarbons (PAHs) through active smoking and passive smoking. Health risks of exposure to PAHs due to tobacco smoke can be evaluated by using urinary hydroxylated PAHs (OH-PAHs) as biomarkers.

Method: Nine OH-PAHs were selected as target compounds, including 2-naphthol (2-OHNap), 2-hydroxyfluorene (2-OHFlu), 2-/3-/4-/9-hydroxyphenanthrene (2-/3-/4-/9-OHPhe), 1-hydroxypyrene (1-OHPyr), 6-hydroxychrysene (6-OHChr) and 3-hydroxybenzo[a]pyrene (3-OHBaP). Urine samples were collected three times from a healthy male smoker (27 years old) during a day. All the samples were firstly hydrolyzed, and then pre-treated by solid phase extraction, and finally analyzed using HPLC-FD.

Results: 2-OHNap and 2-OHFlu were detected in all samples, and the concentrations were  $2.94\pm 0.03$ – $41.80\pm 3.40$  nM and  $1.24\pm 0.06$ – $14.61\pm 5.62$  nM, respectively. 1-OHPyr, 3-/9-OHPhe and 6-OHChr were found in most samples at the concentrations of  $1.59\pm 0.37$ – $4.54\pm 0.40$  nM,  $1.16\pm 0.23$ – $2.38\pm 0.32$  nM,  $2.17\pm 0.21$  nM and  $0.23\pm 0.07$ – $0.59\pm 0.00$  nM, respectively. However, 2- and 4-OHPhe, and 3-OHBaP were not detectable by using the present analytical protocol. Besides, urinary concentrations of 2-OHNap, 2-OHFlu, 1-OHPyr, 3-/9-OHPhe and 6-OHChr increased with the increase of cigarette consumption over time, further indicating smoker's exposure to PAHs through tobacco smoke.

Conclusions: For PAHs exposure to tobacco smoke, 2-OHNap and 1-OHFlu are probably dominated OH-PAHs metabolites, and thus would be potential selective PAHs biomarkers of tobacco smoke. Meanwhile, characterization of other OH-PAHs (e.g., 1-OHPyr, 6-OHChr, OH-Phes) might provide supplementary information for identifying PAHs exposure from tobacco smoke.

**P3-4**

**DEVELOPMENT & OPTIMIZATION OF GC-MS BASED GLOBAL METABOLITE PROFILING OF ADHERENT MAMMALIAN CELLS**

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Introduction: The human metabolome is thought to comprise >100,000 low molecular weight compounds representing a direct signature of metabolic state. Gas-chromatography coupled mass spectrometry (GC-MS) provides the means to detect a wide range of metabolites for the global assessment of metabolic phenotypes. This presents an opportunity to study features of tumour metabolism relating to angiogenesis and metastasis. However, capturing an accurate representation presents a challenge particularly for the evaluation of mammalian adherent cell types.

Objectives: To obtain metabolic profiles of human adherent endothelial and breast cancer cell lines using GC-MS and differentiate between different growth conditions; in addition to assessing the efficiency of novel developed method versus conventional methods, to accurately capture the metabolome.

Methods: We applied GC-MS to breast cancer MCF7, MB-MDA-436 and endothelial HMEC-1 cell-lines, cultured in RPMI media supplemented with 0.05% or 10%FBS. Cells underwent detachment with trypsin or a cell scraper before quenching (60% MeOH) and extraction (100% Aq. MeOH at -40°C). Samples were lyophilised and derivatised with methoxyamine and MSTFA (N-methyl-N trimethylsilyltrifluoroacetamide) before being analysed on a TRACE DSQ GC-MS System and identified using the GOLM and NIST databases.

Findings Despite a 36% reduction in leakage in trypsinized cells (compared with cell scraped cells), a marked difference in the distribution of metabolic classes were observed, with an approximate two fold increase in the proportion of amino acids and phosphates. This suggests metabolomic fluctuations in response to trypsin. Cell scraping with further modification is therefore recommended for rapid sample preparation. Profiles obtained from cancer cell-lines were more concordant than HMEC-1 and distinct patterns for altered conditions with an approximate 1.5 fold increase in yield for fully confluent and serums starved cells. These preliminary experiments demonstrate the application of GC-MS on adherent mammalian cells; further optimisation is required for future work in detecting metabolic characteristics of tumourgenesis.

## SERUM METABOLOME ANALYSIS IN PANCREATIC CANCER PATIENTS

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Pancreatic cancer is characterized by rapid tumor progression and early metastasis. Although the only curative treatment for pancreatic cancer is surgical resection, more than 80% of pancreatic cancer patients have a locally advanced or metastatic tumor that is unresectable at the time of diagnosis. To develop the more efficient diagnostic method for pancreatic cancer, we used gas chromatography/mass spectrometry (GC/MS)-based serum metabolomics as the novel diagnostic application.

The sera from pancreatic cancer patients (PC), healthy volunteers (HV) and chronic pancreatitis patients (CP) were collected at multiple institutions. In the training set study, which included 43 PC and 42 HV, 18 metabolites displayed significantly altered levels in serum between these groups. ROC analysis was used to calculate area under the ROC curve (AUC), sensitivity, and specificity values for these metabolites in order to evaluate their diagnostic performance. 1,5-Anhydro-D-glucitol displayed the highest AUC value as an individual biomarker (0.83499), and its sensitivity and specificity values were 86.0% and 71.4%, respectively. However, there were no metabolites whose sensitivity and specificity values were both over 80%. To construct a more effective diagnostic model, we performed multivariate analysis. First, 4 metabolites were selected via the stepwise method; i.e., xylitol, 1,5-anhydro-D-glucitol, histidine, and inositol. Using these metabolites, which did not display multicollinearity, a diagnostic model for pancreatic cancer was established using multiple logistic regression analysis. The constructed model possessed high sensitivity (86.0%) and specificity (88.1%) for PC in the training set. The utility of the model was confirmed in the validation set study, which included 42 PC, 41 HV, and 23 CP; i.e., it displayed high sensitivity (71.4%) and specificity (78.1%). Furthermore, in the validation set study, the sensitivity (77.8%) of the constructed model in resectable pancreatic cancer was higher than those of CA19-9 and CEA (55.6%, and 44.4%, respectively) and it showed lower false-positive rate (17.4%) in the case of chronic pancreatitis than these conventional tumor markers (30.4%, and 43.5%, respectively). These results suggest that this approach is a promising method for improving the prognosis of PC via its early detection and accurate discrimination from CP.

**AN NMR METABOLOMICS APPROACH FOR THE DIAGNOSIS OF LEPTOMENINGEAL CARCINOMATOSIS**

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Leptomeningeal carcinomatosis (LC) is the third most common metastatic complication of the central nervous system. However, the current modalities to reliably diagnose this condition are not satisfactory. Here we report a preclinical proof of concept for a metabolomics-based diagnostic strategy, using a rat LC model incorporating glioma cells that stably expressed green fluorescent protein. Cytological diagnoses gave 66.7% sensitivity for the 7-day LC group and 0% for the 3-day LC group. MR imaging could not diagnose LC at these stages. In contrast, NMR-based metabolomics on cerebrospinal fluid (CSF) detected marked differences between the normal and LC groups. Predictions based on the multivariate model provided sensitivity, specificity and overall accuracy of 88-89% in both groups for LC diagnosis. Further statistical analyses identified lactate, acetate, and creatine as specific for the 7-day LC group, with glucose a specific marker of the normal group. Overall, we demonstrated that the metabolomics approach provided both earlier and more accurate diagnostic results than cytology and MR imaging in current use.

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## EFFECT OF EXTERNAL BEAM AND <sup>131</sup>I-MIBG THERAPIES ON HUMAN GLIOMA CELL METABOLISM

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Gliomas are the most common primary brain tumours that arise from the glial cells of the brain and nervous system. Radiotherapy is the main therapeutic treatment for this cancer in which ionizing radiation is used to kill the cancer cells and shrink tumours by damaging the cells' DNA, thereby stopping these cells from continuing to grow and divide. Targeted radionuclide therapy, on the other hand, is a systemic treatment in which a molecule labelled with a radionuclide delivers a toxic level of radiation to disease sites. Previous studies in our group have shown that tumour cells exposed to media from cells treated with the radiopharmaceutical <sup>131</sup>I-MIBG show decreased clonogenic capacity in a dose dependent manner. This indicates that these radiopharmaceuticals elicit a radiation induced biological bystander effect (RIBBE) on recipient (non-irradiated) cells which is distinct and more cytotoxic than generated by beam irradiation. Thus our main object of this work was to elucidate the effect of these treatments on donor and recipient tumour cells metabolism. There have been few investigations of the use of metabolomics for assessment of treatment effect on human glioma cells, as both a predictive measure of efficacy and as a pharmacodynamics marker. In this work we discovered some metabolites that are unique to, or elevated or reduced in Glioma cell cultures during treatments and studied the levels of these biomarkers in response to various doses and treatments in order to assess treatment and doses efficacies against these cells. We analysed the effect of two different treatments external beam  $\gamma$ -radiation with those resultant from exposure to 3 radiohaloanalogs of metaiodobenzylguanidine (MIBG): <sup>131</sup>I-MIBG (low-linear-energy-transfer [LET]  $\beta$ -emitter) at different dose ranges: 0, 1, 4 Gy and 0,1, 3 & 6 MBq/mL for external and internal radiation respectively on human glioma UVW/NAT donor and recipient cells metabolism in vitro. Statistically significant differences were observed between the treated and the untreated cells with some human metabolic pathways exhibiting significant elevation in the treated cells. These data also suggested that external and internal radiation exposure may significantly impact the cell metabolism in different ways.

**LIPIDOMIC ANALYSIS DEMONSTRATES THAT THE ANTI-LEUKEMIC THERAPY OF BEZAFIBRATE AND MEDROXYPROGESTERONE ACETATE REDUCES *DE NOVO* PHOSPHOLIPID SYNTHESIS**

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Acute myeloid leukaemia (AML) is an aggressive cancer predominantly affecting patients >60 years old. These patients are often too frail to tolerate chemotherapy and survival rates remain dismal. What are required are less toxic therapies. Our strategy is to use drug redeployment. This approach exploits available low toxicity drugs in disease settings other than those for which they were developed. We have demonstrated that the combination of the lipid lowering drug, Bezafibrate, and the contraceptive, medroxyprogesterone acetate, (BaP) kills AML cells *in vitro* in association with the induction of reactive oxygen species (ROS) but is not toxic to non-leukemic myeloid cells. A Phase II trial of BaP showed clinical activity AML in the absence of toxicity, and a follow up Phase II trial of BaP at higher doses that better reflect those at which the drugs are maximally active is in progress. In the study reported here we have utilised high-resolution Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) to investigate BaP-induced changes to the AML cell lipidome. The aims were to increase the understanding of the molecular mechanism of BaP and to identify specific lipid signatures of BaP exposure that might be used as biomarkers of BaP responses in patients. AML cell lines (HL60 and K562) were exposed to 0.5 mM Bez and 5  $\mu$ M MPA for 24 hours and then quenched at -40°C. Lipids were extracted and analysed by direct-infusion FT-ICR MS. Highly consistent, significant changes to cellular phospholipid profiles were observed after treatment of both cell lines. This included a widespread increase in the unsaturation of phospholipid fatty acyl residues. Significant increases in the average number of double bonds within the fatty acyl groups of diacyl-phospholipids were observed in HL60 (3% increase,  $p=2\times 10^{-4}$ ) and K562 (8% increase,  $p=4\times 10^{-11}$ ) cell lines after 24 hr treatment. BaP treatment of HL60 cell lines in the presence of 1-<sup>13</sup>C D-glucose, revealed that incorporation of glucose into diacyl-phospholipids was significantly decreased 5% ( $p=0.005$ ). Since newly synthesised phospholipids are mostly more saturated than existing ones, the observed decrease in phospholipid synthesis is likely responsible for the increase in unsaturation of acyl groups. We hypothesise that this in turn renders the cells more vulnerable to lipid peroxidation-mediated apoptosis in response to ROS generated by BaP treatment.



## **METABOLIC CHARACTERIZATION OF A METASTATIC PROSTATE CANCER STEM CELL SUBPOPULATION**

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Metastasis represents the most devastating stage of malignancy and the leading cause of death by cancer. For most carcinomas, progression towards metastasis is accompanied by the cellular process known as epithelial-mesenchymal transition (EMT) as well as the participation of a minority of malignant cells known as cancer stem cells (CSCs) or tumour-initiating cells (TICs), endowed with unique self-renewal and tumour propagating capacity.

Taking into account that metabolic reprogramming has been linked to the major hallmarks of cancer, it is expected that cell subpopulations involved in tumour invasion and metastasis show an altered metabolic pattern. We have initiated the metabolic characterization of two related cell subpopulations isolated from PC-3 cell line. They display very divergent features regarding their transcriptional program, as well as their invasive and metastatic properties, resulting in one cell subpopulation endowed with CSC properties and another one expressing the EMT program.

Our studies have been focused on the characterization of the metabolic differences between the CSC and the EMT phenotypes, both present in our dual cell model of highly related populations with clearly distinct metastatic potentials. So far, we have analyzed and compared the metabolism of both cell subpopulation at the level of glycolysis, pentose phosphate pathway and aminoacids. Our goal is to understand the metabolic reprogramming underlying metastasis, looking for those metabolites or metabolic pathways that could be playing an essential role in the most aggressive and metastatic CSC subpopulation.

## PROGNOSTIC VALUE OF <sup>1</sup>H NMR SPECTROSCOPY IN THE TREATMENT OF NON-SMALL CELL LUNG CANCER

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**Introduction:** Lung cancer is the leading cause of cancer mortality worldwide. Despite progress in treatment of lung cancer we experience unacceptable toxicities without a significant increase in overall survival (OS) and progression free survival (PFS). Clinical trials do not answer the question of who will benefit the most of therapy and who may be harmed. The goal of this study is to find an answer on this question by metabolic profiling of plasma of untreated lung cancer patients.

**Objective:** Differentiating responder and non-responder NSCLC patients based on differences in their metabolic profiles.

**Methods:** This project investigates subjects with the diagnosis of NSCLC independent of stage and who are candidate to receive chemotherapy. All patients have a newly diagnosed NSCLC. Participants are asked to give one fasted sample of blood. Samples are collected before the start of the treatment and analysed by <sup>1</sup>H-NMR spectroscopy using a 400 MHz spectrometer. Slightly T<sub>2</sub>-weighted spectra were acquired using a CPMG pulse sequence and are referred to TSP resonance at 0.015 ppm. The integration values of 110 spectral regions are analyzed by a Mann-Whitney test to identify those which differ significantly between responders and non-responders, followed by an orthogonal partial least squares discriminant analysis to investigate whether a discriminating classifier can be constructed. In this way we aim to distinguish a metabolic profile that differs between responders and non-responders. The patient is followed-up by CT scan after every 2 cycles of chemotherapy and every 3 months after completion of the first line treatment or earlier if clinical arguments for relapse appear. Disease progression and response to therapy are defined following the RECIST criteria of 2009. Responders are defined as patients with a PFS of at least 1 year during a minimal follow-up of 1 year. Non responders are patients with progression within 1 year of follow-up.

**Results:** No significant difference between the metabolic profile of responders (n =18) and non-responders (n =29) of chemotherapy. This might be the result of a non-homogeneous small group and interference of medications for co-morbidities.

**Conclusion:** In this small patient cohort, responding and non-responding NSCLC patients could not be differentiated based on the metabolic profiles obtained by <sup>1</sup>H-NMR spectroscopy. This probably due to the heterogeneous composition of the subgroups.

**LC-HRMS BASED METABOLOMICS AND LIPIDOMICS PHENOTYPING FOR THE STUDY OF MAMMARY CANCER SIGNATURE IN CANINE SERUM**

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Sample treatment is extremely important in metabolomics studies because it affects both the observed metabolite content and the related biological interpretation. Until now, no universal serum sample preparation protocol suitable for metabolomics studies was reported as consensual. In general, protein removal by organic solvent precipitation or through filtration is used as minimum sample pretreatment for serum analysis by liquid chromatography – mass spectrometry. In addition, solid phase extraction is sometimes used for the removal of lipidic content. These sample pre-treatments are normally used to minimize matrix effects and ion suppression phenomenon when the analysis of polar metabolites is the objective. However, the lipidic content of the samples is partially or totally discarded with these protocols, while this one may be very of high informative value. Indeed, numerous studies have demonstrated that disruption on lipid metabolism and/or signalling is associated to the progression of several cancers.

In this work, a metabolomics phenotyping approach by Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS) has been developed for a comprehensive characterisation of both metabolic and lipidic content of canine serum. Samples are extracted using Chloroform:MeOH:Water, resulting in fractionation of polar compounds (MeOH phase) and lipidic content (chloroform phase). Complementary LC-HRMS metabolomics profiles can be obtained by the analysis of polar metabolites by reversed phase and hydrophilic liquid chromatography (HILIC) while lipidomics profiles are achieved by a reversed phase LC-HRMS/MS method using all ions fragmentation (AIF) and ESI polarity switching.

The developed methodology was successfully applied to the study of canine mammary cancer. The serum metabolome and lipidome of pet dogs developing spontaneous malignant mammary tumors or benign tumors and of health controls were studied. The preliminary results obtained confirmed the interest of combining metabolomics and lipidomics profiles for characterizing a disease signature from serum samples.

**P3-12**

**TARGETING CELL CYCLE REGULATION IN CANCER THERAPY**

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Cell proliferation is an essential mechanism for growth, development and regeneration of eukaryotic organisms; however, it is also the cause of one of the most devastating diseases of our era: cancer. Given the relevance of the processes in which cell proliferation is involved, its regulation is of paramount importance for multicellular organisms. Cell division is orchestrated by a complex network of interactions between proteins, metabolism and microenvironment including several signaling pathways and mechanisms of control aiming to enable cell proliferation only in response to specific stimuli and under adequate conditions. Three main players have been identified in the coordinated variation of the many molecules that play a role in cell cycle: i) The cell cycle protein machinery including cyclin-dependent kinases (CDK)-cyclin complexes and related kinases, ii) The metabolic enzymes and related metabolites and iii) The reactive-oxygen species (ROS) and cellular redox status. The role of these key players and the interaction between oscillatory and non-oscillatory species have proved essential for driving the cell cycle. Moreover, cancer development has been associated to defects in all of them. Here, we provide an overview on the role of CDK-cyclin complexes, metabolic adaptations and oxidative stress in regulating progression through each cell cycle phase and transitions between them. Thus, new approaches for the design of innovative cancer therapies targeting crosstalk between cell cycle simultaneous events are proposed.

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**DETERMINATION OF AMINO ACID IN URINE SAMPLES COLLECTED FROM PATIENTS WITH PROSTATE CANCER AND BENIGN PROSTATE HYPERPLASIA, BEFORE AND AFTER DIGITAL RECTAL EXAMINATION**

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Prostate cancer is the second most common cause of cancer related death and leading type of cancer diagnosed in men. Although many new patients are diagnosed each year, diagnostic procedures are lacking and need to be improved to increase sensitivity and specificity. Currently, for screening purposes and diagnostics, serum PSA levels and digital rectal examination are used to determine if patient presents with prostate cancer. PSA levels can be helpful with differentiating healthy and sick patients but it needs to be noted that sensitivity and specificity of this test are poor. The aim of our research is to find an improved biomarker for prostate cancer. Our strategy involved identifying and quantifying a number of amino acids in urine samples collected in the morning prior to and after digital rectal examination. Derivatized amino acids were quantified by liquid chromatography mass spectrometry then statistical analysis was performed using MetaboAnalyst 2.0. The diagnostic potential of selected amino acids was analysed by creating ROC curves. We observed that patients with prostate cancer present with higher urinary levels of arginine, homoserine, proline and tyramine. Stronger statistical value was observed in samples collected after digital rectal examination. Although we were able to observe relevant differences in concentrations of selected amino acids, their diagnostic potential was evaluated to be worse than the current PSA standard.

**BREATH ANALYSIS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY IN THE DETECTION OF LUNG CANCER AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE**

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Introduction: Organic Volatile Compounds (VOCs) generated in metabolism can be detected in exhaled air. Several diseases entail altered patterns of exhaled VOCs due to an alteration in normal metabolism. Specifically, abnormal pulmonary and systemic metabolism in lung disorders is expected to result in an altered exhaled VOCs pattern. Therefore, we analyzed the pattern of exhaled VOCs in the breath of healthy subjects, COPD patients and lung cancer patients to assess the diagnostic potential of breath analysis.

Aims: We present a method to identify VOCs profiles in breath samples in order to separate healthy volunteers from COPD and lung cancer patients.

Methods: A total of 81 breath samples were analyzed, including 22 from healthy controls, 29 from patients with COPD, 12 from patients with lung cancer and 18 from patients with both lung cancer and COPD. End-exhaled breath samples (1000mL) were obtained using Tenax/Carbosieve Sorbent Traps and a custom Tidal Breath Sampler (TBS). Analysis were carried out with a Focus Gas Chromatographer coupled to a DSQII single quadrupole electron impact Mass Spectrometer from Thermo Fisher. The spectra obtained were processed with MZmine software, and a Sequential Floating Feature Selection (SFSS) algorithm was used to detect the patterns that classified each group. Results robustness were tested by bootstrap validation.

Results: Analysis of the spectra showed differences between COPD, lung cancer and healthy VOCs profiles, even when variability between subjects in the same group was high.

Conclusions: Exhaled breath VOCs analysis using GC/MS is a non-invasive method with promising potential in distinguishing VOCs patterns from disease and healthy breath samples.

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**IDENTIFICATION OF THE CHEMICAL SHIFTS OF A SERIES OF METABOLITES APPEARING IN THE <sup>1</sup>H-NMR SPECTRUM OF BLOOD PLASMA BY SPIKING**

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Background: Instead of using reported chemical shift values of metabolites, which differ seriously from study to study due to differences in e.g. biofluid, temperature and pH, the chemical shifts of metabolites could be determined more accurately by spiking the biofluid itself.

Objective: To compare the discriminative power of spectral regions of plasma metabolites derived from literature with those obtained by spiking between lung cancer patients and controls.

Methods: Fasting plasma samples, collected from a 44-year-old healthy female, were spiked. One plasma sample was used as reference. The remaining plasma samples were spiked with 37 known metabolites (i.e. components of the glycolysis and Krebs cycle, and all amino acids) at a known concentration (1 mg compound/100 µl plasma). Subsequently, 10 µl spiked plasma was added to 200 µl plasma and 600 µl D<sub>2</sub>O and prepared for analysis with <sup>1</sup>H-NMR spectroscopy. The <sup>1</sup>H-NMR spectra were recorded on a 400 MHz Varian Inova spectrometer using a CPMG pulse sequence with water suppression. TSP was used to calibrate the ppm scale at 0.015. The chemical shifts and J-coupling patterns of the spiked metabolites were determined, allowing to rationally define integration regions. An OPLS-DA multivariate analysis was carried out on 78 lung cancer patients and 78 controls in order to compare integration regions based on diverse literature chemical shift information and spiking.

Results: Sensitivity increased from 81% to 86% and specificity increased from 88% to 95% when using integration regions on plasma spectra defined by spiking.

Conclusion: Spiking experiments seem necessary in order to more accurately identify the chemical shifts of plasma metabolites in a <sup>1</sup>H-NMR spectrum. Moreover, it could lead to an enhancement of the discriminative power of the cluster analysis and a better understanding and/or explanation of the clinical relevance of study findings.

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**OPTIMIZATION AND VALIDATION OF A METABOLOMIC METHOD FOR ANALYSIS OF PROSTATE CANCER CELL CULTURES (LNCaP) USING EXACTIVE-ORBITRAP MASS SPECTROMETER**

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In this study, a method for carrying out metabolomics profiling of tissue cultures with an optimized extraction procedure and validation of an analytical method using Liquid chromatography-mass spectrometry (LC-MS). The data was processed with MZ-Match and ToxID software and analysed with multivariate statistics. Cell culturing, quenching, metabolite extraction, and the LC/MS settings were optimized aiming at a reliable, unbiased, sensitive, and high throughput metabolomic protocol. Due to the heterogeneity of the metabolome and the inherent selectivity of all analytical techniques, development of unbiased protocols is highly complex. The experiments show that any changes in any of the protocol parameters may affect the response of many groups of metabolites. Metabolite extractions were performed using three solvents: methanol, acetonitrile and water (50:30:20, v/v/v). This approach was subsequently validated using ~ 200 standard compounds from different chemical classes and prostate cancer cells (LNCaP). Hydrophilic interaction chromatography (pHILIC) and the MS was operated on both positive and negative electrospray ionization modes. 20mM Ammonium carbonate was used as mobile phase A and acetonitrile as mobile phase B. Two ranges of calibration standards were prepared in the range of 1–500 ng/mL or 1000-20000 ng/ml and the LOD and LOQ for each metabolite were determined. Precision and reproducibility of the standards were below  $\pm 5\%$  cross run (n=6). An MS/MS library was obtained for the standards and the metabolites in the samples. Effects of storage conditions on metabolite profiles and stability study were assessed using multivariate statistics (PCA). The protocol was successfully employed and allowed high-throughput profiling of metabolites covering the major metabolic pathways.



**P3-17**

**METABOLOMICS STUDIES INTO THE REGULATION OF METABOLISM BY THE p53 PATHWAY**

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The function of p53 is best understood in response to genotoxic stress, but increasing evidence suggests that p53 also plays a key role in the regulation of metabolic homeostasis. p53 directly influences various metabolic pathways, enabling cells to respond to metabolic stress. These functions are likely to be important to restrain the development of cancer, but could also have a profound effect on the development of metabolic diseases. However, the metabolic roles of many p53 target genes, such as TIGAR, are less well understood. Furthermore, approximately 50-70% of cancers express mutant forms of p53, which have generally lost p53's pro-apoptotic functions, but can gain functions that contribute to transformation and metastasis. Possibly, cells expressing mutant p53 also retain some of the pro-survival and metabolic adaptation functions of p53, but little is known about the regulation of cellular metabolism by mutant p53, especially under conditions of metabolic stress. Therefore, we have developed LC/MS-based metabolomics methods to examine how these less-studied players in the p53 pathway affect metabolism, including glycolysis, the TCA cycle, glutaminolysis, the PPP, nucleotide biosynthesis and fatty acid synthesis and oxidation. To study changes in the levels of the metabolites involved in these pathways, we use a variety of stable isotope labelled nutrients in combination with various extraction and separation methods, including HILIC and reversed-phase chromatography. This approach enables us to perform both steady-state metabolomics and metabolic flux studies to investigate up- or down-regulation of pathways in response to metabolic stress. The protocols developed have been applied successfully to study the p53-dependent metabolic response to serine starvation and are currently being used to investigate the metabolic changes induced by mutant forms of p53 and to decipher the function of TIGAR. Our approach contributes to a comprehensive picture of the regulation of metabolism by p53 and - as p53 appears to play a central role in metabolic adaptation – helps identify the pathways that cells use to survive metabolic stresses in the context of (mutant) p53.

**A NMR METABONOMIC APPROACH TO EXPLORE EARLY BIOMARKERS OF HEPATOCELLULAR CARCINOMA IN THE EUROPEAN PROSPECTIVE INVESTIGATION INTO CANCER AND NUTRITION (EPIC)**

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Hepatocellular carcinoma (HCC), ranked sixth in incidence and third in cancer-related mortality worldwide, is highly malignant, usually diagnosed at late stages and has often poor prognosis with limited treatment options. Thus, there is a need to identify early diagnostic biomarkers of HCC. It is also plausible that metabolic imbalances, reflective of obesity, diabetes, or lifestyle habits play a central role in HCC etiology.

A NMR metabonomic study was undertaken in a case-control study nested within EPIC, a large prospective cohort of over 520,000 subjects from 10 Western European countries, on serum samples from healthy participants at the time of enrolment and blood collection. After an average of 7.6 years of follow-up, 112 first primary incident HCC cases were identified and matched to control subjects (1:2; by age, sex, study centre). A total of 336 serum samples were analyzed by high-field <sup>1</sup>H NMR spectroscopy. A metabonomic approach was employed to explore for biomarkers that may be relevant either to early diagnosis of HCC or indicative of etiologically relevant exposures.

In a preliminary phase, a statistical method was developed based on Principal Component and Partial R<sup>2</sup> analyses to highlight main sources of variability in the metabolomic data. The method assessed the contribution of age, sex, body mass index, country of origin, as well as smoking, self-reported diabetes status at baseline, fasting status at blood collection, alongside factors related to sample processing to variability of the metabolomic profiles. From this analysis, country was found to display the largest variance contribution (8.0%).

Some serum metabolomic profiles capable of discriminating between matched cases and controls were identified, particularly with stratification for analysis by time of follow-up from blood collection to diagnosis, or sex. The potential of this prospective study to identify diagnostic or etiologic biomarkers may have relevant public health implications and lead to an enhanced understanding of HCC development.

## **NMR-BASED METABOLITE PROFILING FOR EARLY DETECTION AND NON-INVASIVE DIAGNOSIS OF GASTRIC CANCER**

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Diagnosis and monitoring of gastric cancer (GC) on the basis of non-invasive measurements is difficult clinically. Thus, we used a metabolomics approach to investigate altered metabolic patterns in urine from patients with gastric cancer and find urinary metabolic biomarkers for the non-invasive diagnosis of gastric cancer, and compare the urinary and tissue metabolic profiling of GC patients.

Urine samples (n=100) from GC patients and healthy individuals, and 30 pairs of matched tumor and normal stomach tissues were collected. Multivariate analysis was performed on the urinary and tissue metabolic profiles, which were acquired using <sup>1</sup>H NMR and <sup>1</sup>H HR-MAS spectroscopy, respectively.

Multivariate statistical analysis showed a significant separation between GC patients and healthy individuals in the urinary and tissue data. The metabolites perturbed in the urine of GC patients were related to amino acid and lipid metabolism, consistent with the changes in metabolism of GC tissue. Furthermore, the presence of GC in the external validation of the urine model was predicted with high accuracy; patients with early gastric cancer were also predicted correctly.

These data illustrate that a urinary metabolomics approach can be effectively used for early diagnosis of GC, and balanced metabolite profiles between systemic environment and tumor microenvironment not only provide insights into the mechanisms associated with GC but also allow reliable identification of potential markers for GC

## **NMR METABONOMIC INVESTIGATION OF A E3N MATCHED CASE-CONTROL BREAST CANCER PROSPECTIVE COHORT**

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E3N is an epidemiological cohort study that was initiated in 1990 to investigate factors associated with the most common types of cancer. It involves about 100,000 women living in France who were born between 1925 and 1950 and are covered by a national health insurance plan for teachers and co-workers. Many data were collected from these volunteers: participants completed self-questionnaires every 2 to 3 years on their lifestyle (diet, smoking habits, hormonal treatments, etc) and the evolution of their health. Biological data were also included with a collection of blood samples of 25,000 volunteers before diagnosis and stored for subsequent biological analysis. Currently, among 100,000 women in the E3N cohort, 5,455 validated cases of breast cancer were identified, amongst which approximately 900 had given a blood sample. Using a case-control design, 801 breast cancer prospective cases with available plasma samples were identified and matched with an equal number of control subjects.

<sup>1</sup>H NMR (Nuclear Magnetic Resonance) investigation of this E3N breast cancer sub-cohort was carried out at 600 MHz on 1,602 plasma samples collected in citrated tubes at inclusion in the study, with the objective to identify early or predictive biomarkers of breast cancer.

We present here the first results of the NMR metabonomic analysis. First, we compare technical and clinico-pathological data between cases and controls to exclude potential biases related to patient selection, using a descriptive statistical analysis. To detect true risk factors or early pathological changes for E3N volunteers, potential sources of bias must be identified and possibly corrected before further analysis. We thus explore main sources of systematic variation in our metabonomic dataset, including technical parameters (collection center, time before centrifugation, storage time...) and biological factors (BMI, smoking status, menopausal status...). In addition, we evaluate more specifically the impact and metabolic fingerprints associated to the identified systematic variations by supervised multivariate statistics.

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**DETERMINATION OF HETES IN PROSTATE CANCER SERUM SAMPLES USING UHPLC-MS/MS**

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Association of the arachidonic acid (AA) pathway with prostate cancer (PCa) has indicated that there are various key targets within this pathway, that can provide possibly novel therapeutic opportunities for treating PCa. In this study, we aimed to evaluate differences in the concentration of AA, as well as five hydroxyeicosatetraenoic acids (HETEs) in serum samples from patients diagnosed with PCa and controls using UHPLC-MS/MS. Sample preparation included precipitation of the proteins with cold methanol, and the metabolites were separated in 7 minutes by RP-LC using an organic solution gradient. Detection was performed by electrospray ionization in negative mode using multiple reaction monitoring in a triple quadrupole mass spectrometer. Linearity of the method, recovery and stability were tested. Median concentrations in PCa patients and controls for 5-HETE, 8-HETE, 11-HETE, 12-HETE and 15-HETE were found as 29.6 pg/ $\mu$ L, 22.6 pg/ $\mu$ L, 24.6 pg/ $\mu$ L, 4.2 ng/ $\mu$ L and 66.6 pg/L, respectively. No significant differences in concentration of HETEs were observed among the controls. Remarkably, concentrations of up to 100 times elevated in 6 out of 20 patients in the group of PCa patients were observed. Elevated concentration of 5-HETE, 8-HETE, 11-HETE and 15-HETE were observed for the same subgroup of patients. Endogenous levels of AA and 12-HETE between the cancer group and control groups showed no statistical difference ( $p > 0.05$ ). No correlation between high concentrations of HETEs and clinical information, such as PSA level and Gleason Score, was observed in the PCa group. Why a certain group of patients show this elevated level is still unknown, we try to make links to available microarray data and mass spectrometry- based proteomics data.

## IDENTIFICATION AND QUANTIFICATION OF INTRACELLULAR METABOLITES INVOLVED IN TRICARBOXYLIC ACID CYCLE METABOLISM

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We present a highly selective and sensitive method for identification and quantification of intracellular metabolites involved in tricarboxylic acid cycle (TCAs) metabolism (including the configuration of the enantiomers of (L/D)-2-hydroxyglutaric (2-HG), (L/D) glutamic and (L/D) glutamine acids) by means of liquid chromatography–tandem triple quadrupole tandem mass spectrometer (LC–MS/MS). The simultaneous separation of 6 negatively charged intermediates is performed using ion-pairing chromatographic separation and the enantiomeric chiral separation of (L/D) - 2-HG acid was achieved using a ristocetin A glycopeptide antibiotic silica gel bonded column. The six enantiomeric forms of the compounds were well separated with baseline resolution at 11.42/12.41 min for the L/D-2-HG, 11.80/12.11 min for the L/D-glutamic and at 10.31/11.18 for the L/D-glutamine. Method validation provided limits of detection (LODs) for L/D-2HG acid calculated according to a signal-to-noise (S/N) ratio of 3:1. These were all below 8µM with the exception of malate which was <10µM. The calibration curves showed good linearity mainly over three orders of magnitude with a correlation coefficient R<sup>2</sup> > 0.99.

Additionally, we developed and applied a novel liquid chromatography-mass spectrometry method for studying changes in TCA cycle intermediates and their associated metabolite concentrations. To subsequently apply these in cell and in vivo based studies identifying and quantifying oncometabolites. This method used a hybrid stationary phase with an Acquity HSS T3 column (hydrophilic C18 retention mechanism) coupled to ACE-PFP (pentafluorophenyl modified C18). This provides a unique retention most likely based on hydrophobic and hydrophilic interactions between the stationary phase and the analyte. Under the optimized chromatographic conditions, the two isomers were well separated with citrate eluting at ca. 3.84 min followed by the isomer isocitrate at ca. 4.74 min and also fumarate at 4.66 min followed by the isomer maleate at 5.84 min.

These LC–MS/MS methods were developed and applied to the analysis of cancer cell tissues to investigate changes in citric acid cycle intermediates and the identify selected enantiomer concentrations. Here we describe the methodology used and give examples from the analysis of selected wild-type and modified cancer cell lines which show highly specific enantiomeric changes taking place in mutant cell lines.

**USING METABOLOMICS TO INVESTIGATE THE INDUCTION OF NON-ALCOHOLIC FATTY LIVER DISEASE IN A RAT MODEL OF HEPATOCELLULAR CARCINOGENESIS**

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Non-alcoholic steatohepatitis (NASH) is a progressive form of NAFLD associated with worsening cirrhosis and hepatocellular carcinoma (HCC). The pathogenic mechanisms underlying such hepatic pathologies remain to be fully elucidated. Improved metabolic characterisation of NASH may uncover diagnostic and prognostic disease markers or identify novel targets for treatment. In this study, a metabolomics approach was utilised to compare a rat model of NAFLD induced by a choline deficient (CD) diet with control animals. In addition rats were treated with a thyroid hormone analogue, GC-1, to assess the potential of this intervention as a therapeutic for NAFLD. A comprehensive metabolomics strategy combining <sup>1</sup>H nuclear magnetic resonance (NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS), ultra performance liquid chromatography-mass spectrometry (UPLC-MS) and direct infusion mass spectrometry was employed to investigate metabolite changes in the liver.

CD diet significantly increased the total fatty acid concentration by increasing medium and long chain fatty acids. Consistent with the fatty acid profile, we observed marked accumulation of acylcarnitine species in the livers of animals on a CD diet which may be attributed to incomplete mitochondrial  $\beta$ -oxidation. Total lipid profiling revealed increased unsaturated (5-8 double bonds) of acyl chains within triacylglycerides (TAG) in the livers from the CD group. This liver tissue was also characterised by increased glycolysis and ketogenesis, and decreased gluconeogenesis. In addition, oxidative stress was increased as measured using the surrogate oxidised methionine. A 2 week co-feeding with GC-1 lowered the total fatty acid content and lipid accumulation in the CD diet livers. There was a dramatic increase in the concentration of betaine in the GC-1 treated liver, a reported lipotrope which may prevent or reduce accumulation of fat in the liver. Furthermore, positive correlations between the increase of betaine and several acylcarnitine species have been found.

**ASSOCIATIONS BETWEEN IN VITRO SURVIVAL TO CHEMOTHERAPY, MINIMAL RESIDUAL DISEASE AND CONDITIONED CULTURE MEDIA METABOLOME OF PRIMARY ACUTE LYMPHOBLASTIC LEUKEMIA**

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Childhood acute lymphoblastic leukemia (ALL) is the most prevalent type of cancer in children. Although outcome has improved considerably with advancements in therapy, around 20-30% of patients relapse, in most cases due to a drug-resistant phenotype of leukemia cells. Previous studies have shown that in vitro leukemia resistance to chemotherapy is associated with worse patient outcome. Newly-diagnosed leukemia cells from 21 ALL patients were cultured in RPMI-10 (RPMI-1640 with 10% FBS) for 24h in absence (CTRL) or presence of prednisolone (PRED) or asparaginase (ASP), two major chemotherapy agents against ALL. As expected, leukemia cells from patients with positive ( $\geq 10^{-3}$ ) minimal residual disease (MRD) at the end of induction therapy (Day 28) had both increased in vitro resistance to ASP and PRED and in vitro survival in RPMI-10, as determined by Annexin V labeling of death and apoptotic cells. The metabolome of leukemia conditioned media, collected after 24h of culture, was assessed by <sup>1</sup>H-NMR. In total, 43 metabolites were identified and quantified at the micromolar range. Treatments (CTRL, PRED, ASP) were analyzed in separate. For comparisons, median survival rates were used as a threshold to classify patients' samples into resistant (R) or sensitive (S) groups. Mann-Whitney test ( $p < 0.05$ ) revealed 27 differing metabolites among R and S groups in ASP; 2 in PRED (hypoxanthine and isopropanol) and 1 in CTRL (acetone). When sample's were categorized according to their MRD status, 3 metabolites in ASP (alanine, dihydrothymine and hydroxyproline), 1 in CTRL (acetone) but none in PRED were successfully associated to this clinical feature. Ingenuity Pathways Analysis software was used to construct an integrative network from ASP metabolites.



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**ROLE OF TGF- $\beta$  INDUCED EPITHELIAL MESENCHYMAL TRANSITION (EMT) IN PANCREATIC CANCER METABOLISM**

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Although TGF- $\beta$  (Transforming growth factor) has been known to profoundly augment metastasis and chemo resistance in many tumor epithelial cell and growth suppressive effects on normal epithelial cells, by inducing epithelial to mesenchymal transition (EMT), the precise changes on the metabolism are not known. The goal of this study was to identify and characterize metabolic changes associated with EMT in pancreatic cancer. PANC1 and BxPC3 cell lines were treated with TGF- $\beta$  which resulted in the reduction of epithelial marker E-cadherin and increase in N-cadherin and Vimentin that are the markers of the mesenchymal phenotype. Phase contrast microscopy revealed changes in cell morphology indicative of EMT in PANC1 cells. We subsequently performed untargeted metabolomic profiling in the pancreatic cell lines before and after treatment with TGF- $\beta$  using UPLC-ESI-QTOF-MS (Ultra performance Liquid Chromatography – Electrospray Ionization- Quadrupole-Time of Flight Mass Spectrometry). Feature extraction was performed using XCMS followed by multivariate analysis for selection of significant features ( $p \leq 0.05$ ). Functional pathway analysis revealed the induction of pathways associated with oxidative stress and cell differentiation. The validation of metabolites participating in these pathways and their role in mediating pancreatic cancer progression is ongoing.

### GLUTATHIONE SUCCINATION IN FUMARATE HYDRATASE DEFICIENT CELLS

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Loss of function of fumarate hydratase (FH), the mitochondrial tumor suppressor and tricarboxylic acid (TCA) cycle enzyme, is associated with a highly malignant form of papillary and collecting duct renal cell cancer. The accumulation of fumarate in these cells has been linked to the tumorigenic process. The high level of fumarate leads to the chemical succination of glutathione. The comprehensive metabolomics study of cell lines and kidney tissue discovered the succinicGSH substantially high in fumarate hydratase deficiency category. Consequently, the oxidative stress was observed higher in Fh1<sup>-/-</sup> cells. Furthermore, from stable isotopologue analysis, it is observed that the biosynthesis rate of glutathione and cystine uptake are increased in Fh1<sup>-/-</sup> cells, which consequently make these cells more sensitive to cystine deprivation.

**USE OF MASS SPECTROMETRY IMAGING COMBINED WITH METABOLOMICS STUDY TO EVALUATE DRUG EFFICACY AND IMPACT ONTO BIOLOGICAL ENVIRONMENT**

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The pharmacokinetics and pharmacodynamics (PK/PD) are important information to understand drug efficacy and toxicity in drug development. Pharmacokinetic data can be provided by Mass Spectrometry imaging which can not only localize drug within tissue, but also determine its concentration. Unfortunately, no pharmacodynamics information is usually generated by mass spectrometry imaging (MSI). The combination of targeted High Resolution MSI experiment with untargeted metabolites identification and distribution on the same biological sample might address this issue. This presentation concerns the study of the endogenous metabolite composition in brain tumor models in vivo.

High resolution mass spectrometry (Solarix FTICR 7.0T from Bruker) was used to perform metabolomics and imaging experiment on tissue (in negative or positive mode). The ionization source coupled to FTICR were the LESA-nano-ESI (Advion) and the MALDI with SmartbeamII laser (Bruker). the target molecules were endogenous metabolites in Glioblastoma xenografts in mouse brain, including tumors carrying a mutation in the gene isocitrate dehydrogenase (IDH). Visualization and Quantitation from LESA and MALDI data was generated by proprietary Quantinetix software (ImaBiotech).

Lactate and its metabolites, the 2-hydroxyglutarate (2-HG) and the ketoglutarate have been targeted in Xenograft Glioblastoma tissues. The latter two molecules are intermediates in the Krebs cycle and have recently been implicated in glioma development. The quantification and profiling analyses of brain tumor related metabolites were achieved with a LESA/MALDI combining approach (validated by LC-MSMS). Higher concentration of 2-HG was observed in Glioblastomas carrying the IDH1 mutation. The metabolite was only detected in the tumor region and not in the surrounding brain area, using the tissue extinction coefficient approach with LESA-ESI-FTICR analyses. Additional endogenous metabolites were followed in order to obtain potential readouts of tumor in tissue. This experiment emphasized the detection of 14 metabolites with particular distributions according to specific regions of the brain tumor section.

In conclusion, the new process which combines QMSI and metabolomics provides valuable information to support not only pharmacokinetics but also pharmacodynamics. investigations.

**STANDARDIZING THE SAMPLE HANDLING PROTOCOL FOR METABOLIC PROFILING OF A *HeLa* CELL CULTURE**

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Immortalized mammalian cell lines have been widely used in molecular biology and functional genomics as an easily manipulated model system. Effective quantitative investigation of the cellular physiology dynamics using these cell cultures based on omic analyses requires that the experimental design is properly adjusted so that any required sample handling before cell quenching has a minimal effect on cellular physiology. Sample handling of adherent immortalized cell lines may involve cell detachment and suspension in selected buffer, while cell washing from the growth medium is imperative, especially in metabolic profiling analysis. In this study, we investigated the effect of three cell handling protocols on the metabolic profile of a *HeLa* cell culture grown for 48h at 70% confluence in high glucose/high pyruvate DMEM + 10% fetal bovine serum. Protocols 1 and 2 were based on the conventional method for the collection of adherent cultured cells excluding trypsinization. In this method, mechanical cell detachment and suspension is carried out in an isotonic buffer followed by multiple washing and centrifugation steps before the addition of methanol for cellular physiology quenching (protocol 1). In protocol 2, cell detachment and suspension was performed in the cell growth medium. In protocol 3, *HeLa* cells were repeatedly washed while attached to the plastic culture plate; methanol for metabolism quenching was added upon removal of the buffer after the final washing step. All protocols were performed using as washing solution(s) either phosphate buffer saline (PBS) or saline solution (0.9% NaCl) or selected combinations of the two. Different durations of the washing steps were also tested. Gas chromatography-mass spectrometry was used to measure the intracellular and medium profiles at the various steps of the sample handling procedure. All measurements were interpreted in the context of the known cancer cell metabolic physiology and the Warburg effect. Comparative evaluation of the metabolic profiles indicated as mildest for cell physiology the protocol in which cell detachment before quenching is avoided and the fastest effective washing procedure is pursued; in this case, the effect on cell physiology is independent of the washing solution(s) used. As such, this protocol should be preferred for the metabolomic analysis of adherent mammalian cell cultures.

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## **SECRETOMIC ANALYSIS OF POTENTIAL RESISTANT MARKERS IN HUMAN UTERINE CANCER CELLS**

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Drug resistance has been the obstacle to cause failure in chemotherapy for decades. In this study, we used doxorubicin-sensitive uterine sarcoma cell, MES-SA, and self-developed two different levels of doxorubicin-resistant cells: MES-SA/DxR 2  $\mu$ M and MES-SA/DxR 8  $\mu$ M followed by increased Doxorubicin treatment for secretomic analysis. The secreted proteins play crucial roles in pathological processes including cancer differentiation, angiogenesis, migration and invasion. In order to analyze doxorubicin resistance-induced secreted protein alterations, the global analysis of secreted fractions of resistance cells is essential to fully understand the mechanism of resistance regulations. In this study, 89 differentially secreted proteins have been identified representing 53 unique gene products. These secreted proteins mainly involve in cell proliferation, metabolism and redox homeostasis. In the followed-up studies, RNA interference and overexpression strategies will be applied to verify the roles of the identified proteins in the formation of drug resistance. Further studies will investigate the obtained potential resistant-associated proteins in a serial of resistant cell lines and uterine patient's specimens before they can be served as resistant biomarkers. To sum up, the secreted protein analysis will provide another dimension to combat against drug resistance and some of potential secreted proteins will be evaluated to be diagnostic and prognostic biomarkers in resistance detection. The current cell line models will be extended into a metabolomics analysis for further drug resistant research.

**DISCOVERY OF NEW METABOLIC BIOMARKERS FOR THE DIAGNOSIS OF PANCREATIC DUCTAL ADENOCARCINOMA (PDAC)**

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Background and objective: Pancreatic cancer (PDAC) is characterized by poor prognosis with 5-year survival rates of less than 5% and an increasing worldwide incidence. Early symptoms are rare and nonspecific. We have therefore conducted discovery and confirmation studies to identify metabolite plasma biomarkers for the detection of pancreatic cancer and the differentiation from chronic pancreatitis.

Material and methods: The retrospective study was conducted in three phases: An initial pilot study on plasma samples was followed by analysis of a second plasma sample collection and a serum sample collection from pancreatic cancer, chronic pancreatitis and liver cirrhosis patients as well as matched healthy blood donors. Metabolomic profiles of plasma and serum samples were generated applying high quality polar and lipid GC-MS and LC-MS/MS technology (MxP™ Broad Profiling). In addition, targeted platforms for steroids and lipids (MxP™ Steroid, MxP™ Lipids) were applied. Up to 477 metabolites were analyzed semi-quantitatively or quantitatively. Statistical data analysis was done by linear models (ANOVA) on log<sub>10</sub> transformed data considering age, gender, BMI and sample storage time as fixed effects. A panel of 10 metabolites was selected for the creation of a diagnostic biomarker. The selection of metabolites was done via classification by using the Random Forest (RF) algorithm with forward feature selection. The predictive ability of the biomarker was evaluated through the estimation of ROC characteristics and AUC values from Bootstrap-based Cross-Validation.

Results: Within the three consecutive studies, some lipids were consistently and significantly increased in the pancreatic cancer group relative to the corresponding pancreatitis group whereas certain amino acids and amino acid related metabolites were consistently and significantly decreased. The multimarker panel identified by Random Forest consisted of 10 metabolites and provided an AUC=0.85 when discriminating between pancreatic cancer and pancreatitis. When the CA19-9 data (AUC=0.82) was included in the analysis, an AUC= 0.94 was reached.

On the basis of these data a multicenter study has been initiated to validate the biomarker candidate.

## IMPACT OF DROUGHT STRESS ON THE METABOLITE PROFILES OF BARLEY KERNELS

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Due to global climate changes, cultivated crops are increasingly exposed to drought conditions. The resulting water deficit may lead to complex responses affecting agronomic performance. Considering the role of metabolites as end products of cellular processes, they should also reflect the response of plants to this type of abiotic stress. A gas chromatography-mass spectrometry (GC-MS)-based metabolite profiling method, previously shown to be suitable for comprehensive analysis of genetic and environmental impact factors on plants, was applied to investigate changes in the metabolite profiles of barley kernels in response to drought stress.

Different genotypes were grown in a field trial under (i) normal weather conditions as control and (ii) under simulated drought conditions, using a Rain-Out-Shelter. The same barley genotypes were also grown in a second field trial at another location. Both experiments were performed in three seasons (2010-2012). Samples were subjected to a GC-MS metabolite profiling procedure based on the extraction and fractionation of a broad spectrum of non-polar (*e.g.* triglyceride-derived fatty acids, free fatty acids, fatty alcohols, sterols) and polar (*e.g.* sugars, sugar alcohols, acids, amino acids and amines) low molecular weight-constituents.

The assessment of the profiling data by means of multivariate and univariate statistical analyses revealed a differently pronounced impact of drought stress on the barley metabolites depending on the genotype. The metabolite profiles of the polar constituents were strongly influenced by drought conditions. In contrast, no significant differences were observed for the non-polar compounds. The metabolic responses to drought stress were strongly dependent on the growing year.

## **METABOLITE PROFILING OF CROPS: ASSESSMENT OF FOOD QUALITY AND SAFETY**

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Metabolites are the end products of cellular processes and represent the ultimate reflection of the response of biological systems to genetic and environmental changes. Metabolomics-based approaches aspire to provide a comprehensive picture by extracting, detecting, identifying and quantifying a broad spectrum of metabolites present in complex biological systems. The unbiased and non-targeted screening of metabolic profiles in combination with appropriate statistical tools enables the evaluation of major impact factors on food quality and safety.

Different crops including cereals (maize, rice, barley) and legumes (soybeans, mung beans) were subjected to a metabolite profiling approach based on GC/MS. The objective was to investigate the impact of genetic background (different cultivars), breeding strategy (genetic engineering, mutation breeding), environmental conditions (growing location, season), farming practice (organic farming, conventional farming), stress (drought), and food processing (malting, sprouting) on the respective crop metabolic phenotype.

The employed extraction and fractionation methodology allowed a comprehensive coverage of a broad spectrum of low molecular weight metabolites ranging from lipophilic (fatty acid methyl esters, hydrocarbons, free fatty acids, fatty alcohols, sterols, tocopherols) to hydrophilic (sugars, sugar alcohols, organic acids, amino acids, amines) compounds. The resulting GC metabolite profiling data were assessed by means of multivariate and univariate statistical methods such as PCA, HCA and ANOVA. The presented metabolite profiling was shown to be suitable to the investigation of different plant systems. The data generated in the course of the GC/MS-based profiling can provide valuable data regarding the safety status and the nutritional quality of raw and processed foods.



**MULTI-RESPONSE OPTIMIZATION OF METABOLOMICS PROTOCOL BASED ON RESPONSE SURFACE METHODOLOGY**

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Given the complexity of the metabolome exhibiting a wide spectrum of chemical and physical properties, finding the best compromise for identification and quantification of the metabolites is often very challenging. Searching for this optimal setting through a conventional one-variable-at-a-time procedure makes the task daunting. Multi-response optimization based on response surface methodology could serve as a systematic alternative approach. This procedure allows searching for optimal parameters while simultaneously considering gross detection enhancement of various metabolic classes. We applied a multi-response optimization approach based on Derringer's (desirability) function to optimize the derivatization step of the GC-MS protocol. For studying the effect of derivatization parameters on metabolite response, RSM based on Box-Behnken Design with six factors and three levels was employed. Peak area of different metabolic classes, derivatization products and artifacts were used as response parameters. Subsequently, second order polynomial models were fitted to the responses and each response was transformed into an individual desirability index. After defining the optimization criterion of each response, the individual desirability's were combined into a single global desirability index using geometric mean. Finally, a search was performed on this index for all possible combination of factors. The significance of each factor and interaction between them on model quality was assessed by analysis of variance. Considering linear effects, the volume of BSTFA, methoxyamine hydrochloride, methoxymation temperature and duration had major effect on most metabolites. In contrast, silylation temperature did not show a dramatic effect. Moreover, the contour plots display interesting interactions among the factors. Based on global desirability values, the best optimal set of derivatization parameters were selected and validated on apple tissue.

**METABOLOMIC DIVERSITY IN THE LEAVES OF DIFFERENT VARIETIES OF *PIPER BETLE***

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*Piper betle* L. (Piperaceae) leaves (also known as betel leaf or 'paan') are largely used as masticatory in South East Asian countries. The leaves are also medicinally and economically important. Different local varieties are available for consumption. Such varieties differ from each other in aroma and taste. The objective of the present study was to characterise the chemical constituents of the varieties available in West Bengal following metabolomics approach. Eight local varieties of betel leaves e.g., Bangla, Bagerhati, Manikdanga, Meetha, Kalibangla, Chhaanchi, Ghanagete and Haldi were collected from different locations of West Bengal, India, during the month of October, 2012. Metabolic profiling of these varieties was performed using GC/MS analysis. Ninety nine metabolites (21 amino acids, 19 organic acids, 3 fatty acids, 5 phenols, 7 sugar alcohols, 19 sugars, 13 essential oils and 12 other metabolites) were identified by comparison of the MS fragmentation patterns with NIST library and with Fiehn GC/MS Metabolomics RTL Library Software (2008). The relative variability within the different varieties was explored using Principal Component Analysis. Some varieties separated distinctly from the others. A few metabolites responsible for the variation could be identified. The heterogeneity between the eight varieties was also studied using hierarchical cluster analysis of the metabolite profile. The varieties could also be differentiated on the basis of essential oil constituents.

**P4-5**

**COMPARATIVE METABOLIC PROFILING OF RICE GRAINS REVEALS PRIMARY METABOLITES ARE CORRELATED WITH SECONDARY METABOLITES**

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Gas-chromatography coupled with time-of-flight mass spectrometry (GC-TOFMS) was used to analyze the relationships between primary metabolites and phenolic acids in rice (*Oryza sativa* L.), including six black cultivars and one white cultivar. The metabolite profiles were subjected to data mining processes, including principal component analysis (PCA), Pearson's correlation analysis, and hierarchical clustering analysis (HCA). PCA could fully distinguish between these cultivars. HCA of these metabolites resulted in clusters derived from common or closely related biochemical pathways. There was a positive relationship between all phenolic and shikimic acids. Projection to latent structure using partial least squares (PLS) was applied to predict the total phenolic content based on primary metabolite profiles from rice grain. The predictive model showed good fit and predictability. The GC-TOFMS-based metabolic profiling approach could be used as an alternative method to predict food quality and identify metabolic links in complex biological systems.

**METABOLIC PROFILING OF LYCIUM CHINENSE FRUITS USING GAS CHROMATOGRAPHY-TIME-OF-FLIGHT MASS SPECTROMETRY FOR QUALITY ASSESSMENT BASED ON VARIETY**

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*Lycium* species have been used as food and as medicinal plants for centuries in East Asian countries. They have become increasingly popular in Europe and North America since the beginning of the century, because of their health-enhancing effects. However, its functional components have not been elucidated. In this study, we identified 55 primary metabolites in *L. chinense* fruit, including both hydrophilic metabolites and lipophilic compounds such as carotenoids, tocopherols, and phytosterols. The metabolite profiles obtained from 11 cultivars were applied to data mining processes including principal component analysis (PCA) and hierarchical clustering analysis (HCA). PCA showed that the Cheongdang (LM-3) cultivar was distinct from the others. The correlation results of a total of 55 compounds revealed strong correlations between metabolites that participate in closely related pathways. The Cheongdang cultivar appears to be suited for functional food production. The results of this study suggest that metabolite profiling combined with chemometrics can be used as powerful tool for assessing food quality. This study provides valuable information regarding future conventional breeding programs for *L. chinense* containing carotenoids, tocopherols, and phytosterols. The Cheongdang cultivar could be of high dietary value.

**THE DETERMINATION OF SUBSTANTIAL EQUIVALENCE FOR CAROTENOID BIOFORTIFIED RICE USING COMPARATIVE ANALYSIS OF METABOLIC PROFILING**

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Safety assessment of genetically modified (GM) food is based on the concept of substantial equivalence (SE), developed by the Organization for Economic Co-operation and Development (OECD) and further elaborated by the Food and Agriculture Organization/World Health Organization (FAO/WHO). Metabolomics is of interest because the composition of low-molecular-weight molecules includes important nutritional and toxicological characteristics. Thus, the metabolomics approach has been developed and applied to identify unintended effects on a new gene insertion. To investigate substantial equivalence among carotenoid-biofortified GM rice and five conventional rice cultivars having common white (three) and red (two) grain colors, profiles of 52 polar metabolites were analyzed using gas chromatography time-of-flight mass spectrometry. The results were compared to evaluate the differences among GM and non-GM rice cultivars using principal components analysis. The GM rice is more comparable to its non-transgenic counterpart rice variety according to the closer co-separation than for other cultivars tested. Metabolic profiling using GC-TOFMS is a useful tool for investigating compositional similarities between GM and conventional crops. This tool also provides a potential method for assessing undesirable changes in GM crops that enhance a nutrient or create novel nutrients through the intentional modification of metabolic pathways.

**A NEW METHOD FOR PLANT METABOLOMICS STUDY BASED ON CAPILLARY ELECTROPHORESIS-TIME OF FLIGHT MASS SPECTROMETRY**

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Many metabolites in plant are highly polar and ionic. Their separation using GC-MS and LC-MS can be problematic because of their inherent defects. However, the electrically driven CE-TOF-MS with its high efficiency, few organic solvent consumption and good selectivity is particularly suitable for the analysis of these polar and charged analytes in complex biological matrices. For this reason, a CE-TOF-MS method was developed to investigate polar metabolic profiling in plant.

It is well recognized that the pretreatment of samples is the first step for metabolomics analysis. Design of experiments (DOE) was used to optimize the extraction solvent system. A series of fourteen protocols were evaluated based on the response surface representation of area, the peak height and the number of the detected metabolites. The optimal extraction protocol of metabolic profiling was found to be chloroform/methanol/water system. Method validation was carried out to evaluate the analytical characteristics. The RSD of inter- and intra- precision at low, middle and high spiking level of internal standards are less than 11.5%. The samples are stable within 72 hr (e.g., RSDs of most of the detected peak areas < 15%). The extraction reproducibility of 86% compounds has RSDs less than 15%. The developed method is successfully applied in study of tobacco leaf metabolomics. A total of 149 metabolites were identified including sugar phosphates, nucleoside, nucleic acid and coenzyme. The metabolic profiling from CE-TOF-MS can clearly discriminate tobacco leaves of different flavours and geographical origins. The relationship among plant metabolic pathways, geographical origins and its growth is well elucidated.

**METABOLOMICS OF DEFENSIVE CHEMISTRY: MS-BASED CHARACTERIZATION OF SESQUITERPENE LACTONE- AND HYDROXYBENZENEACETIC ACID- DERIVED DEFENSIVE METABOLITES OF *TARAXACUM OFFICINALE* ROOT LATEX**

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Plant roots produce an enormous diversity of chemicals which are thought to be important for plant resistance against herbivores and pathogens. Yet, only few studies have conclusively demonstrated a defensive function of root secondary metabolites. We studied the chemical composition of the root latex, of *Taraxacum officinale* as a first step to fill this knowledge gap. Latex was often associated with a defensive function before.

We found two major classes of secondary metabolites: Sesquiterpene lactone glycosides and inositol-4-hydroxybenzeneacetic acid derivatives. The identification and structural elucidation of the individual molecules is a first crucial step to test the mayor defensive role against root herbivores in vitro bioassays.

Chromatographic separation of extracts was carried out using an UHPLC system combined with high-resolution Q-TOF MS detection. Enriched extracts of sesquiterpene lactone glycosides and hydroxybenzeneacetic acid derivates were subjected to detailed fragmentation studies by means of MS2 measurements on an ESI-Q-TOF instrument in positive ion mode. Full scan spectra and MSn product ion spectra were recorded for structural assignment and confirmation on an ion trap instrument.

We created MS2 and MS3 libraries from *T. officinale* root latex in positive ion mode and annotated 90 non-redundant fragments. Based on this annotation we were able to identify diagnostic fragments for the sesquiterpene lactones, for the inositol-4-hydroxybenzene-acetic acid derivates and their side-groups as well as compound-specific ions. We defined a set of rules for deduced neutral losses and characteristic adduct formation. This set of rules in combination with the MS/MS library was applied to characterize extracts of different *T. officinale* accessions and different treatments after herbivory attack.

Based on these fragmentation schemes and rules we were able to propose 8 novel inositol-4-hydroxybenzeneacetic acid derivates and 3 taraxinic acid derivates.

The established workflow, solely based on mass spectrometry, speeds up metabolite re-identification in new biological matrices and is readily extendable, after preliminary fragmentation studies, to many more compound classes.

**P4-10**

**ANALYSIS OF BARLEY (*HORDEUM VULGARE* L.) PROTEOME AND METABOLOME SUBJECTED TO DROUGHT STRESS WITH MASS SPECTROMETRIC METHODS**

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Barley is one of four most important cereals in worldwide production, used for food, feed and malt production. It is considered as a model system due to short life cycle and good morphological, physiological and genetic characterization. Drought is one of the greatest factor limiting plant growth and the productivity of crops.

The aim of conducted research was comparison of changes in barley proteome and metabolome under drought stress. An attempt of linking metabolites synthesis with corresponding enzymes (proteins) expression was undertaken.

Changes in protein and metabolite expression patterns in response to water deficit were monitored in four different genotypes of barley (Maresi, Cam/B1/Cl, Sebastian and Stratus). Plants were grown in greenhouse under controlled environmental conditions and subjected to drought stress. Leave samples were harvested after 10 days of drought.

Comparative analysis of proteins was studied by 2D gel electrophoresis and MALDI-TOF mass spectrometry. Proteins for qualitative and quantitative analysis were isolated by phenol extraction.

Qualitative and quantitative analyses of barley metabolite extract samples were performed using 6890N gas chromatograph (Agilent) – GCT Premier mass spectrometer (Waters) controlled using Waters MassLynx software version 4.1.

Implemented techniques allowed identification 52 proteins in leaves of Maresi variety, Cam/B1/Cl revealed 32 proteins in leaves, in two other varieties were recognized about 45 proteins. These proteins were classified on the basis of biological function in the cell. Proteins belonging to different classes, identified in leaves demonstrated changes in expression level. Only few proteins belonged to various classes of enzymes involved in metabolic pathways of defined metabolites.

Identification of 65 primary and secondary metabolites was achieved. Plants exposed to water deficit revealed changes in metabolite profiles in comparison with control plants, particularly in abundances of some amino acids and sugars.

Some of the results obtained at proteomic and metabolomic level were correlated and are presented on the poster.



P4-11

**TOWARD BETTER ANNOTATION OF PLANT METABOLOME: ISOLATION AND STRUCTURE ELUCIDATION OF SECONDARY METABOLITES FROM *ORYZA SATIVA* (RICE)**

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In recent years, metabolomics has rapidly developed in plant science. It has been used in the investigation of biological mechanisms related to genetic and/or environmental factors. Liquid chromatography-mass spectrometry (LC-MS) is a common approach for metabolite identification using authentic standards by applying fragment patterns of tandem mass spectrometry (MS/MS) spectra in combination with the retention time or on-line UV spectrum data. High-resolution MS/MS spectral data reported in literatures and databases are also useful to metabolite annotation. However, there are over 200,000 metabolites in plant kingdom, many of them remain unknown, and even the known secondary metabolites are not always commercially available. Therefore, in MS based metabolite profiling, substantial number of metabolites detected as MS peaks are still not well characterized. Thus, metabolite identification is a major bottleneck in non-targeted metabolomics studies. Thus, isolation and structural elucidation of (un)known metabolites by combining a variety of spectroscopic methods such as nuclear magnetic resonance (NMR) are important in plant metabolomics. For the better annotation of plant metabolome in general, we aim to elucidate structures of isolated compounds from the model plants using MS/MS and NMR methods. In the present study, we have investigated the isolation and identification of secondary metabolites from *Oryza sativa* (rice), which is the best world-important crop. Thirty six compounds including three novel flavonoids were isolated from the leaf of *O. sativa*. Majority of isolated compounds are the glycosylflavonoids of tricetin, apigenin, and chrysoeriol as aglycones. The purified compounds and MS/MS data will enrich our spectral database, ReSpect <<http://spectra.psc.riken.jp/>>, and thus benefit for the better annotation of plant metabolome.

**METABOLOMICS AND CHEMOMETRICS STUDY TO MONITOR THE BIOCHEMICAL CHANGES AND  $\alpha$  – AMYLASE INHIBITORY ACTIVITY DURING POST-HARVEST RIPENING IN *ACHRAS SAPOTA* L. FRUITS**

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*Achras sapota* L. (syn. *Manilkara zapota* (L.) P. Royen) commonly known as Sapodilla, is one of the fruit crops in West Bengal, India. The unripe fruits, very firm and astringent in taste, ripen after plucking. The aim of the present investigation was to determine the changes in metabolic composition during post-harvest ripening that result in the formation of taste and nutritional quality and to correlate the metabolites with  $\alpha$ -amylase inhibitory activity. Mature, unripe fruits of *A. sapota* (variety Cricket Ball) were collected right after their harvest. These fruits were stored at room temperature over a span of 10 days. Each day, the methanol extract of the homogenized flesh of fruits were assayed against  $\alpha$  – Amylase *in vitro*. A metabolomics approach was adopted to monitor the biochemical changes during post-harvest ripening using gas chromatography-mass spectrometry (GC-MS). During the study a total of 75 metabolites (18 organic acids, 16 amino acids, 8 phenolics, 7 fatty acids, 23 sugar and sugar alcohols, and 3 other components) which showed significant differences at different stages of ripening, could be identified. All the data underwent a variety of chemometric analyses, including principal component analysis (PCA) and partial least squares analysis (PLS), to identify the metabolic differences between ripening stages and the metabolites influencing the enzyme inhibitory activity. The fruits showed maximum activity on Day 1. In the subsequent days there was reduction in activity profile with the lowest being observed on Day 10. Based on the variable importance plot (VIP) and PLS coefficient plot, a number of key metabolites were identified. Citric acid, malic acid, raffinose and sucrose were among the constituents influencing most in the enhancement of  $\alpha$  – Amylase inhibitory activity in the fruits while an inverse relationship was found with proline, glycine, 4-guanidinobutyric acid and valine. Thus the analyses revealed that a variation in the composition of these significant constituents accounted for a marked alteration in the activity of the fruits.

**METABOLIC EXPLORATION OF ANOXIA-INDUCED WAX-ESTER SYNTHESIS IN *EUGLENA GRACILIS***

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Saturated wax esters from *Euglena gracilis*, typically composed of myristic acid (C14:0), attract attentions as a candidate for biodiesel feedstock. The wax ester fermentation is strongly evoked by anoxia, whereas key events underlying the metabolic shift toward the lipid biosynthesis are poorly understood. A wild type *E. gracilis* Z strain and a chloroplastless mutant bleached by streptomycin were sampled at 0, 4 and 24h after the anaerobic shift. Metabolites were extracted with methanol:chloroform:2%acetic acid (5:2:1) from lyophilized samples. Apolar fraction from phase separation was subjected to GC-MS analyses to compare the accumulation profile of wax esters and fatty acid methyl esters (FAME). Profiling of 56 different wax esters demonstrated the clear initiation of wax ester fermentation within 4h after the cessation of oxygen supply. Both strains exhibited similar wax ester profiles with C14:0 FAME and C13:0 fatty alcohols as the dominant lipids, while different PUFA (polyunsaturated fatty acids) profiles were detected, possibly reflecting their difference in organelle (membrane lipids). On the other hand, the RNA-seq data for the same samples did not show alteration of the global transcriptomic landscape, implying immediate metabolic response toward the wax ester fermentation without gene regulatory shifts.

**THE EFFECT OF GENETICS AND ENVIRONMENT ON THE METABOLOME OF COMMERCIAL MAIZE HYBRIDS USING LC/MS: A MULTISITE STUDY**

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It is imperative to understand biological variation in expression of metabolites due to genotype, environment, or both to assess the potential of metabolomics to supplement compositional analysis for substantial equivalence assessment of genetically modified crops. We recently applied GC/TOF-MS and advanced multivariate analysis to extensively study and elucidate biological variation of many metabolites due to environment and genotype in 695 forage and 654 grain maize samples collected from 50 non-GMO DuPont Pioneer commercial maize hybrids grown at 6 North America locations. The results revealed that from a total of 156 and 185 metabolites measured in grain and forage samples, respectively, the environment had far more impact on the forage metabolome compared to the grain metabolome. Fifty percent of the metabolites were affected by the environment compared to less than 2% by the genetic background. However, GC/TOF-MS detects only primary metabolites. We here report on extending the metabolome coverage in these grain and forage samples using LC/MS. High resolution FT-ICR MS facilitates putative identification of statistically interesting unknown plant secondary metabolites. The results of this study will not only complement our prior GC/MS work, but will further our understanding of the biological context that must be placed on metabolomics data if used to supplement compositional analysis for substantial equivalence assessments.

**THE GC-MS METABOLOMICS APPROACH TO FIND BIOACTIVE COMPONENTS OF BLUEBERRY, RASPBERRY AND BLACKBERRY**

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Blueberry (*Vaccinium corymbosum* L.), Raspberry (*Rubus idaeus* L.) and Blackberry (*Rubus* sp.) have essential dietary minerals and phenolic compounds and been used health functional food as antioxidant, antimicrobial, anticancer and anti-inflammatory. Bioactive components of berries have studied on horticulture and nutrition depending on their growing condition. The goal of this study was applied metabolomics approaches to develop metabolic profiling in blueberry, raspberry and blackberry with gas chromatography-mass spectrometry (GC-MS) and mass profiler professional. Mass spectrometry based metabolite profiling in combination with multivariate data analysis was introduced to quantity changes in metabolic patterns. Blueberry, raspberry and blackberry were sequentially extracted with hexane, ethyl ether, ethyl acetate and methanol and analyzed by GC-MS. The data of whole component of berries were processed by principle component analysis (PCA) as multivariate analysis to determine whether separate classes of extraction solvents. PCA score plot was obtained from hexane and ethyl ether extracts with good discrimination between berries. And then, loading plot analysis performed to increase accuracy of data processing was collected 139 peaks. A hierarchical cluster analysis using ANOVA test ( $p < 0.001$ ) was categorized 28 peaks of main component in berries. Fatty alcohols and fatty acids were commonly found in berries. Although we have not yet identified the component of berries, fatty acids in berries are known as beneficial for human health in preventing heart disease.

**METABOLOMIC FINGERPRINTING EMPLOYING UPLC-TOFMS FOR THE IDENTIFICATION OF THE ORIGIN OF AGRICULTURAL PRODUCTION SYSTEMS**

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A growing market share and a premium price of organically grown products are the background for the need to develop analytical methods which aim to discriminate conventionally and organically grown agricultural products. The interest of consumers in organic products mainly stems from health and environmental considerations. Mass spectrometry-based fingerprinting techniques are increasingly being used to study how the metabolome of an organism changes as a result of biotic and/or abiotic stresses. In this study, the metabolite fingerprint of organic and conventional carrots (*Daucus carota* L.) was analysed using ultraperformance liquid chromatography-time-of-flight-mass spectrometry (UPLC-TOFMS). Using this method, a comparison of the metabolome of organic and conventional carrots harvested in 2005, 2006, 2007 and 2008 from three different environments revealed significant and consistent differences in a number of metabolites. By applying the partial least squares discriminant analysis, 91% of organic carrot samples were correctly classified. Our study shows that a UPLC-TOFMS fingerprinting methodology is a valuable approach to investigate and understand specific metabolic alterations as a result of changes in environmental conditions such as the influence of different agricultural production systems in the plant physiology.

P4-17

**DISCRIMINATIVE ANALYSIS OF OIL PALM SPEAR LEAF METABOLOME OBTAINED FROM OIL PALM GROWN UNDER DIFFERENT PLANTING CONDITIONS**

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The presence and level of an organism's metabolome reflects the phenotypic response to environmental condition and changes while describing its genotype. Scarcity of arable land leads to crop improvement through biotechnology and also planting on peat. A sound and systemic corroboration is required to discriminate environmental effects on oil palm planted on different settings. LC-MS-based metabolomics and multivariate statistical analysis were carried out on oil palm clones to allow the discovery of patterns discriminating the specimens under study. The demarcation between the oil palm clones planted in the field and in containment will serve as baseline data for biosafety screening while analysis of oil palm clones planted on peat and mineral soil facilitates the understanding of environmental influence especially soil type onto plant chemicals biosynthesis.

P4-18

## **METABOLIC FINGERPRINTING FOR OIL PALM BASAL STEM ROT DISEASE DIAGNOSTICS**

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Basal stem rot (BSR), caused by the wood-rotting fungal pathogen *Ganoderma boninense*, is considered the most lethal disease of oil palm in the world. The available control measures for BSR disease such as cultural practices, mechanical and chemical treatment have not proved satisfactory to cure the disease. However, little information is available on both the genetic and biochemical basis of plant resistance to BSR. To gain a better understanding on defense mechanism to *Ganoderma boninense*, the metabolite profiles of methanolic extracts of root tissues from partially tolerant and susceptible parental palms were compared using liquid chromatography coupled with a Q-TOF mass spectrometer. Chemical constituents ranging from sugars to flavonoid derivatives were profiled and identified by tandem mass spectrometry. Data analysis was performed using multivariate analysis to reveal chemical constituents that discriminate between the datasets. These findings generated a substantial amount of metabolites data for oil palm and contributed to the identification of candidate compounds related to oil palm tolerance, thus will improve the current understanding of BSR caused by *Ganoderma boninense*. The data also offers huge potential to highlight biomarkers and support of pathology investigations in oil palm *Ganoderma* research program.



**METABOLITE PROFILE IN SUGARCANE VARIETIES, HYBRIDS AND SPECIES WITH HPLC-DAD COMBINED WITH MULTIVARIATE ANALYSIS**

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Metabolomics approaches enable the assessment of a broad range of metabolites and have been documented to have great phenotyping value and diagnostic analyses in plants. Considering this, the present work describes the application of a method developed by our group for the metabolomic analysis of sugarcane. A total of 16 genotypes comprising of 11 varieties, 2 hybrids and 3 noble and wild species were collected from the sugarcane collection center - "Centro de Cana, Instituto Agronômico – (IAC)" SP, Brazil. Sixteen genotypes of sugarcane were previously classified into three groups based on the different phases of sugarcane genetic breeding. Immediately after the harvest, the leaves were frozen under liquid nitrogen and stored at -80 °C. The samples were lyophilized and milled in a cryogenic mill. The extracts were then prepared as reported by Leme, 2011. Aliquotas (20 µL) of each extract were analyzed by HPLC-DAD using a Kinetex<sup>®</sup> C18 column eluted by gradient mode as follows: MeOH/Water 10:90 to MeOH/Water 50:50 in 45 minutes, then to MeOH 100% in 15 minutes. The data pretreatments and Principal Component Analysis (PCA) were done using Matlab<sup>®</sup>. Principal Component Analysis (PCA) was performed to investigate the correlation between chromatographic profiles aiming to identify molecular markers. In the first step, chromatograms of all the samples were normalized and subjected to PCA. A 4-components model explained 94.3% of the variance, with the first two components covering 85.4%. By the scores and loadings plot, it was possible to identify homorientin as the main molecular marker of the sugarcane leaves followed by chlorogenic acids and the flavones schaftoside, isovitexin and tricin-7-O-neohesperidoside. Due to the complexity of the chromatogram with more than 100 peaks, a new model was constructed using the relative area of peaks. The data set was normalized and autoscaled. In this new model, other variables were found to be important to the genotypes discrimination mainly due to the discrimination between *S. spontaneum* and modern varieties of sugarcane, essentially showed by the larger abundance of chlorogenic acids inherent in this specie than in modern varieties. From these results, we can infer that metabolomic approaches could help in genetic breeding programs.

## IMPROVED LEGUME CROPS FOR A SUSTAINABLE FUTURE

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The food supply chain has come under increasingly varied pressures in recent times, with an unstable climate and increasing world population providing perhaps the most significant challenges. Changing consumption patterns in relation to food and feed crops along with increasing quality expectations from consumers and retailers are further challenges for the agricultural sector. This presentation will discuss how post genomics technologies are beginning to enter the plant breeding arena potentiating the rapid development of new crop varieties. Legumes are used as an example of a sustainable crop with positive environmental attributes, the potential for reducing the European protein deficit and an established scientific base tracing back beyond Mendelian genetics.

Molecular phenotyping is the term that has been coined for the use of principally metabolomics and proteomics, when applied to breeding traits. The concept involves using non-targeted analytical measurements to support the plant breeding process by correlating genetic and phenotypic attributes. Specific examples will be presented where we have developed methodologies to examine the association between genotype and sugar composition in pea seeds. Similarly, nutritional and flavour attributes will be considered in relation to natural and induced genetic diversity for the selection of breeding materials. This concept will be extended to look at how an integrated systems biology approach will produce new plant materials that are better able to withstand the challenges of a changing climate, such as the divergent profile of plant diseases and the increased likelihood of extended periods of drought.

P4-21

**METABOLOME ANNOTATION IN BIOMASS CROPS: uHPLC-MS, AND NMR STUDIES OF SECONDARY METABOLITES IN WILLOW**

John Baker, Michael Beale, Jane Beale

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Rothamsted Research maintains The UK National Collection of willow (*Salix* sp.) varieties as well large genetic mapping populations designed for breeding of improved bioenergy crops. The result is that many thousands of genotypes of *Salix* are available for metabolomics screening - not only in relation to biomass traits but also as in the area of novel chemical products for both heavy (biorefining) and fine (pharmaceutical) chemical applications.

The poster will present the metabolic complexity of secondary metabolism in selected high biomass genotypes of *Salix viminalis* as revealed by uHPLC coupled to very high resolution ESI-MS carried out on an Orbitrap Elite mass spectrometer. Using correlative analysis, of fractions generated by reverse phase preparative HPLC, by 600MHz NMR and LC-MS-MS techniques we have been able to identify a large number of mainly phenylpropanoid and flavonoid products that make up the *Salix* secondary metabolome. We can now confidently annotate some 25% of the top 500 retention-time/empirical formula features in willow uPLC-ESI-MS runs. This data is invaluable in identifying biomarkers arising from genetic and environmental treatments that are emerging from multivariate analysis of data from metabolomics screens by NMR and direct infusion MS, that form the core of a high throughput screening operation at Rothamsted.

Thus, the empirical formulae and MS<sup>n</sup> data emerging from the high resolution LC-MS instrumentation now form an important and integral part of a metabolomics operation that is focussed on gene and biomass/bioenergy trait discovery in *Salix* (and its close relative Poplar).

P4-22

**CROPPING CARBON – A STRATEGIC RESEARCH PROGRAMME, USING METABOLOMICS, TO OPTIMISE WOODY BIOMASS CROPS FOR BIOENERGY AND INDUSTRIAL APPLICATIONS**

Claudia Harflett, Jane Ward, Michael Beale, Ian Shield, Steven Hanley, Angela Karp

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Increasing global energy consumption has led to concerns over energy security and environmental impacts such as rising greenhouse gas emissions. The UK aims to obtain 15% of its energy needs from renewable resources by 2020. Perennial energy crops e.g. Willow trees and *Miscanthus* grasses, represent a viable energy alternative to fossil fuels and can alleviate concerns over climate change and energy security if we maintain high and consistent quality biomass yields.

Using a metabolomics approach, we can investigate the carbon flow and pathways in these high biomass crops to maximise above ground carbon harvest, whilst achieving an optimal balance between harvestable and sequestered carbon in perennial cropping systems.

To understand the optimal growth strategy of efficient high yielding willow and *Miscanthus* cultivars and genotypes requires extensive knowledge of the 1° and 2° metabolites involved, the balance between assimilated and sequestered carbon and nitrogen and the biochemical pathways involved. Studying the metabolites present in different plant parts (e.g. leaves, roots, buds and stems) at different growth stages and under different environmental conditions helps reveal interactions between newly assimilated and recycled carbon.

Using extensive genetic resources in The National Willow Collection and established mapping populations the project aims to identify mQTLs with relevance to biomass and novel products.

The project therefore includes a substantial metabolomics effort addressing one of the grand challenges of modern crop science.

## **METABOLITE PROFILING STUDY OF BURDOCK ROOTS IN RESPONSE TO COPPER STRESS**

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*Arctium lappa* L. (Asteraceae), known as burdock, has long been cultivated as a popular vegetable for dietary use and folk medicine worldwide, as a diuretic and antipyretic tea as well as hypertension, gout, arteriosclerosis, hepatitis and other inflammatory disorders. Stress in plants could be defined as any change in growth condition(s) that disrupts metabolic homeostasis and requires an adjustment of metabolic pathways in a process that is usually referred to as acclimation. Exposure to heavy metals is one of the most prevalent environmental stresses encountered by plants.

In this study, metabolite profiling coupled with multivariate analysis was applied to achieve a holistic view of the copper stress response in burdock roots and conduct detailed pathway analysis using <sup>1</sup>H NMR and GC-MS analysis. PCA model from NMR data showed significant separation between control and copper-treated burdock roots. Copper-treated burdock roots were characterized by the increased phenols and decreased primary metabolites such as sugars, amino acids and organic acids. These results suggest that it is related to the activation of phenylpropanoid pathway and growth inhibition. GC-MS analysis showed the increased levels of unsaturated fatty acids and decreased levels of sterols in the copper-treated group. These changes could be considered as the result of the damage and loss of membrane integrity.

This study demonstrates that metabolomic profiling is an effective analytical approach to understand metabolic pathway associated with copper stress in burdock roots.

**DE NOVO RNA SEQUENCING AND METABOLITE PROFILING TO IDENTIFY GENES INVOLVED IN ANTHOCYANIN BIOSYNTHESIS IN KOREAN BLACK RASPBERRY (*RUBUS COREANUS* MIQUEL)**

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The Korean black raspberry (*Rubus coreanus* Miquel) is commonly consumed as fresh fruit or processed foods, but it is also an enriched source of traditional herbal medicine. Korean black raspberry (KB) as an unripe fruit is used in traditional herbal medicine and its ripe fruit is used as a processed food, such variety in stage specific usage of KB has been assumed due to the changing metabolite profile at different stages of fruit ripening. So far, molecular and biochemical changes during its fruit maturation are poorly understood. To analyze biochemical changes during fruit ripening process at molecular level, firstly, we have sequenced, assembled, and annotated the transcriptome of KB fruits. Over 4.86 Gb of normalized cDNA prepared from fruits was sequenced using Illumina HiSeq™ 2000, and assembled into 43,723 unigenes. Secondly, we have reported that alterations in primary metabolites such as sugars, organic acids and amino acids and secondary metabolites including anthocyanins and proanthocyanidins are the major factors facilitating variations in these stages of fruits. In addition, we have demonstrated the positive correlation between the expression of anthocyanin biosynthetic genes and the anthocyanin accumulation during ripening of KB. Furthermore, the ability of RcMCHI2 (*R. coreanus* Miquel chalcone flavanone isomerase 2) gene to complement *Arabidopsis transparent testa 5 (tt5)* mutant supported the feasibility of our transcriptome library to provide the gene resources for improving plant nutrition and pigmentation. Taken together, these datasets obtained from transcriptome library and metabolic profiling would be helpful to define the gene-metabolite relationships in this non-model plant.

**TOBACCO SEEDS EXPRESSING A MUTATED FORM OF ARABIDOPSIS CYSTATHIONINE  $\Gamma$ -SYNTHASE EXHIBIT HIGHER LEVEL OF METHIONINE AND ALTERED LEVELS OF SEVERAL METABOLITES**

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Methionine is a fundamental metabolite in plant cells used as a precursor to essential metabolites. Its low level in crop plants limits their nutritional quality. The main regulatory enzyme of methionine biosynthesis pathway is cystathionine  $\gamma$ -synthase (CGS). To reveal the role of CGS in methionine accumulation in seeds, tobacco plants were transformed with the *Arabidopsis* feedback insensitive form of CGS (*T-AtCGS*) under the control of the seed specific promoter of Legumin B4. Although the level of free methionine was not significantly altered in these seeds, the content of protein-incorporated methionine was significantly elevated. Notably, the levels of most other protein-incorporated amino acids have also increased resulting in higher content of total amino-acids. These results suggest that methionine availability limits protein synthesis in seeds. To reveal the effect of high level of methionine on primary metabolites in the seeds, metabolic profiling was performed using GCMS. The transgenic seeds had increased levels of Glycerol, Xylitol, Lyxonic acid, Gluconic acid, Galactonic acid, Melibiose and Adenosine compared to the wild type, while Pantothenic acid and 3 unknown metabolites were reduced.

P4-26

## CHANGES IN METABOLITE PROFILE IN FODDER GRASSES IN PLANT ADAPTATION TO COLD

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Quality and quantity of metabolites are changing in fodder grasses in plant adaptation to cold stress conditions. Plant samples were collected on the third, fifth and twelfth day of the adaptation to cold. Monosaccharides were observed in higher amounts than oligosaccharides at the first two time-points with no significant changes of GC-MS studied metabolite profiles. A dramatic increase of sucrose level and amounts of phenylalanine, tyrosine and lysine were observed at the last time point.



**P4-27**

**IDENTIFICATION OF 6-C-(6''-O-GLYCOSYL)-GLYCOSIDE OF FLAVONES AND TRIACYLATED POLIAMINES PRESENT IN TRACE AMOUNTS IN BARLEY (*HORDEUM VULGARE*)**

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In order to establish the detailed chemical profile of barley leaves, extract from a large amount of material from variety Maresi was submitted to preparative polyamide column chromatography and reversed phase C18 HPLC. Collected effluent containing trace compounds was analyzed by HPLC-MS. Such analytical approaches enable to identify 109 metabolites including phenylpropenoic derivatives of flavone apigenin, luteolin and chrysoeriol glycosides and polyamines putrescin, hydroxyputrescin, describe for the first time in barley.

## THE EFFECT OF GENOTYPE AND ENVIRONMENT ON STEROL CONCENTRATION IN TOBACCO

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Sterols are essential molecules for all eukaryotic organisms. Tobacco sterols are present in tobacco smoke and appear in plasma of mammals exposed to cigarette smoke. Tobacco sterols may be important in the pathogenesis of smoking-induced lung and vascular diseases. Influence factor of Sterol concentration in tobacco was a concerned question, which attracts many scientists to study on. In this study, seven kinds of sterols including stigmasterol,  $\beta$ -sitosterol, cholesterol, brassicasterol, campesterol, lanosterol and chenodexychoic acid in tobacco leaves were determined by GC-MS. Three cultivars HongDa, K326 and Zhongyan100 were cultivated in Xiangxian (North of China), Zunyi (Area of the upper Yangtze River) and Dali (South of China) respectively. The data were analyzed by Partial least squares discriminate analysis (PLS-DA) using the SIMCA-P 13.0. PLS-DA result showed a clear separation of compositions among the three regions, while not among the three cultivar groups. The results showed that the sterols in tobacco leaves of Xiangxian, Zunyi and Dali were obviously different. The concentration of total sterols in tobacco of Xiangxian was significantly higher than other two regions. By contrast, the concentration of total sterols in tobacco of Zunyi was lowest. The  $\beta$ -sitosterol content in tobacco leaves obtained from Dali is very high, while the other two regions are rare. The concentration of sterols of HongDa, K326 and Zhongyan100 from same region was roughly the same. The results suggested that the effect of environment play a more important role on sterol concentration in tobacco than genotype factor.

## **METABOLITE PROFILING AND HYPERSPECTRAL IMAGING FOR SCREENING OF PATHOGEN RESISTANT SUGAR BEET GENOTYPES**

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The fungal pathogens *Cercospora beticola*, *Rhizoctonia solanii* and the viral-caused disease Rizomania are responsible for severe yield loss of sugar beet worldwide. Breeding programs aiming for pathogen resistant lines are a major strategy to minimize yield loss and also the cost for fungicide application. To speed up the selection for resistant lines during the breeding process an early detection of disease symptoms is essential. Therefore we want to establish a hyperspectral imaging system, that can 'see' disease symptoms before the human eye can. The camera measures signals in the Short Wave Infrared Range (SWIR) from 970 to 2,500 nm. Image data of control and infected leaves at different points in time after inoculation are used to calibrate a prediction model.

In addition, untargeted metabolite analysis with LC-PDA-Q-TOF-MS (liquid chromatography coupled with photodiode array detection and quadrupole time-of-flight mass spectrometry) is utilized to provide data in form of the amount of molecular markers, to calibrate the prediction model. An extraction and an LC-MS based method have been adapted to be suitable for the detection of phenylpropanoids in sugar beet leaves. For the data evaluation various proprietary and open source software is tested. We will distinguish between young and older developmental stages of the leaves. The metabolite data will be further used for metabolite profiling of various sugar beet genotypes with different levels of resistance to the above mentioned pathogens. There will be a focus on the characterization of metabolites of the phenylpropanoid pathway in sugar beet leaves, as a mean to evaluate stress response. Elevated levels of phenylpropanoids are often associated with biotic stress response and therefore are a target to get insight into defence and resistance strategies of sugar beet. We chose a metabolomic approach to be closer to the phenotype, as genomic and proteomic data not always correspond to the actual metabolite situation in the plant.

**EFFECT OF HEREDITY, ENVIRONMENTS AND DEVELOPMENTAL STAGE ON ALKALOIDS CONCENTRATION IN TOBACCO**

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Nicotine, nornicotine, myosmine, neonicotine were major alkaloids in tobacco. Many alkaloids have strong physiological functions to human body. In this study, ten kinds of alkaloids including nicotine, nornicotine, myosmine, anabasine, nicotyrine, anatabine, 2,3'-bipyridyl, 4,4'-bipyridyl, cotinine and N-formylnornicotine in tobacco leaves were analyzed by GC-MS. Three cultivars HongDa, K326 and Zhongyan100 were cultivated in three regions respectively. Five developmental stages were observed to study the influence of growth and development factor on the concentration of alkaloids in tobacco. As a result, the concentration of tobacco alkaloids went down at first and then went up from vigorous growth period to mature period with the lowest point appearing at squaring stage, and risen sharply after topping. By principal components analysis (PCA) cluster analysis, it could be discovered that samples were more inclined to cluster together according to the regions. Meanwhile, grouped by different regions, the partial least squares discriminate analysis (PLS-DA) of the data showed a good validation result, which indicated that the environmental deviation performed a greater impact on the concentration of alkaloid than genotype differences.

**METABOLITE AND GENE EXPRESSION PROFILES OF *RICINUS COMMUNIS* SEEDLINGS REVEAL A COORDINATED SHIFT IN CARBON-NITROGEN METABOLISM IN RESPONSE TO AN INCREASE IN TEMPERATURE**

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*Ricinus communis* possesses a highly adaptive tolerance to most diverse climates including high temperature and drought conditions. Metabolite and gene expression profiles were performed to determine metabolic changes during seedling establishment of three *R. communis* genotypes (IAC80, MPA11 and MPB01) in response to a temperature shift from 20°C to 34°C. Metabolite profiling was performed by measuring primary and secondary metabolites in roots and leaves of young seedlings using GC-TOF-MS and HPLC platforms. Carbohydrate content was reduced in both roots and leaves in response to the temperature shift, while amino acid content increased. In response to an increase in temperature, *R. communis* seedlings rapidly mobilize storage carbohydrates, such as starch, to provide soluble sugars as a response to enhanced demands for energy. Variation in metabolite contents of glycolytic and Krebs cycle intermediates confirms the strong relationship between these metabolic pathways and indicate that, in response to increasing temperature, *R. communis* seedlings activate catabolic pathways to generate energy through the oxidization of acetate. Preliminary gene expression analysis of genes encoding for key enzymes involved in carbohydrate metabolism as measured by RT-qPCR did not provide evidences to support this hypothesis yet, but further detailed analysis is being performed. Relative gene expression of the elongation factor 1-beta (EF1B), a component of the eukaryotic translation elongation factor-1 (EF1) complex, which plays a central role in the elongation cycle during protein biosynthesis, suggests that protein biosynthesis is up-regulated in response to an increase in temperature. The present work adds new insights to the understanding of *R. communis* adaptation to different temperatures during seedling establishment.

## GENOTYPE AND OZONE TREATMENT GENERATED DIFFERENCES IN PHENOLIC PROFILES OF EURAMERICAN POPLAR

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Tropospheric ozone is a phytotoxin that causes oxidative stress in plants. Genotypes differ in their ability to tolerate ozone due to differences in stomatal conductance or detoxification capacity. We studied the role of phenolic compounds in ozone tolerance of three Euramerican poplar (*Populus deltoides x nigra*) genotypes (Cima, Robusta and Carpaccio).

Poplar cuttings were cultivated in pots in phytotronic chambers exposed to filtered air or ozone treatment (120 ppb each day for 13 hours). Leaf samples were collected 2, 4, 11, 15 and 17 days after the start of ozone treatment. Analyses of phenolic compounds were made by HPLC-MS.

The three genotypes differed from each other in their growth and responses to ozone. Robusta showed the greatest number of visible injuries. Greatest growth reductions by ozone were measured for Cima, while Carpaccio was the most tolerant of the three genotypes.

The genotypes clearly differed from each other in their phenolic profiles. Cima had more flavonoid glycosides than the other two genotypes.

Ozone treatment changed phenolic concentrations so that in later samplings control and ozone treated leaf samples were clearly separated. Several phenolics increased in response to ozone.

**UNTARGETED STABLE ISOTOPE-ASSISTED METABOLIC PROFILING BY LC-HRMS REVEALS NOVEL CONJUGATES OF THE MYCOTOXIN DEOXYNIVALENOL IN WHEAT**

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This study will present a novel approach for the untargeted screening of metabolisation products of xenobiotics in plants using liquid chromatography – high resolution mass spectrometry (LC-HRMS). By the use of a mixture of non-labelled and fully-labelled xenobiotic precursors, a distinction between all MS signals originating from the xenobiotic and signals resulting from e.g. matrix background, solvent and noise is feasible.

The developed stable isotope assisted metabolic profiling approach is exemplified with the application of uniformly  $^{13}\text{C}_{15}$  labelled and non-labelled mycotoxin deoxynivalenol (DON) to wheat plants. Two wheat lines Remus (susceptible) and CM-82036, resistant to infection with the fungus *Fusarium graminearum*, were challenged by injection of the prepared DON mixture in two flowering wheat ears after 0, 24, 48, 72 and 96 hours resulting in a total amount of 100  $\mu\text{g}$  DON per ear. 108 hours after the first inoculation, treated ears were harvested, immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until analysis. Frozen plant samples were homogenised, extracted and analysed by LC-HRMS.

The recently developed software algorithm MetExtract was successfully used to automatically detect DON derived LC-HRMS signals originating from the non-labelled and fully-labelled xenobiotic precursor. Data evaluation resulted in the assignment of a total of nine different DON conjugates. Besides the well-known DON-3-O-glucoside, additionally DON-glutathione (GSH), DON-S-cysteinyl-glycine, DON-S-cysteine, as well as a putative DON-hexitol, DON-malonyl-glucoside and DON-acetyl-glucoside. All of these DON-conjugates have been annotated based on accurate mass measurements (max. deviation less than  $\pm 5\text{ppm}$ ). Moreover, MS/MS measurements of these compounds were carried out to elucidate and confirm the structures of the detected DON-conjugates.

This contribution will explain the analytical methodology and the complete workflow in detail. Moreover, putative molecular structures of the identified DON conjugates will be presented and the formation of these DON-biotransformation products in different wheat lines over a time period of 96 hours after inoculation will be discussed and correlated to the *Fusarium* resistance level of the respective wheat line.

## GC-MS BASED TARGETED PROFILING OF *FUSARIUM* – WHEAT INTERACTIONS

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Fusarium Head Blight (FHB, scab) is a fungal plant disease commonly infesting wheat. It is responsible for severe harvest losses throughout the world and a relevant hazard for food safety and security. A major virulence factor of the fungus *Fusarium graminearum* is the trichothecene mycotoxin deoxynivalenol (DON), which effectively inhibits eukaryotic protein translation.

The presented study was carried out to investigate fungal virulence and plant resistance in the FHB disease. In this experiment the parent wheat lines CM-82036 (resistant) and Remus (susceptible) were treated with either (i) *F. graminearum*, (ii) deoxynivalenol or (iii) water. Besides, four near isogenic wheat lines (NILs), which differed in the two major resistance QTLs against FHB (Qfhs.ndsu-3BS & Qfhs.ifa-5A) were treated in the same way. Samples of the treated plants were taken after 0, 12, 24, 48 and 96 hours and immediately shock frozen in liquid nitrogen. Ground wheat ears were extracted with acidified aqueous methanol and purified by liquid/liquid extraction with chloroform. The methanol/water phase was analyzed using GC-MS after an automated two step derivatisation employing methoxyamine (MOX) and N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA). GC-MS chromatograms were deconvoluted and further processed with the MetaboliteDetector software. A targeted approach which currently covers more than 100 polar metabolites was chosen to identify substances differentially expressed according to treatment and/or wheat genotype.

In this contribution we aim to identify the correlation between FHB resistance and the primary metabolites determined by our GC-MS method. Thereby we intend to gain deeper insights on fungal virulence and plant resistance towards FHB. We will present detailed GC-MS results of this metabolomics experiment with the goal to identify wheat metabolites, which are closely linked to infection with *F. graminearum* or inoculation with DON.



**TARGETED METABOLOMIC ANALYSIS OF DIURNAL CHANGES IN CENTRAL CARBON METABOLISM TO IMPROVE BIODEGRADABLE PLASTIC PRODUCTION IN TRANSGENIC SUGARCANE**

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Polyhydroxyalkanoate production in plants would decrease petroleum dependency by producing a renewable supply of biodegradable plastics. The simplest of these polymers, polyhydroxybutyrate, has been successfully produced in a number of plant species including sugarcane. This introduces a novel, irreversible carbon sink, which taps into central carbon metabolism via acetyl-CoA and potentially affects the delicate balance between carbon assimilation, storage and growth that needs to be maintained in plants.

Previously we have shown that carbon storage compounds such as starch and sucrose are decreased in transgenic sugarcane lines producing high levels of polyhydroxybutyrate (PHB). Starch is accumulated while photosynthetic carbon fixation occurs during daylight and is degraded to supply carbon and energy for respiration throughout the night.

Metabolomic analysis was used to investigate adjustments in central carbon metabolism in response to plastidic PHB production in sugarcane leaves during a diurnal cycle with a 12 hour photoperiod. In the first instance targeted quantitative analysis of central carbon and amino acid metabolism was performed using LC-MS/MS and HPLC-FLD. Univariate and multivariate analyses were then applied to identify differences in primary metabolism between a wild-type and two transgenic PHB-producing lines of sugarcane and across the diurnal time course. The metabolomic output was also correlated to starch and PHB to investigate the metabolic relationships with the primary metabolome.

**SEED-SPECIFIC EXPRESSION OF A FEEDBACK-INHIBITION INSENSITIVE FORM OF CYSTATHIONINE- $\Gamma$ -SYNTHASE (CGS) INCREASES THE PRODUCTION OF MET AND OTHER AMINO ACIDS IN TRANSGENIC *ARABIDOPSIS THALIANA* SEEDS**

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In order to study the regulation and importance of methionine (Met) in plant seeds, *Arabidopsis* plants were transformed with the Met/SAM feedback insensitive *Arabidopsis thaliana* cystathionine- $\gamma$ -synthase (AtCGS) gene fused to a seed-specific promoter. AtCGS is the main regulatory enzyme in Met synthesis through the Asp-family pathway. Increase in CGS expression was detected in the transgenic seeds, leading to enhanced accumulation of soluble Met (up to 27-fold) compared to the wild-type seeds. Interestingly, this increase was accompanied with a dramatic increase in the contents of most other free amino acids. To reveal the impact of these elevations, protein hydrolysis of the seed storage proteins will be performed. To track the impact of altered Met levels and identify related novel metabolic networks, GC-MS analysis will be conducted. Since major perturbations were observed in amino acid levels, the transgenic seeds will be subjected to microarray analysis to identify gene expression programs influenced by altered Met metabolism. Notably, qRT-PCR analysis showed no changes in the expression levels of the two major catabolic enzymes of Met, SAM synthase 3 (SAMS3) and Met- $\gamma$ -lyase (MGL). This research will provide comprehensive knowledge about the consequences of altered Met levels on the seed's transcriptome and metabolome.

## ON-GOING METHOD DEVELOPMENTS IN BORDEAUX METABOLOME FACILITY

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Bordeaux Metabolome Facility (BMF) is dedicated to plant metabolomics from chemical to data analyses. Besides routine analyses, this facility has developed methods to improve metabolome and lipidome coverage, facilitate metabolite identification, and increase throughput from extraction to data processing. This poster focuses on three on-going method developments:

- (1) Lipidomics has been established at BMF in 2010 (a). To improve lipid coverage, a method involving LC-QTRAP-MS has been developed for quantification and characterization of triacylglycerols.
- (2) Identification is a key point in metabolomics to link biomarkers discovery to biological information. A pipeline has been developed to identify metabolites by a combination of LC-MS and LC-NMR (b), as exemplified by metabolite identification in tomato organs.
- (3) In order to increase the throughput of <sup>1</sup>H-NMR metabolomic profiling, sample preparation has been improved through robotization of extraction and titration, eventually revealing the bottleneck of data processing. Therefore, an automated binning tool for NMR spectra has been developed to speed up bin design and integration calculations (c). Moreover, chemical information can be highlighted by bin clustering and use of reference spectra libraries, thus providing a helpful support for compound identification.

In the near future, these three types of development will allow BMF to achieve new ambitious plant metabolomics projects.

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**METABOLOMICS APPLICATIONS TO ELUCIDATE THE ROLE OF METABOLITES IN BI-/MULTITROPHIC PLANT INTERACTIONS**

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Plants are continually challenged by pests and pathogens and, being sessile, must be able to respond and fight their ground. Many plants have developed a very sophisticated 'chemical arsenal' which may have the effect of repelling the attacker. Fungal spores and mycelium as well as pathogenic bacteria are also known to be killed by such compounds or have their growth retarded and reproduction impaired. Moreover, metabolites that are emitted can the enemy's enemy, such as predators, nematodes, and parasitic wasps. We are using metabolomics to define this chemical arsenal. We will give an overview of where metabolomics can help to detect key metabolites involved in defence mechanisms. Once we have identified these key metabolites we can exploit them in follow up research focused upon developing new strategies for improving crop production. For instance, current approaches use these biomarkers to develop early warning systems in a large commercial green house and growers can use them to assist in improving their sustainable disease and pest control strategies. By linking data from metabolite profiling with genomics and transcriptomics data metabolomics we are also finding molecular markers important for future breeding approaches towards new crop plant varieties which are less dependent on agrochemicals.

**P4-39**

**EFFECTS OF INFLUENTIAL FACTORS (GERMPLASM, ENVIRONMENT, TRANSGENES)  
ON METABOLITE VARIABILITY IN COMMERCIAL CROPS**

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Influential experimental factors such as environment stress, growing location, germplasm, tissue type, development stage, and specific transgenes all affect crop metabolism. There is a need in commercial crop development to quantify the relative contributions to compositional and metabolite variability of these different factors as well as reveal potential interaction effects. Newer statistical tools such as Principal Variance Component Analysis (PVCA), and related techniques, have been used at Monsanto to clarify and visualize the effects of these experimental factors, their relationships and relative magnitudes to drive data interpretation. We have employed these tools in crops to rank and make key conclusions about the effect size and impact of environmental, genetic, and transgenic effects on metabolite variability.

**MOLECULAR AND MORPHOLOGICAL INSIGHTS INTO POLYPLOIDY OF POPLAR TREES**

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Poplar is a highly adaptive, robust and fast growing tree widely cultivated in short rotation plantations. As one of the most planted non-food crops it is used for the production of biofuels of second and third generation. Polyploid plants, carrying multiple sets of chromosomes, can show higher genetic flexibility leading to better environmental adaptation, stress tolerance or growth vigor. Polyploid poplar clones were shown to have useful traits in matters of biomass production and fibre characteristics and are therefore interesting subjects for further investigations. Poplar trees of different genotypes cultivated on soil are used to study effects of polyploidy on their morphological and molecular phenotype using metabolomics and proteomics techniques. Leaf extracts of diploid, tetraploid and mixoploid trees were subjected to metabolite profiling using a combined GC-MS and LC-MS platform covering primary as well as secondary metabolites [1,2]. Exact m/z features of secondary metabolites provided by nanoUPLC-Orbitrap MS/MS analyses were quantified by a modified in-house version of ProtMAX [3,4]. Data dependent MS<sup>2</sup> fragmentation of precursor ions were used to obtain further structural information. Integration of metabolomics-, proteomics- and morphological data as well as statistics were conducted using the COVAIN toolbox [5]. Distinct effects of polyploidy on poplar trees will be discussed and compared between plants grown on soil as well as in tissue culture.

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**P4-41**

**PROFILING FREE AND WALL BOUND METABOLITES OF *PHOENIX SYLVESTRIS* FRUIT**

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Phoenix sylvestris (L.) Roxb. fruit is an underutilized fruit of West Bengal, India. With an aim to investigate the bioactive constituents of the fruit, the metabolite profiles of the free and wall bound constituents from the mesocarp tissue were analyzed by GC-MS. The mesocarp tissue was extracted in 50% Methanol. 23 Metabolites (8 organic acids, 3 fatty acids, 7 sugars and sugar alcohols, 5 phenolic constituents) could be identified from this extract. From the residue, the cell wall bound (ether linked and ester linked) metabolites were extracted after sequential hydrolysis. 22 Ester linked metabolites and 17 ether linked metabolites were identified. The ester linked metabolites included 3 organic acids, 9 fatty acids and 10 phenolic compounds. The ether linked metabolites included 1 organic acid, 5 fatty acids and derivatives and 11 phenolic compounds. All the fractions were tested for  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibitory activities. Out of the three fractions, ether linked fraction showed highest activity. A few active constituents could be identified.

**APPLICATION OF METABOLOMICS TOOLS IN DIFFERENTIATION OF FRUITING BODIES OF *AMANITA MUSCARIA* AND THE CORRELATION WITH TYPE OF TOP-SOIL**

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A metabolomics approach was applied to fly agaric (*Amanita muscaria*) collected in Poland. The main purpose of this study was improving the knowledge about its chemical profiles in dependence on given top-soil properties taking into account different structural parts of mature fruiting bodies.

The tissue of *A.M.* PBS extract of caps and stems were analyzed by <sup>1</sup>H NMR method. The top-soil parameters were analyzed using physicochemical parameters and chosen micro- and ultra-elements were determined by AAS. Basing on <sup>1</sup>H NMR spectra 24 metabolites were assigned from mushroom homogenates.

The performed metabolic profiling and target analysis allowed to elaborate PCA and OPLS-DA models, which show separation between the morphological parts (cap and stem) of *A.M.* in relation to environment.

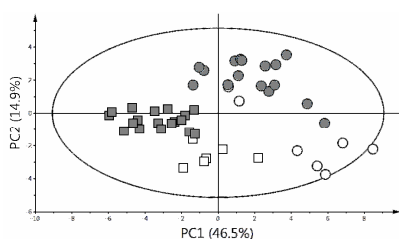


Figure: PCA analysis showing separation between cap (circles) and stem (squares) versus top-soil properties (white mineral soil; grey mineral-organic soil).

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## NMR BASED TAXONOMICAL IDENTIFICATION OF HALLUCINOGENIC AND POTENTIALLY HALLUCINOGENIC MUSHROOMS

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For most morphological investigations freshly collected species and/or living cultures are more suitable than dried material. However, this is not the case in forensics mycology, where material designed for investigation is often in poor condition (e.g. powdered). Therefore, ascertaining taxonomic identity of hallucinogenic mushrooms using available taxonomic tools is often difficult. Moreover, some harmless fungi are superficially rather similar to hallucinogenic ones, thus accurate identification is crucial for law enforcement.

Eight fungi species belonging to the four most popular hallucinogenic and potentially hallucinogenic genera (*Coprinus*, *Gymnopilus*, *Amanita* and *Stropharia*) were analyzed by means of <sup>1</sup>H NMR spectroscopy. The multivariate data analysis of mushroom homogenates were implemented (PCA and OPLS-DA) basing on whole spectral profiles. All four genera were well separated using PCA and perfectly separated, when utilized OPLS-DA method. Twenty three compounds were identified and nine of them have been considered for further univariate analysis. In some cases even a single biomarker concentration was sufficient for discriminating purposes. Differentiation among species belonging to the same genera resulted in high quality, validated models,  $Q^2 Y (>0.8)$ .

Proposed method allows for quick and accurate classification of the most popular hallucinogenic mushrooms. The metabolic fingerprints of collected material were found to be characteristic not only for genera but also for particular species. This results present metabolomics as a competitive approach to the currently practiced morphological studies, which should be considered as useful technique in forensics mycology related sciences.

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**ANALYSIS OF ISOPRENOID PATHWAY METABOLITES BY LC-MS**

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Isoprenoid pathway metabolites such as the isoprenoidphosphates and isoprenoid-pyrophosphates are central metabolites leading to sterols, dolichols, ubiquinones, prenylated natural products and proteins. The development of new methodologies for the preparation as well as the separation and quantitation of pure phosphorylated isoprenoid metabolites is key for detailed investigations of these pathways on a molecular level. It is therefore of much interest to develop new high-performance separation methods which are able to determine the whole range of isoprenoid-(pyro)phosphates. The analysis of a whole range of synthetic metabolites of isoprenoid pathways by LC-MS methods will be presented.

New HPLC-MS methods for the simultaneous analysis of the stereoisomeric dimethylallyl- and isopentenyl-(pyro)phosphates respectively have been established using the cyclo-dextrine-based stationary phase Supelco Cyclobond 2000 and a buffer/acetonitrile eluent in HILIC mode. Whereas the separation of these polar single unit isoprenoid-(pyro)-phosphates is based on selective ionic interactions on a cyclodextrin-based stationary phase, the separation of the larger isoprenoid-(pyro)phosphates has been achieved with IPC-UHPLC-MS using ion-pair chromatography on a Supelco Ascentis Express C8 column and dihexylamine acetate. The resolution of this IPC has been improved significantly with Fused Core particles. This IPC method turned out to be very versatile and applicable for a large range of other phosphorylated and polar metabolites.

## THE METABOLOMICS STUDY OF DIFFERENT NICOTINE CONVERSION TOBACCO LEAF AND FLOWER

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Nicotine is the major alkaloids in *Nicotiana tabacum*. Nor nicotine is the secondary tobacco alkaloid that is produced by the N-demethylation of nicotine, which process is called nicotine conversion. In most commercial varieties, nicotine conversion rate is very low and can be ignored. As for burley tobacco, nicotine conversion is a serious question. The nicotine conversion mechanism have been clarified by Siminszky<sup>1</sup>. Apart from nicotine changed to nor nicotine, other metabolites alteration may account for the decrease of tobacco leaf quality. The tobacco leaf metabolomics study is necessary to carried out.

A high nicotine conversion mutant was found in our lab which linked to white-petal mutation. Based on the UPLC-Q-TOF/MS, the metabolomics of leaf and flowers of the mutant and it's wide type tobacco were analysed. Two type of tobacco leaf metabolomics can be easily distinguished by using PCA method, while the petals metabolomics can't be distinguished. And using PLS-DA method to analyse the data, the petals metabolomics can be effectively distinguished and the leaf metabolomics were distinguished more separately. Thus, we can propose that the leaf have more dramatically metabolites variation comparing with their corresponding petals. Through comparing two-dimensional volcano plot, select  $P < 0.001$  and the fold change  $> 3$  metabolites as differences metabolites. Using this standard, 257 candidate substances were selected as differences of leaf and 84 candidate substances were selected as differences of petal. And search the METLIN database, some significantly change metabolites were deduced including some key substances which related with tobacco leaf quality. While, reference standards database is needed to confirm our finding.

- 1 Siminszky, B., Gavilano, L., Bowen, S. W. & Dewey, R. E. Conversion of nicotine to nor nicotine in *Nicotiana tabacum* is mediated by CYP82E4, a cytochrome P450 monooxygenase. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 14919-14924, doi:10.1073/pnas.0506581102 (2005).

**TARGETED METABOLOMICS EMPLOYED FOR THE STUDYING OF 'PINOT NOIR' GRAPE SKIN PHENOLICS AS INDUCED BY CANOPY MICROCLIMATE MANIPULATION**

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Apart from variety specifics, the occurrence of grapevine secondary metabolites is largely determined by geo-climatic conditions in which the plant is grown. Even if regional macroclimate conditions basically cannot be influenced, the canopy microclimate of cluster area on the other hand can be manoeuvred by implementing some viticultural practices (e.g. leaf removal) into the vineyard environment, especially if performed at different phenological stages of grape berry development. Earlier research efforts to reveal the effect of canopy microclimate manipulation on grape quality parameters were mainly focused only to few targeted compounds. Contrary, newer analytical approaches (such as metabolomics), are offering much wider possibilities to study plant secondary metabolism within such purposely-induced microclimate shifts. A field trial was thus designed in 'Pinot Noir' vineyard in order to reveal related alternations of multiple classes of skin phenolics, including some very rarely studied ones to date. Different accumulation trends during grape berry development were detected not only between groups but interestingly also between individual compounds within those groups. Canopy microclimate manipulation had a significant effect in case of anthocyanins and flavonols, particularly early peaking flavonols. Despite the fact that flavonols and anthocyanins share the same biosynthetic pathway, it seems possible to positively affect both classes since the peaking behaviour of many individuals during berry development is distinct. Stilbenes were generally highest in case of closed canopy, most probably due to better conditions for *B. cinerea* development. Within benzoates, syringic acid was the only representative still showing significant changes in favour of early leaf removal at harvest. Other classes of polyphenols, such as flavones, flavan-3-ols, flavanones and hydroxycinnamic acids essentially reduced their concentration from the time of veraison, with differences between treatments often obtained only in earlier stages of maturation. However, in total 31 out of 72 detected phenolic compounds still showed significant differences at harvest point in comparison to the control for at least one of the three leaf removal approaches.

P4-47

**CHEMOTYPING UNDER FIELD CONDITIONS: ANALYSIS OF METABOLOME DIVERSITY IN THE BIOENERGY CROP *MISCANTHUS***

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Society is demanding more green chemicals which need to be sustainably sourced. *Miscanthus* is a C<sub>4</sub> perennial grass which has increasing value within the field of bio-refinery; in addition to providing fuel, *Miscanthus* can be used as an alternative to food crops and petroleum-based feed stocks to make a variety of bulk, intermediate and speciality chemicals. The identification of high value components within energy crops can help to increase value in the processing chain. Indeed the identification of such chemicals could significantly increase the adoption of bioenergy crops and reduce the need for subsidies. A large and diverse population of wild accessions of *Miscanthus* were grown in a single field plot. Mass spectrometry (MS) metabolite fingerprinting methods, in conjunction with supervised data mining, were used to validate leaf sampling methods to ensure the experimental procedure was not confounded by differences in plant architecture, developmental phase or uncontrolled environmental influences. Following identification of the main environmental sources of metabolome variance, the same approach was used to explore metabolome differences associated with genotype. Major inter-specific differences (*M. sinensis* versus *M. sacchariflorus*) were evident in flow infusion-MS fingerprints. Several metabolome subgroups were evident within the set of *M. sinensis* genotypes, with geographic origin identified as a major driver of these differences in chemical content. Current experiments are investigating the links between genotypes and chemotype to identify genes responsible for metabolic diversity within *Miscanthus*.

## WHERE CAN YOU BENEFIT FROM EXTREMELY HIGH RESOLUTION MASS SPECTROMETRY IN METABOLOMICS RESEARCH – ONLY A NUMBER’S GAME?

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Recent developments in FT-ICR-MS instrumentation demonstrated mass spectral resolution of up to 20 Million [1]. Apart from pure technical feasibility and highly specialized applications, FT-MS found its way into many research fields including metabolomics enabling novel workflows virtually impossible to achieve before. This presentation highlights the impact offered by the capability to resolve isotopic fine structures in several applications, namely unknown identification, “S-omics”, fluxomics and imaging.

Unknown identification is one of the major bottlenecks in Metabolomics. It typically starts with generating the correct molecular formula from accurate mass and isotopic pattern information. The isotopic fine structure is decomposing the isotopic peak cluster into the individual elements e.g. M+1 into N15, C13, typically achieved at an MS resolution >250,000 FWHM. This information enables to “read out” the correct elemental composition for the target compounds. Currently software tools to evaluate isotopic fine structures are developed for an automated unambiguous formula generation.

Experiments targeted at discovering biochemical pathways by tracing stable isotopic labels will benefit largely from resolved isotopic fine structures, as it will do in fluxomics.

Isotopic fine structure data was recently applied to detect sulphur containing compounds in a plant metabolite extract – a further workflow enabled by high resolution MS data [2].

In addition to detecting biomarkers in homogenized samples, the localization of metabolite species by recent advances in MALDI imaging techniques received increasing attention for bridging the gap to the biological context. The combination of MALDI with ultra-high resolution MS systems solves the typical problem of matrix interferences by “simply” resolving them into distinct signals.

The goal of this talk is to trigger a discussion which other metabolomics related questions might be answered by ultra-high resolution MS and which software tools will be required to quickly access the isotopic fine structure and link it into different workflows.

[1] Nikolaev et al. 2011, JASMS 22(7):1125-33

[2] Nakabayashi et al. 2013 Anal Chem. 85(3):1310-5

P5-2

## **UNDERSTANDING HUMAN METABOLISM AND HEALTH BY NMR-BASED METABOLOMICS**

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Metabolism is essential to understand human health. To characterize human metabolism, a high-resolution read-out of the metabolic status under various physiological conditions, either in health or disease, is needed. Metabolomics offers an unprecedented approach for generating system- and/or compartment-specific biochemical definitions of a human phenotype through the capture of hundreds of metabolites in a single measurement. The emergence of large cohorts in clinical studies rises the demand of technologies able to generate and manage a large amount of measurements, in an automated fashion, in the most robust and reproducible way.

NMR is an established metabolomics tool for obtaining metabolic phenotypes. Using non-invasive human samples (urine, blood and stool), NMR is able to provide quantitative and qualitative metabolite information directly from  $^1\text{H}$  NMR profiles, in a high throughput way.

In terms of technology, we will describe the establishment of a robust flow injection analysis system coupled to a NMR in the analysis of 1,000s of urine samples. Samples were acquired at a throughput of  $\sim 20$  min/sample. From the obtained urine profiles, the quantification of  $>30$  metabolites, essentially belonging to the central carbon metabolism and gut microbial host co-metabolism is feasible.

All obtained metabolic read-outs can therefore be considered for further pathway modelling and/or biological interpretation in metabolic studies, such as nutritional interventions, growth and development of infants, and ageing.

**ETHNIC DIFFERENCE IN CERAMIDE PROFILES OF SKIN STRATUM CORNEUM**

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In the stratum corneum of skin, ceramides represent the major constituent of the free extractable intercellular lipids and play a significant role in maintaining the water permeability barrier of the skin. Ceramides are sphingolipids consisting of sphingoid bases, which are amide-linked to fatty acids. Up to now, 16 ceramide subclasses have been identified in human stratum corneum. In this study, we developed the lipidomic platform for the skin lipid profiling and identification using chip-based direct infusion nanoelectrospray tandem mass spectrometry. Using this platform, we identified 21 skin ceramides in human skin stratum corneum, and compared ceramide composition among three ethnic groups (Korean, Malay, and Vietnamese). However, there were no significant ethnic differences in skin ceramide profiles.



P5-4

**EXHALED BREATH METABOLOMICS: ADDING TO DISEASE DIAGNOSIS, CHRONOBIOLOGY AND INDIVIDUALIZED HEALTHCARE**

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**Background:** Exhaled breath contains relevant metabolites that may reflect the biochemical activity within a subject. However, in contrast with other biofluids (e.g. plasma), the analysis of breath in metabolomics remains far less explored. We present here some recent examples of how real-time breath analysis may contribute in the fields of disease diagnosis, chronobiology and individualized healthcare.

**Methods:** We modified the entrance of a commercial quadrupole time-of-flight (Qtof) mass spectrometer to allow for the real-time analysis of breath via secondary electrospray ionization-mass spectrometry. We have studied: i) differences between chronic obstructive pulmonary disease (COPD) and case controls; ii) temporal fluctuations in breath composition; iii) differences in breath signatures at an individual level.

**Results: Diagnosis of COPD.-**We found a panel of discriminant features that allowed for the accurate prediction of disease/non-disease states.

**Chronobiology.-**We captured temporal fluctuations in the composition of human exhaled breath that may allow for the prediction of internal body time.

**Personalized breathprints.-** Consistent with previous urinary metabolomic studies, we found an invariant part in breath, characteristic for each person, which can be extracted from the analysis of multiple samples from each single subject.

**Conclusions:** We conclude that the real-time mass spectrometric analysis of exhaled metabolites may contribute to address some of the most relevant topics in metabolomics, which are currently investigated through the analysis of body fluids other than breath.

**CRYSTALLOGRAPHIC SCREENING OF METABOLITE BINDING AS A TOOL FOR DETERMINATION OF UNKNOWN PROTEIN FUNCTIONS (AND, POSSIBLY, NOVEL METABOLITES)**

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Most transformations in the metabolome are facilitated by proteins. Defining the roles of these proteins is important to our understanding of metabolic processes. However, a significant fraction of sequenced genomes encode for proteins of unknown function.

We developed a method to determine a protein function based on the identification of its natural ligands (substrates, effectors, etc.). Proteins have evolved to efficiently bind their cognate ligands in the cell, and to ignore unrelated metabolites. However, comprehensive screening of protein binding to all compounds in the metabolome is a formidable task.

In order to identify natural ligands, we employ a two-phase approach that takes advantage of structural relationships among metabolites. In phase I, the binding of compounds in a diverse metabolic library to a target protein is studied using protein crystallography to detect compounds that are structurally similar to a natural ligand and possess its protein-binding determinants. In phase II, the binding of metabolites structurally related to the hits from phase I is evaluated to identify those that bind to the protein with high affinity and are likely to be involved in the protein function.

This method was applied to proteins of unknown functions representing three protein families, PF01256, YjeF\_N, and DJ-1/PfpI. In phase I, ligand binding in the predicted active sites was observed in crystals of all tested proteins. In phase II, a focused search of the metabolic space identified metabolites with a high binding affinity. The PF01256 proteins were established to catalyze ADP/ATP-dependent NAD(P)H-hydrate dehydration, a previously described orphan activity.

The other potential application of our method is identification of novel metabolites when they serve as ligands of target proteins. In such cases, phase II should include modelling to optimize affinity of the hits discovered in phase I using techniques employed in fragment-based drug design. The modifications tested in this optimization should be limited to metabolically meaningful groups. Thus, the suggested approach has applications in both proteomics and metabolomics.

**METHOD DEVELOPMENT AND VALIDATION FOR RAT SERUM FINGERPRINTING WITH CE-MS: APPLICATION TO VENTILATOR-INDUCED-LUNG-INJURY STUDY**

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Ventilation with high tidal volumes may induce lung injury or pre-injured lungs: a condition termed ventilator-induced lung injury (VILI), which has been studied using rats as experimental animals. The purpose of this research was to illustrate acute lung injury (ALI) through developing and demonstrating the reliability, suitability, accuracy and precision of a new method for serum fingerprinting using capillary electrophoresis coupled to electrospray ionization - time of flight - mass spectrometer (CE-TOF-MS). This method combines the simplicity of preparation (ultrafiltration) for serum samples with the capability to separate complex matrices with high resolution. Method validation was performed for a wide range of compounds in a pooled serum – covering the full electropherogram of rat serum – whereby metabolites such as carnitine, choline, ornithine, alanine, acetylcarnitine, betaine and citrulline were selected. In addition, 34 compounds were also confirmed in the rat serum pool. Subsequent application of the method to each serum sample followed by univariate and multivariate statistics, revealed 18 statistically significant compounds out of 1163 molecular features and five of them were found to distinguish VILI from control group. Specifically, an increase in the amount of choline, asymmetric dimethyl arginine (ADMA) and ornithine was observed, while leucine and arginine were reduced with VILI. This study shows the reproducibility of CE-MS profiling and opens the possibility to identify biomarkers in order to avoid ALI.

## A STUDY OF THE PROPERTIES OF SILICON HYDRIDE BASED COLUMNS IN RELATION TO METABOLITE PROFILING

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In comparison with silica gel the properties of silicon hydride are very different with regard to chromatographic retention. In silicon hydride most of the surface silanol groups have been replaced by with Si-H. In addition the basic Si-H surface may be modified by hydrosilation where the Si-H bond is reacted with a terminal alkene in the presence of a platinum catalyst. In the current study three silicon hydride columns, Silica C (which has no modifying ligands on the Si-H surface), Phenyl Hydride and UDC Cholesterol, were studied for their ability to retain standard mixtures containing 80 standards and for their properties with regard to profiling the urinary metabolome. The mobile phase used 10 mM ammonium acetate in water as mobile phase A and acetonitrile as mobile phase B with detection by an Orbitrap Exactive MS. The columns offer a mixed retention mode having both reversed phase properties and hydrophilic interaction like properties and this made it possible to carry out sequential runs from high organic to low organic followed by reinjection of samples moving from low organic back to high organic. The two sequential sets of conditions provided conditions favourable to the retention of polar and non-polar compounds respectively. The Silica C column was most retentive for polar compounds and the phenyl column was the least retentive. Even relatively non-polar compounds such as pantothenic acid were more strongly retained in the Silica C column in HILIC mode with the alkyl chains on the surfaces of the Phenyl Hydride and UDC Cholesterol columns reducing the interaction with the Si-H surface. The columns provided coverage 4-500 metabolites in urine recognizable by database searching. The Silica C column provided best performance in HILIC mode whereas as the Phenyl Hydride column offered good selectivity in reversed phase mode for steroid glucuronide conjugates and xenobiotics such as the glucuronides and sulphates of phenolic acids. The current efforts focus on optimising a the mobile phase modifiers to provide an optimum range of capacity factors for a wide range of metabolite types as well as trying to understand the exact mechanism for retention on this interesting family of chromatography columns.

## NON-TARGETED METABOLIC PROFILING OF HUMAN URINE SAMPLES

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The metabolome is difficult to characterize; there is a wide dynamic range of concentrations of metabolites, which are chemically and structurally diverse with various polarities and sizes. Creating a single analytical method for all these components is challenging. With global profiling techniques, the aim is to detect and quantify as many metabolites as possible. Information dependent acquisition (IDA) provides useful MS/MS information, based on the user selected criteria to prioritize candidates for fragmentation. Both the TripleTOF @ 4600 and TripleTOF @ 5600+ systems are capable of delivering high resolution, accurate mass data with high acquisition rates, enabling MS and MS/MS information to be acquired in a single acquisition. Speed is crucial to the workflow, enabling MS/MS information to be collected for as many features as possible, providing a comprehensive interrogation of the samples.

Urine samples were collected from male and female subjects. The female group was collected into two further groups of pre- and post-menopausal. For global metabolite profiling LC/MS data was acquired across these urine samples using the profile workflow. Data were opened into MarkerView™ Software for simultaneous feature finding, alignment, and statistical analysis to highlight metabolites of interest. For interesting peaks, the MS and MS/MS data were further evaluated using Formula Finder and the Structural Elucidation tool within PeakView Software. PeakView™ calculates the elemental composition / formula using precursor and fragment masses, links the MS with the MS/MS data for confirmation and gives possible structural information on the formula of interest. Here we highlight some interesting metabolite changes amongst a healthy group of individuals and demonstrate the power of a high resolving MS/MS system.

## THE METABOLITES ANALYSIS OF SERUM USING FAST-GC-MS/MS WITH TANDEM COLUMN

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Introduction: Gas chromatography/ mass spectrometry (GC-MS) and a gas chromatography-tandem mass spectrometry (GC-MS-MS) are highly suitable techniques for metabolomics analysis because of the high separation, reproducible retention times and sensitive and selective mass detection. In metabolomics studies, measurements of many samples are required and the samples have to be analyzed within 24 hours after sample treatments because of sample degradations. To increase the sample throughput, we have tried to shorten the analysis time of serum by fast GC-MS/MS using a short medium-bore capillary column with a thicker stationary phase in place of the inactive retention gap and a short narrow-bore capillary column (Tandem Column).

Method: A human serum sample is extracted to 250  $\mu$ L of methanol / water / chloroform (2.5:1:1) and the supernatant were subjected to methyloximation and trimethylsilylation (TMS) derivatization prior to analysis. GC-MS (Scan) and GC-MS/MS (MRM) analysis were performed on a GCMS-TQ8030 (Shimadzu, Japan). Two types of capillary columns, a BPX5 (1.3 m x 0.25 mm I.D., Df=0.5  $\mu$ m, SGE, Australia) and a BPX5 (4 m x 0.15 mm I.D., 0.25  $\mu$ m, SGE, Australia) were joined linearly using an SGE Sil-Tite<sup>TM</sup>  $\mu$ -Union column connector. The temperature program was started at 100 °C and was increased to 340 °C at 50 °C/min and held for 0.35 min at 340 °C (Total time 5.5 minutes).

Result: In the analysis results of fast-GC/MS and fast-GC/MS/MS with Tandem column, 20 targeted metabolites, which were included the TCA cycle metabolites, were measured from 0.4 to 3.1 minutes. In the scan data in serum, the metabolites like methionine and malic acid were interfered by other substances. In MRM data in serum, all targeted metabolites were separated and the %RSDs of the peak area for 6 replicate analyses were 0.41 - 8.52 % and the contributions of calibration curves from 0 to 10  $\mu$ g / ml were more than 0.999. The lower limit of quantification (LOQ) for fast-GC/MS/MS in 20 targeted metabolites was under 50pg/ml.

Conclusion: These results demonstrate the fast GC-MS/MS with the combination of two short columns can shorten the analysis time of serum from 30-60 minutes (Conventional GC-MS) to 5.5 minutes and quantifies the 20 targeted metabolites accurately. Such useful fast-GC/MS/MS systems in metabolomics studies will be widely distributed all over the world in the near future.

**UNTARGETED METABOLOMICS WORKFLOW USING UHPLC/QUADRUPOLE ORBITRAP MASS SPECTROMETER AND SIEVE 2.1**

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Plasma samples were deproteinized with organic solvent. Endogenous metabolites were reconstituted in methanol/water (1:9) containing isotopically labelled internal standard. A 15-minute water/methanol gradient was implemented on a Dionex Ultimate 3000 HPG (high-pressure gradient) UHPLC using reversed-phase C18 column at 450 $\mu$ L/min and 55°C. ESI positive, negative and polarity switching full MS scan ( $m/z$  80-1000) and MS/MS were acquired on a Thermo Scientific Q Exactive Orbitrap operating at resolution 70,000. Obese versus lean rats' plasma LC/MS data was processed using Thermo SIEVE 2.1 by executing background subtraction, component detection, peak alignment, differential analysis. Statistical results and putative IDs were generated. Metabolites of interest were identified via MS/MS and mass spectral database matching.

ZDF plasma samples from 4 fatty rats and 4 lean rats were analyzed in an alternating order. The 8 were pooled to make a QC sample. D5-hippuric acid was spiked into samples at 10  $\mu$ g/mL. In the whole batch of analysis spanning overnight, <10% intensity variation of IS and the majority of metabolites in QC sample were observed. The RT shift of IS was within  $\pm 6$  seconds and the mass accuracy was usually < 3ppm, demonstrating the excellent LC reproducibility and outstanding mass accuracy and signal stability of MS. Post-run normalization was thus not needed.

Data was processed using SIEVE 2.1 and XCMS online. SIEVE extracted ~8000 features while XCMS extracted ~22,000 features. The overlapping features showed the same statistical results. Examination of the excess numbers from XCMS revealed that many of these were background noise, indicating the importance of background subtraction for metabolomics data. A panel of dysregulated metabolites (ratio >2,  $P < 0.01$ ) were characterized, for example, the increase in certain phospholipids, fatty acids, acylcarnitines in fatty ZDF rat plasma. The changes agreed well with literatures. Metabolites were identified through accurate mass and MS/MS spectral matching to online libraries, e.g., METLIN and m/zCloud.

**A PLATFORM TO IDENTIFY ENDOGENOUS METABOLITES USING A NOVEL HIGH PERFORMANCE Orbitrap AND THE mzCLOUD LIBRARY**

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The structural identification of metabolites represents a significant challenge in metabolomics study. Multistage mass spectrometry (MS<sup>n</sup>) is a powerful tool for compound identification that goes beyond identifying the exact same compounds, to discovering additional compounds with structural homology to those in the library. Combining with high resolution accurate mass (HRAM) measurement, MS<sup>n</sup> is highly effective in identifying the unknown but biologically relevant compounds in metabolomics studies.

Presented here is a new platform to identify endogenous metabolites using a novel high performance Orbitrap hybrid instrument in conjunction with UHPLC and an MS<sup>n</sup> library. The benefit of 400K ultra high resolving power was demonstrated with a mixture of human metabolite standards in an infusion experiment. For UHPLC-MS experiment, MS<sup>n</sup> spectral trees were acquired data dependently in parallel to the HRAM full scan. Various breadth and depth of the MS<sup>n</sup> trees were tested to maximize the information content. CID and HCD dissociation methods were compared. The data shows that the instrument is capable of acquiring multiple spectral trees within the time scale of UHPLC peak width.

The mzCloud is an online library of HRAM MS<sup>n</sup> spectral trees for small molecules. Both CID and HCD MS<sup>n</sup> spectral trees were acquired from metabolite standards under + and - ionization modes. It integrates functionalities including scoring based on spectral tree identity or similarity measurement, and fragmentation interpretation annotation, etc.

Using the NIST "human metabolites in plasma" sample, stereoisomers leucine and isoleucine (C<sub>6</sub>H<sub>13</sub>NO<sub>2</sub>, m/z 132.1019) were chromatographically separated, and the annotation was based on their endogenous concentration ratio (approximately 1:2) and the MS<sup>2</sup> spectral pattern difference in mzCloud library. In urine samples, two peaks with exactly the same m/z (132.1019) preceding the leucine pair were observed. Their identities were unknown. The MS<sup>4</sup> data acquired during the UHPLC run provided deeper fragmentation information for these peaks, which matched two other amino acids with highest spectral score in mzCloud library. This platform demonstrates the capability of **de novo** identification of endogenous metabolites in parallel to high-throughput LC/MS metabolic profiling.



## ANALYTICAL PLATFORM FOR METABOLOME/LIPIDOME ANALYSIS OF MICROBIAL CELLS

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Metabolomics/lipidomics has recently become a powerful tool in biological research, providing the functional readout of the cellular state that can be linked to a particular phenotype<sup>1</sup>. These links make possible to understand upstream biological information (from genes, transcripts and proteins), which may lead to the discovery of the biomarkers for disease in biomedical research or fundamental insights into the cellular biochemistry.

Our research focus is on the study of yeast metabolism by using systems biology approach with the focus on fundamental science and applications to industrial research. For the industrial perspectives, the group is currently interested in developing yeast cell factories for the production of biofuels and biochemical products. For this purpose, we have recently implemented the analytical platforms for characterizing the wide range of compounds that are produced during the development process.

We present here several high-throughput analytical methods that we have developed in-house<sup>2</sup> and apply routinely for our research purposes. Our analytical platform covers from an initial state of the analysis (sampling) through the end analysis. Up to date, we are able to analyze several different metabolites (targeted analysis by GC-MS) that are highly interested e.g. lipids, fatty acids, amino acids and organic acids. We are currently developing a method for untargeted analysis based on high resolution LC-MS with an aim to profile as many as possible of metabolites in yeast sample.

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**DEVELOPMENT OF A HYDROPHILIC INTERACTION CHROMATOGRAPHY - MASS SPECTROMETRY (HILIC-MS) METHOD FOR IMPLEMENTATION AND EXPANSION OF A NON - TARGETED METABOLOMICS PLATFORM SCREENING HUMAN URINE AND PLASMA**

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In the past Hydrophilic interaction liquid chromatography (HILIC) was not a widespread chromatographic technique, rather a niche application. Currently HILIC is gaining popularity in separation science due to its unique separation capabilities (Cubbon, Antonio et al. 2010). Reversed phase liquid chromatography coupled to mass spectrometry (RP-LC-MS) is a well-known and widespread technology used as a workhorse in almost all disciplines of analytical chemistry. In non-targeted metabolomics it enables the detection of medium- to non-polar analytes in biofluids and extracts (Forcisi, Moritz et al. 2013). Highly polar compounds (e.g. non-aromatic amino acids, monosaccharides, small di-carboxylic acids, phosphoesters of nucleotides) remain undetected. In this work we present new opportunities that HILIC offers due to the close to orthogonal retention behavior, compared to reversed phase chromatography. HILIC enables the study of highly polar classes of metabolites without the necessity of changing the whole instrumental analytical setup, since HILIC solvents and buffers are compatible to standard HPLC-MS systems. The aim of the project is the development of a robust HILIC-MS method to integrate to an already established RP-LC-MS metabolomics platform. In this way the range of covered metabolites will be considerably expanded. The integration of high resolving power analytical techniques and chemometrics enables the detection of biochemical patterns describing metabolic phenotypes.

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**A STANDARDIZED METHOD FOR BREATH MOLECULAR PROFILING TO DISCOVER DISEASE SPECIFIC BIOMARKER**

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Exhaled breath contains numerous VOCs, which represents metabolic phenotype of a subject. Although GCxGC-TOF-MS is efficient enough to capture high dynamic range, limited application for exhaled breath molecular profiling is available in literature. Therefore, we undertook a study to standardize method for breath molecular analysis. We observed that BIOVOC could play a major role in breath sample collection in resource limited settings. Volume of ~390 ml exhaled breath trapped using a multibed matrix i.e. Carbotrap 300 thermal desorption tube (TDT) captures most of the molecular details.

TDTs are heated at 320°C for 6 mins to desorb the molecules and trapped at a cool injection system at -35°C. From cold trap (CIS) molecules were released using a temperature gradient of 12°C/sec to a final temperature of 350°C and transferred to the primary column (RTX-1, 30m; 35°C to 200°C @ 10°C/min). These separated molecules were transferred to a secondary column (RTX-Wax. 1.5m) through a dual thermal modulator (Hot pulse: 0.7 sec, Cold time: 1.3 secs). A TOF-MS at a scan speed of 200 Hz identified more than 300 molecules with high similarity index (>600 at S/N>100) in the breath matrix of a healthy subject. ChromaTOF software was used for data acquisition and processing. High precision (RSD<20%) of the standardized method was observed using standard VOC mix. This method is fast (~30 mins) and reproducible that could be useful for breath molecular profiling for disease specific biomarker discovery

**A COMPREHENSIVE EVALUATION OF VARIOUS PRECIPITATION AND EXTRACTION PROCEDURES FOR UNTARGETED BLOOD PLASMA PROFILING BY UPLC-MS**

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Blood delivers oxygen and nutrients to organs through systemic circulation and mirrors the global physiological state of the organism. Profiling the plasma metabolome is used to identify biomarkers associated with risk factors involved in the development of the metabolic syndrome, such as hypertension, hyperglycemia, and hypercholesterolemia. Due to the complex composition of blood, comprising lipoproteins, free fatty acids and small molecular-weight metabolites, exploratory untargeted ultra-high performance liquid chromatography-mass spectrometry (UPLC-MS) profiling offers an overview of the molecular species present in a sample. Sample preparation is critical for the successful analysis of these molecular species and may impact the quality and reproducibility of the data. This present study aims to compare eight pretreatments for lipid extraction. Two different approaches were employed: protein precipitation and liquid-liquid extraction. Four protein precipitation protocols were evaluated using: methanol, acetonitrile, isopropanol and isopropanol-acetonitrile. Additionally, four liquid-liquid extraction protocols were evaluated, combining methanol with chloroform, dichloromethane, methyl-tert butyl ether or isopropanol with hexane. The eight sample preparation procedures were assessed using a set of qualitative and quantitative criteria, to define the advantages and limits of each pretreatment. The parameters tested include simplicity/rapidity, protein removal efficiency, recovery efficiency, specificity of molecular species recovered, repeatability of procedure.

We found that protein removal was more efficient by precipitation (99%) than extraction (95%). Additionally, isopropanol appeared to be the most repeatable procedure (61.7% of features with CV<15%) and enabled a broad coverage of plasma lipid species to be identified. These results suggested that isopropanol precipitation was the most robust sample preparation method for untargeted lipid profiling using UPLC-MS. This sample preparation method is not limited to untargeted profiling and could be easily amenable for targeted UPLC-MS/MS profiling.

P5-16

**COMPARING DIFFERENT ANTICOAGULANTS FOR SAMPLING BLOOD PLASMA USING GC-TOF-MS AND LC-QTOF-MS/MS METABOLOMICS**

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Plasma is the primary source of material used in many clinical and preclinical metabolomic studies. Very few studies compare the use of different anticoagulants and their effects on metabolomic profiles in terms of systematic errors they introduce. We here compare blood 10 ml aliquot samples that were taken from a single model animal *Sus domesticus* using four different blood withdrawal methods: citrate, EDTA, heparin anticoagulations and serum clotting as positive control. Each tube was extracted six times as technical replicates using 15  $\mu$ l aliquots for profiling primary metabolism by GC-TOF-MS and 20  $\mu$ l for profiling complex lipids using CSH-QTOF MS/MS. Peak finding and mass spectral deconvolution were conducted by the Leco ChromaTOF software and subsequent BinBase metabolite annotations for GC-TOF-MS, and using mzMine2 and Agilent MassHunter Quant for CSH-QTOF MS/MS data, using LipidBlast, Metlin and MassBank for compound annotations. We present results from the different platforms including statistical assessments, biochemical pathway annotations and metabolome coverage, and robustness analysis for each of the anticoagulants. Preliminary observations indicated that citrate plasma is the least optimal use of anticoagulants. Overall conclusions will be presented in the form of guidance how to best conduct prospective human cohort studies and projects on animal models.

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## USING CAPILLARY ION CHROMATOGRAPHY MASS SPECTROMETRY FOR METABOLOMIC APPLICATIONS

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Ion chromatography (IC) has been used extensively to separate and determine ionic and charged small molecules. When coupled with mass spectrometry (MS), the combined techniques provide confirmatory identification, structural interpretation, and higher sensitivity in complex matrices. With its unique selectivity, IC has been successfully applied to the identification and quantification of targeted and untargeted charged metabolites in biological samples. Capillary IC-MS (Cap IC-MS) furthers the capability of IC with respect to metabolite identification and quantification by improving the system sensitivity and stability as well as reducing the amount of sample required.

Here we demonstrate the targeted analysis of metabolites. The analytes were separated on a capillary packed (0.4 mm ID) or monolith (0.25 mm ID) ion exchange column using an electrolytically generated hydroxide gradient by a Reagent-Free IC (RFIC) instrument. The analytes were detected by MS/MS in selected reaction monitoring mode (SRM) for selective and sensitive quantification. At the IC-MS interface, the mobile phase was continuously desalted online. Using this configuration, targeted metabolites can be quantified at fmol levels with a small number of  $\mu\text{L}$  sample consumption, and linearity can be maintained over two orders of magnitude.

**MUSCLE: A NOVEL MULTI-PLATFORM, USER-FRIENDLY SOFTWARE TOOL FOR THE ROBUST, OBJECTIVE AND AUTOMATED OPTIMISATION OF TARGETED LC-MS ANALYSES**

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The development of mass spectrometry based analyses of metabolites can be time consuming and thus expensive. In addition, transferring existing GC-MS or LC-MS methods between different instruments also places a considerable burden on the analyst. This bottleneck significantly impedes our ability to establish new bioanalytical methods for metabolite analyses. Previously we developed a closed-loop strategy for the automated optimisation of metabolomics analyses on specific platforms, but our original software implementation was time consuming to program and specific to each platform and software. Here we report the development of a multi-platform, user-friendly software tool (MUSCLE) for the robust and automated optimisation of targeted metabolite analysis using LC-MS/MS. MUSCLE provides an interface to allow a user to create a set of keyboard and mouse instructions, called an Application Control Script (ACS), which instructs MUSCLE how to change both the LC and MS instrument parameters within the application software package. This approach is designed to work across all instrument manufacturers' software and hence LC-MS/MS instruments. MUSCLE can then automatically optimise the LC-MS parameters using an evolutionary algorithm, against a set of pre-defined objective functions, i.e. detect as many metabolites as possible from the peak list provided in as short an LC run time as possible. An ACS is designed to be re-usable, so that MUSCLE users can apply them to multiple experiments as well as share them with other analysts. We have validated MUSCLE on two independent LC-MS/MS instruments, including a Thermo Fisher Scientific UHPLC TSQ Vantage, for the targeted analysis of multiple steroids. Following an optimisation experiment lasting several 10's of UHPLC-MS analyses, this fully automated approach discovered a set of LC and MS parameters that offered a faster analysis than achieved by a manual optimisation of the same multi-steroid analyses.

## NON-TARGETED SCREENING IN AQUEOUS MEDIA USING GC×GC-TOFMS

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Comprehensive two-dimensional gas chromatography coupled to fast acquisition time-of-flight mass spectrometry (GC×GC-TOFMS) is a widely established method for non-targeted analyses. However, for tobacco and tobacco related research, the inherently complex chemical space usually requires the use of a number of different experimental strategies.

The development and implementation of a non-targeted GC×GC-TOFMS approach for aqueous samples, e.g. cigarette smoke trapped in phosphate buffered saline (PBS) or microsomal incubation samples is described. The presented approach comprises minimal sample preparation for lowest risk of changing chemical constituent profiles and enables a broad coverage of volatile compounds without the need of using additional methods.

Aqueous trapping solution is directly injected on-column and analyzed by GC×GC-TOFMS using a combination of water resistant ionic-liquid based analytical columns. By avoiding any solvent delay, this setup allows the detection of highly volatile furans amongst other constituents. A set of furans was used to test the reproducibility, linearity and robustness of the chromatographic separation. Non-targeted screening of complex matrices such as cigarette smoke trapped in aqueous media is also discussed.

This method represents a novel approach, which defies the common belief that aqueous media are incompatible with gas chromatography.



**LIPIDOMIC ANALYSIS USING HIGH RESOLUTION TIME OF FLIGHT MASS SPECTROMETRY – LOOK NO COLUMN NEEDED**

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High resolution mass spectrometry (HRMS) has become a preferred tool in comprehensive differential analysis for metabolomic profiling associated with disease states. In its many forms it is used in the direct analysis of samples (no chromatography) with great results for lipidomics. The successful direct analysis of complex lipid extracts requires high resolution (> 50,000) and low mass accuracy (< 1ppm). Few analytical platforms meet these criteria. Plasma samples are investigated using direct infusion analysis to differentially profile lipids from diseased and control rats in the Zucker model. As a separate challenge polar lipid extracts from mouse liver are also interrogated using high resolution MS. The ability to leverage mass spectral data having resolving power of up to 100,000 and mass accuracies below 1 ppm is discussed in the context of potential biomarker identification and selective relative quantitation. Specifically the ability to investigate the “unsaturome” (unsaturated forms of the same lipid classes) in these complex samples without chromatographic separation is demonstrated and compared to some results from LCMS using the same MS platform. The ability to characterize lipid classes and differentiate lipids of the same formula but different classes is discussed by examining the fragment ion profiles simultaneously in the same samples. Evaluations and differential analysis is not limited to lipids and is extended to other classes of metabolites. The power and simplicity of direct analysis as a screening tool for metabolic analysis is demonstrated and its strengths, values and limitations discussed.

**P5-21**

## **QUANTITATIVE METABOLITE PROFILING IN PICHIA PASTORIS**

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Quantitative metabolite profiling is the backbone of a cutting edge strategy in the biotechnological production optimization, i.e. the metabolic engineering, linking the primary carbon metabolism to production rates of cell factories. Truly comprehensive, accurate quantitative analysis of all intermediates of the carbon metabolome is a major goal in this context. In order to achieve this, analytical workflows established in our laboratory comprise the use of orthogonal chromatographic separations or even complementary LC-MS/MS and GC-MS/MS analysis.

We will discuss the benefits of on-line two-dimensional chromatography (employing orthogonal separations) in combination with MS/MS detection in order to increase peak capacity, decrease analysis time and in the best case restrict the analysis to one mass spectrometric platform or even method.

Method validation is an integral part of any analytical method development; however in this specific application of intracellular metabolite profiling, it is highly challenging to follow principles of metrology. As a matter of fact, there is a complete lack of reference materials. In some cases, even the purity of calibrants poses severe limitations to absolute quantification. Therefore, we focused on a critical evaluation of our mass spectrometry based procedures applied to primary metabolite profiling in *Pichia pastoris*, including the assignment of measurement uncertainty and accuracy of quantification. Accuracy was addressed by an interlaboratory comparison study proving the comparability of results obtained from the yeast *Pichia pastoris*. It was designed for evaluation of different measurement platforms integrating different quantification strategies with internal standardization.

## METHOD DEVELOPMENT FOR PEPTIDOME CHARACTERIZATION

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The concept of dietary bioactive peptides brings a new dimension that should be considered in the description of dietary protein quality. Bioactive peptides have been defined as protein fragments that have, in addition to their nutritional value, a positive impact on body functions (vascular system, immunity, metabolism ...) and may ultimately influence health. Most dietary proteins contain bioactive sequences, but the corresponding peptides need to be released by proteolysis during digestion, and absorbed from the gut.

The purpose of this study was to develop the analytical tools required for peptide isolation, characterization and identification by mass spectrometry, in plasma and serum samples.

A rapid and robust sample preparation method, based on a protein precipitation using acetonitrile, was optimized. Peptides profiles were then determined using Ultra Performance Liquid Chromatography coupled on-line to a LTQ-Orbitrap Velos mass spectrometer. The separation was performed on an Aeris peptideXB-C18 column and using a 17 min gradient. Data were collected in positive ion mode in scan and data dependent MS/MS modes. Two approaches depending on peptide masses were developed to perform the data processing and the identification. Peptides of masses between 400-800 m/z were identified with proteomic tools: ions were extracted using Progenesis LC-MS software, and Mascot (<http://www.matrixscience.com/>) was used to identify peptides in the merged peaklist generated by Progenesis. For peptides of lower masses between 100-500 m/z, not listed in proteomic databases, metabolomic tools were used: the raw data were transformed to centroid mode and mass corrected before being analyzed using XCMS. The data were peak detected and noise-reduced. Identification was performed using an in-house database built from peptide data extraction from the Human Metabolome DataBase (<http://redpoll.pharmacy.ualberta.ca/hmdb/HMDB/>) and from the BIOPEP website (<http://www.uwm.edu.pl/biochemia>). The validation of the putative identification was performed by analysis of their fragmentation with the combination of CID and HCD.

The developed method allowed us characterizing the peptide fraction in plasma or serum samples, to study the bioavailability of peptides and their kinetics of appearance in blood, following the intake of a complete meal. (<http://redpoll.pharmacy.ualberta.ca/hmdb/HMDB/>) and from the BIOPEP website (<http://www.uwm.edu.pl/biochemia>).

The validation of the putative identification was performed by analysis of their fragmentation with the combination of CID (collision induced dissociation) and HCD (Higher-energy C-trap dissociation) a good compromise will be possible to obtain better fragmentation informations.

The developed method allowed us characterizing peptide fraction from plasma or serum samples, to study the bioavailability of peptides and their kinetics of appearance in blood following the intake of a complete meal.

## TOF-SIMS LIPID PROFILING OF ACUTE MYELOID LEUKAEMIA CELLS TREATED WITH A NOVEL COMBINATION THERAPY

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Time of flight secondary mass spectrometry (ToF-SIMS) is a powerful surface analysis technique which has seen much development in biological applications in recent years. The emergence of such applications has been largely driven by instrument developments in the last decade in which cluster primary ion beams ( $\text{Au}_n^+$ ,  $\text{Bi}_n^+$ ,  $\text{C}_{60}^+$ ) have been employed instead of atomic ions such as  $\text{Ga}^+$  and  $\text{Cs}^+$ . These polyatomic cluster ion beams are capable of generating higher secondary yields from molecular materials, particularly higher mass species and thus increase the sensitivity of the technique making ToF-SIMS a novel tool for probing the surface of biological materials. Recently there have been a number of reports into the localisation of metabolite and lipid species in various mammalian tissues using ToF-SIMS and other mass spectrometry imaging (MSI) techniques. The unique advantage of SIMS amongst MSI techniques is its sub-cellular spatial resolution (typically 1  $\mu\text{m}$ ), potentially allowing single cell metabolite analysis.

Acute myeloid leukaemia (AML) is an aggressive cancer made up of dysfunctional cells from the myeloid lineage. Patients frequently die within weeks of diagnosis if treatment is not administered rapidly; however, high grade chemotherapy currently employed is very toxic and poorly tolerated. Little treatment progress has been made in the last two decades therefore revealing a demand for novel treatments which have high selectivity for tumour cells and lower systemic toxicity than current options.

Drug redeployment, in which existing drugs used for other medical conditions are exploited, is utilised here in a combinatorial therapeutic approach. Bezafibrate, a cholesterol lowering drug, is combined with medroxyprogesterone acetate, a female contraceptive to give a combination, denoted BaP which shows anti-leukaemic activity at low dose and has been well tolerated in clinical trials with no observable haematological toxicity.

Here we report preliminary data from an investigation into the lipid composition of AML cells treated with BaP using ToF-SIMS. The aim is to assess the application of this technology to provide new insights into the chemical effects of drug treatment in cellular membranes and to explore the potential for lipid analysis at the cellular level.

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**THE AUTHENTICATION AND QUALITY CONTROL OF NATURAL PRODUCTS BY USING A NON-TARGETED AND TARGETED NMR SCREENING TOOL**

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There has been significant increase in the usage of natural products such as plant extracts for herbal remedies or phytopharmaceuticals worldwide. This has raised many concerns as increases in the adulteration of natural products with other materials have been detected in the market. In some cases, this is due to species misidentification but in other cases, this is due to financial incentive. Due to the wide diversity and complexity of chemical compounds within botanical material, a demand exists for the analysis of the composition of natural products for proper quality control and authentication. NMR spectroscopy is one of the main analytical techniques for metabolomic studies as it provides a high-throughput, reproducible and detailed methodology in visualizing the chemical profile of a sample. In this study, the development of a NMR-based fingerprinting tool for non-targeted and targeted screening of natural products will be shown. In addition, the application of NMR spectroscopy in understanding the characteristics of different natural products, in identifying key metabolites that classify them to their various groups and in providing a powerful tool to quantify specific metabolites of interest in the samples will be presented. With the increasing demand for robust high-throughput analytical methods, an automated NMR-based screening tool could be a powerful approach for validating the identity, purity, strength, and composition of many natural products.

## STRATEGY FOR EXPANDING A METABOLOMICS SERVICE CORE USING GOOGLE DOCUMENTS

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A range of metabolomics platforms have reached maturity, enabling University service cores to closely work with biologists and clinicians on metabolic problems. At UC Davis, the metabolomics fee-for-service facility is in operation since 2005, with demand for services today being four-fold higher than in 2011. The NIH Common Funds initiative has implemented further metabolomics resource cores across the United States, yielding greater accessibility to metabolomics at more capacity and improved depths and breadths of services.

Nevertheless, such service cores remain relatively small in comparison to established analytical service businesses in industry. Implementing and maintaining Laboratory Information Management Systems (LIMS) is a very costly investment with respect to the software platform itself and concerning expanding the code and utility of a LIMS platform.

At the UC Davis West Coast Metabolomics Center, we have tested a new strategy using free Google Docs as an alternative method to the traditional LIMS. We highlight benefits and disadvantages of this solution in relation to service volume and productivity, client feedback and flexibility. We give examples on service workflows, client and laboratory interactions, links to metabolomic platforms, data and statistical services, and links to a billing system. Highlights and problems in daily service practice of a metabolomics core are discussed. Importantly, our current Google-docs linked LIMS system is integrated within our Center website ([metabolomics.ucdavis.edu](http://metabolomics.ucdavis.edu)) which aids the communication specialist to direct clients to frequently asked questions, our central communication email address ([metabolomics@ucdavis.edu](mailto:metabolomics@ucdavis.edu)), our repository of InChI-linked target metabolites and the diversity of analytical platforms used in the Center.

**IDENTIFICATION OF LIPIDS BY LC COUPLED ION MOBILITY MASS SPECTROMETRY USING IMS-QTOF**

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Complex biological samples are often difficult to analyze. Lipids represent a broad class of compounds that, due to their chemical and structural complexity, are particularly challenging and therefore different analytical techniques are required in order to identify and characterize them. Lipids have many different functions in biological systems, and in recent years they have become increasingly important for their roles in cell-cell communication. We have used a prototype IMS-QTOF system to gain further separation of lipids in complex biological matrices. Lipid extracts from pooled rat spinal cords were dissolved and extracted in chloroform, and injected onto a prototype LC IMS-QTOF system using a water, methanol and isopropanol chromatographic solvent system. The injection volume was adjusted to gain broad coverage of different classes of lipids. The lipids were identified using two data analysis workflows: Agilent Technologies lipidomics profiling software containing MS database and MS/MS spectral library content from METLIN. And in parallel, third party SimLipid software was used to identify specific lipids. Data obtained with and without IMS separation were analyzed and identified using an unbiased feature finding algorithm and lipid database searches. This resulted in several hundred annotated lipid compounds in each sample. For some of the compounds, the IMS data revealed the presence of more than a single isomer. For example phosphatidylethanolamine (PE), was initially identified as one intense peak. After considering the contribution of drift space, two distinct peaks were observed, indicative of at least two compounds with differing cross sections. Preliminary fragmentation experiments suggest that compounds separated in the drift space could have different fragmentation patterns. By elucidating these patterns, the compounds can be identified; making our IMS-QTOF a powerful tool for the analysis of lipids in complex mixtures.

P5-27

## COMPREHENSIVE METABOLOME ANALYSIS OF SMALL VOLUME SAMPLES BY TWO COMPLEMENTARY UPLC-MS METHODS

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Metabolomics, thought as a complement to proteomics and transcriptomics, is still at need for a comprehensive as well as efficient identification and quantification of the metabolites and lipids in any given biological sample, but especially in small volume samples. Here we like to present a complementary set of two capillary scale ultra-performance liquid chromatography (capUPLC)-electrospray ionization-high resolution and accurate mass spectrometry (ESI-HRMS) methods for the comprehensive analysis of metabolites, ranging from very polar (sugars) to very non-polar (lipids) biochemical classes and ranging in concentration levels over several orders of magnitude.

Limits of detection for both methods were shown to be in the low picomolar range for 200 tested reference compounds, covering all major metabolite classes. The simultaneous acquisition of MS and MSMS enables metabolite detection and quantification as well as confirmation of metabolite identities in one run. When applied to methanol extracts of various biological samples (e.g. colon normal mucosa, adenoma tissue and colon cancer tissue from colonoscopy, needle biopsies, serum from small mammals, oocytes) the methods revealed, by the use stable isotope labeled metabolite analogues, excellent robustness and reproducibility, i.e. maintained narrow symmetrical peaks, retention times and detection limits as expected from the analysis of the pure reference compounds. Additionally, it was concluded from the high number of observed  $m/z$  values assignable to data base listed metabolites (KEGG, HMDB, MassBank), that the simple and fast one-phase extraction of metabolites with methanol results in a broad metabolome and lipidome coverage (up to 80% coverage). We recommend the described set of methods primarily for high-throughput metabolomics experiments relying on a few hundreds of low volume samples. As the methods features low solvent consumption and perfect compatibility to nanoESI mass spectrometry platforms, as in use in proteomics and increasingly also in metabolomics and lipidomics, we recommend consideration of this methods also in cases where sample size does not matter but platform flexibility and reduced operation costs are assets.



## NON-TARGETED METABOLOMIC ANALYSIS OF FRESH WATER AND MARINE STICKLEBACK FISH

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Threespine stickleback fish (*Gasterosteus aculeatus*) are found in both freshwater and marine environments and have evolved mechanisms for osmoregulation. We have used an untargeted LC and GC/Q-TOF based metabolomics approach to identify metabolomic differences from tissues obtained from lab raised freshwater and marine fish. The focus is on the GC/QTOF workflow since there is significant overlap between the GC and LC/MS results.

As is often the case in untargeted metabolomics, not all the chromatographically resolved components are known. In order to correctly identify the remaining annotated chromatographic peaks, accurate mass spectrometers such as TOFs with high resolving power can be used to identify empirical formulas. The GC/ Q-TOF also generates accurate mass product ion spectral information to aid in structural elucidation.

The fish pectoral muscle tissues were extracted by the Folch method. The aliquots from the upper aqueous layer were dried by speed vacuum and the active functional groups were derivatized. The carbonyl groups were first protected by methoximation, the samples were then derivatized with MSTFA + 1 % TMCS prior to analysis by GC/Q-TOF. The resulting data were first de-convoluted, metabolites were identified by comparison with a metabolomic library, and finally processed using a multivariate statistical package.

Interestingly, we observed significant changes in the non-essential amino acid profiles but no corresponding changes in the essential amino acids which would be expected from a stress-induced fasting response. A metabolite frequently associated with epigenetic effects was also identified.

This study demonstrates the use of GC/Q-TOFs in an untargeted metabolomics approach to gain insight into biochemical differences between populations that have adapted to different natural environments.

**LIPIDOMIC PROFILING USING SUB-2 $\mu$ M PARTICLE CO<sup>2</sup> BASED SUPERCRITICAL CHROMATOGRAPHY MASS SPECTROMETRY**

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The typical chromatographic methods for analyzing fatty acids and neutral lipids are gas chromatography after derivitization and liquid chromatography-tandem mass spectrometry (LC/MS/MS). However, there are shortcomings associated with each of these methods. For example, GC methods require derivitization of the fatty acids to methyl esters (FAME), which is burdensome, time consuming, and there is a risk of re-arrangement of the fatty acids during derivitization which leaves doubt as to whether the esters formed are from free fatty acids or intact complex lipids. In LC/MS/MS methods, the runs typically involve labor intensive and time consuming sample preparation, and utilize toxic solvents, which are expensive to purchase and dispose. We have developed rapid, high throughput and efficient method for the separation and analysis of free fatty acids and neutral lipids using sub-2 $\mu$ m particle CO<sub>2</sub> based supercritical chromatography. The organic extract from the matrix containing lipids is directly injected onto the system showing a significant saving in solvent, cost and sample preparation time. The separation mechanism is mainly based on the number of carbon chains and the number of double bonds on the acyl chain.

The analysis of adipose tissue extract using the CO<sub>2</sub> based supercritical chromatography mass spectrometry produced a very good separation of the free fatty acids and neutral lipids in less than 5 minutes. The separation mechanism is mainly based on the number of carbon chains and the number of double bonds on the acyl chain. The datasets were processed using TransOmics Informatics for Metabolomics and Lipidomics a new software tool that provides automatic peak detection followed a quantitative comparison and statistical analysis to differentiate those features that are significantly changing and finally identify those features from the mass spectrometry data. Preliminary results showed there is a group separation between the control and PPAR-pan agonist treated samples. Some potential biomarkers that contribute to the group difference have been identified.

**ELECTRON IMPACT AND CHEMICAL IONIZATION HIGH RESOLUTION TIME-OF-FLIGHT MASS SPECTROMETRY ANALYSES OF BLOOD PLASMA SAMPLES**

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The high sensitivity, peak resolution and reproducibility of gas chromatography mass spectrometry (GC-MS) have made it one of the most utilized techniques for plant and animal metabolite profiling. While no single instrument is capable of fully profiling the metabolome, the unique capabilities of high resolution time-of-flight mass spectrometry (HRT) make it an essential tool for metabolomics. HRT provides additional benefits such as reduced analysis times, more effective peak deconvolution and the ability to interrogate rich data sets repeatedly for novel materials. In addition, utilization of high performance TOFMS instruments reduces matrix interferences, and allows for the production of high quality, accurate mass spectral data for precise formula determinations and library database comparisons. Unfortunately, many metabolomic studies have relied primarily on retention indices and spectral library matching to identify analytes. This is acceptable for targeted analysis of known compounds present in existing databases; however, true profiling requires additional information and instrument capabilities (e.g., accurate mass, high resolving power) for known and unknown compound identification. The workflow in this study included analyses by both EI-HRT and CI-HRT to obtain comprehensive profiles of derivatized blood plasma samples from lean, fatty and obese Zucker rats. Mass spectral data was collected in high resolution mode and chromatography was adjusted to maximize the number of metabolites identified. Excellent mass accuracy values (MA < 1.0 ppm) allowed for confident elemental composition determinations for molecular, fragment and adduct ions.

**P5-31**

**A GCXGC/TOFMS METABONOMICS STUDY FOR LIVER TISSUES IN D-GALACTOSAMINE/LIPOPOLYSACCHARIDE INDUCED ACUTE HEPATIC FAILURE MICE**

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A new comprehensive method of two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GCxGC/TOFMS) was established for the study of metabolomics in acute hepatic failure. 982 peaks were obtained from the approach of GCxGC/TOFMS, 89 metabolites were identified, among them 47 metabolites were differentially determined, while 243 peaks from GC/TOF-MS, 51 metabolites were identified. For complex metabolite profiles compared with the traditional GC/TOFMS, GCxGC/TOFMS would be a better way for the characterization of metabolomic profiles.

## **AUTOMATED JUST IN TIME ONLINE DERIVATIZATION FOR GC-MS AND GC MS/MS ANALYSIS OF PHOSPHORYLATED METABOLITES**

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Accurate quantitation of phosphorylated metabolites in biological samples is highly challenging due to the natural occurrence of several isomers (e.g. hexose or pentose phosphates). Their chemical similarity renders chromatographic separation difficult and mass spectrometric fragmentation shows virtually the same patterns for several isomers.

For gas chromatographic analysis two step derivatization (alkoxymation followed by trimethylsilylation), which is commonly in use for metabolite analysis, was automated and carried out on-line for 14 phosphorylated metabolites. Since several metabolite derivatives are known to be unstable, samples are derivatized just in time for GC analysis (no time lag between derivatization and analysis). Using this setup combined with cooled storage of the underivatized samples on the autosampler and automated liner exchange, analytical sequences with a total duration of up to 50 h can be run on a routine base. The use of uniformly <sup>13</sup>C labeled internal standard, derived from cell extracts of *Pichia Pastoris* grown on <sup>13</sup>C glucose, additionally compensates for matrix influences on derivatization.

Detection limits obtained for single quadrupole MS in SIM mode were below 0.1 pmol for all 14 phosphorylated metabolites. By improvement of gas chromatographic separation and implementation of MS/MS strategies lower detection limits as well as excellent separation of several isomers, which could up to our knowledge not yet be separated by GC, could be achieved.

**P6-1**

**PROFILING NEUROPROTECTION MECHANISMS IN NEURONAL CELLS FOLLOWING ACUTE ADMINISTRATION OF METHAMPHETAMINE**

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To study the biochemical effects of a high dose of the illicit psychostimulant drug methamphetamine, rat cortical neurons were cultured and exposed to 1 mM for 48 hours. Metabolites were profiled using GC-MS. Key changes included perturbation of amino acid homeostasis, excitotoxicity and oxidative stress. Multiple amino acid neuroprotective mechanisms were observed, several of which have not been previously associated with methamphetamine exposure. Comparison was also made with caffeine exposure, which caused some similar effects.

**ALTERATIONS IN METABOLIC PATHWAYS AND NETWORKS IN ALZHEIMER'S DISEASE: METABOLOMICS INFORMS GENOMICS**

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Pathogenic mechanisms of Alzheimer's disease (AD) remain largely unknown and clinical trials have not demonstrated significant benefit. Biochemical characterization of AD and its prodromal phase may provide new diagnostic and therapeutic insights. We used a metabolomics platform to profile cerebrospinal fluid (CSF) from mild AD (n=40), mild cognitive impairment (MCI, n=37), and control (n=38) participants; univariate and multivariate analyses to define between-group differences; and partial least square-discriminant analysis models to classify diagnostic groups using CSF metabolomic profiles. A partial correlation network was built to link metabolic markers, protein markers, and disease severity. AD participants had elevated methionine (MET), 5-hydroxyindoleacetic acid (5-HIAA), vanillylmandelic acid, xanthosine, and glutathione vs. controls. MCI participants had elevated 5-HIAA, MET, hypoxanthine, and other metabolites vs. controls. Metabolite ratios revealed changes within tryptophan, methionine, and purine pathways. Initial pathway analyses identified steps in several pathways that appear altered in AD and MCI. A partial correlation network showed total tau most directly related to norepinephrine and purine pathways; amyloid-beta (Ab42) was related directly to an unidentified metabolite and indirectly to 5-HIAA and MET. The methionine and folate pathways were then pursued by a targeted genomic approach using the ADNI-1 cohort. The additive and dominant effects of relevant SNPs were investigated using logistic regression and linear regression for disease status and several imaging phenotypes, including average FDG-PET measures, normalized to pons, from selected regions of interest and Homocysteine levels from plasma and CSF. The overall effect of each gene in gene-based approach was also evaluated using SNP-set-based analysis. In conclusion, MCI and AD appear associated with an overlapping pattern of perturbations in tryptophan, tyrosine, methionine, and purine pathways, and suggest profound biochemical alterations are linked to abnormal Ab42 and tau metabolism. Metabolomics provides powerful tools to map interlinked biochemical pathway perturbations and study AD as a disease of networks failure.

**DEVELOPMENT OF AN IN VITRO METABOLOMIC APPROACH FOR ENHANCED NEUROTOXIC EFFECTS BY ENVIRONMENTAL CONTAMINANTS, WITH EMPHASIS ON NEUROTRANSMITTER PATHWAYS**

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Worldwide, serious concern has arisen about the increased incidence of learning and developmental disorders in children. From a scientific point of view, there is no doubt that exposure to neurotoxic chemicals during early brain development can adversely affect learning and development. Various recent epidemiological studies have indicated that exposure to low doses of environmental biologically active contaminants during human development can have deleterious effects on cognitive development in childhood. The European commission-funded project DENAMIC "Developmental Neurotoxicity Assessment of Mixtures in Children" investigates neurotoxic effects (e.g. learning and developmental disorders) of low-concentration mixtures of pesticides and a number of common environmental pollutants in children. One of the aims is to develop better and sophisticated tools, procedures and testing methods to screen compounds for (developmental) neurotoxicity and to improve assessment of exposures and effects ([www.denamic-project.eu](http://www.denamic-project.eu)). As part of the project, a new alternative assessment strategy based on a combination of in vitro and in vivo assays is under development in order to prioritize compounds for further in vivo testing. To this end, hazard characterization of pesticides and environmental pollutants on a molecular and cellular level is carried out, with emphasis on adverse effects during neuronal development. An array of in vitro assays is used to investigate (developmental) neurotoxic effects, including neuron differentiation in the SH-SY5Y human neuroblastoma cell line, acetylcholinesterase (AChE) inhibition, and transthyretin (TTR) binding. An important aspect is the development of biomarkers for (developmental) neurotoxicity in animal models using (epi-)genomics, proteomics and metabolomics. This paper presents the development of a metabolomic and neurotoxicity approach for the SH-SY5Y cell line. The focus is on four neurotransmitter pathways: Dopamine, Serotonin, Gaba, and Acetylcholine. Analytical methods were developed to detect and quantify the precursors, neurotransmitters and metabolites in the SH-SY5Y cells using a 12 well based system and LC-MS/MS. An LC-HRTOF system was used for untargeted analysis. SH-SY5Y cells were exposed to various pesticides, their metabolites and MeHg to investigate the effects on the neurotransmitter pathways.



**LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY BASED ANALYSIS OF THE CEREBROSPINAL FLUID METABOLOME FOR THE STRATIFICATION OF PATIENTS WITH UNEXPLAINED ENCEPHALOPATHIES**

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Many complex neurological disorders remain of unknown etiology despite extensive biochemical and genetic work-up. These “unexplained encephalopathies” may be caused by inborn errors of metabolism (IEM). Diagnosis of IEMs can usually be accomplished by the biochemical analysis of biofluids to highlight the consequences of an enzymatic or a transport protein defect. However, biochemical analyses available at clinical chemistry laboratories are restricted to well-known metabolites, and many metabolic pathways remain unexplored. In the present study, we aimed at evaluating liquid chromatography coupled to mass spectrometry (LC/MS)-based analysis of the cerebrospinal fluid (CSF) metabolome for the study and characterization of IEMs involving the nervous system. To this end, about 200 CSF samples were selected from a biobank collected from adult patients seen in the neurometabolic unit of the Pitié-Salpêtrière Hospital. The developed LC/MS methods led to the annotation of 430 features and identification of more than 100 metabolites in CSF samples. CSF analysis revealed abnormal fingerprints in patients with confirmed metabolic diseases, and enabled the grouping of 5 samples, given the high number of common abnormalities detected in their fingerprints. Interestingly, two of these samples have already been grouped from a previous NMR study in which a significant elevation of free sialic acid was detected in these samples. This demonstrates that LC/MS based metabolome analysis of CSF samples is relevant for the stratification of patients with unexplained diseases.

## 2,4 DIHYDROXYBUTANOIC ACID AS A POTENTIAL MARKER OF ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is a fatal neurodegenerative disorder manifested by cognitive and memory impairments. More than 35 million people worldwide have AD, at a cost of \$605 billion in care and treatment for patients, as well as lost productivity. Age is the most notable risk factor with the incidence of disease doubling every 5 years after 65 years of age. Abnormalities in  $\beta$ -amyloid and hyperphosphorylated tau proteins are connected with AD but their mechanisms in disease progression are not well understood. In order to prevent or delay the disease onset early markers that set the scene for drug development or lifestyle interventions are needed. In a previous study, we have found that plasma 2,4-dihydroxybutanoic acid (2,4-DHBA) predicts progression to AD.

Here we hypothesized that changes of 2,4-DHBA plasma levels reflect changes in the brain metabolism in progression to AD. We analyzed small polar metabolites in cerebrospinal fluid (CSF) and plasma using the GC $\times$ GC-TOFMS platform for combined quantitative and non-targeted profiling. The samples were collected from 64 subjects of which 6 were healthy controls (HC), 19 had stable mild cognitive impairment (sMCI), 21 had progressive mild cognitive impairment (pMCI) and 18 had already diagnosed AD. HC and sMCI subjects were pooled to form a control group against which the pooled disease group containing pMCI and AD subjects was compared to. We found 7 metabolites with  $p < 0.1$  using the two sided t-test.

Confirming the study hypothesis, the most predictive CSF metabolite was organic acid 2,4-DHBA. Its biochemistry is not well known but it is associated with sugar breakdown in the brain under hypoxic conditions. Our findings show that 2,4-DHBA is a potential marker for AD which compares favourably against the tau protein and  $\beta$ -amyloid risk markers. Further studies are needed to understand biochemistry of 2,4-DHBA and to elucidate the potential link between hypoxia and AD progression.

**UNTARGETED METABOLOMICS METHODS FOR THE EARLY DETECTION OF ALZHEIMER'S DISEASE (AD): POTENTIAL FOR DEVELOPING NEW DIAGNOSTICS?**

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In this study high resolution mass spectrometry (UPLC-QToF-MS) was combined with chemometrics to analyse human plasma from patients attending the Belfast City Hospital Memory Clinic. Groups consisted of patients with mild cognitive impairment (MCI) ( $n=20$ ), patients with MCI who later went on to develop Alzheimer's disease (MCI-AD) ( $n=20$ ), and healthy age-matched controls ( $n=40$ ). Samples were extracted using acetonitrile and analysed using an Acquity UPLC coupled with a Xevo G2 QToF mass spectrometer (Waters). Chromatography was carried out on an Acquity UPLC HILIC (1.7 $\mu$ m x 2.1 x 100 mm) column and detection was in ESI+ acquisition mode. The acquired data were used to build an OPLS-DA statistical model capable of predicting the onset of AD in a well-defined clinical cohort. The importance of this approach for distinguishing prospective AD cases from controls is highlighted by its ability to also clearly distinguish MCI patients (of which ~10% later go on to develop AD) with  $R^2=0.95$  and  $Q^2=0.78$  (rising to 0.84 when gender specific information is co-factored). Using this high resolution mass spectrometry (HRMS) profiling method 2443 ions of interest were used to build the multivariate model however from the S-plots we were able to identify the ions of interest which could be considered as potential blood-based "biomarkers" for the disease. Using this small sub-set of statistically significant ions we were able to build a predictive model capable of distinguishing AD and MCI suffers from healthy age matched controls with a high degree of accuracy. In this study we have optimised an untargeted HRMS method combined with chemometrics capable of predicting AD 2 years before conventional clinical diagnosis. There is huge potential for the metabolites/ions of interest discovered here to provide blood-based diagnostics for MCI and AD, and to improve clinical trials through better patient stratification.

**METABOLIC SIGNATURES OF HUMAN ALZHEIMER'S DISEASE (AD): <sup>1</sup>H NMR ANALYSIS OF THE POLAR METABOLOME IN POST-MORTEM BRAIN TISSUE**

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<sup>1</sup>H NMR spectroscopy is a powerful tool for uncovering small molecule metabolite disturbances that are associated with disease. We recently reported the potential of NMR metabolomics to distinguish animal models of Alzheimer's disease (AD) from wild-type litter mates. Here, <sup>1</sup>H NMR metabolomics studies were conducted on polar extracts of human post-mortem brain tissue which was obtained from the Newcastle Brain Trust (Brodmann 7 region, neocortex region, n=15; age-matched controls; n=15 AD). Lyophilised tissue was milled and 100 mg added to 1 mL of 50% methanol:water in a 2 ml sterile Eppendorf tube. Samples were mixed, sonicated, centrifuged (16,000 g; 4°C; 20 min) and the supernatant collected and dried. Samples were reconstituted in D<sub>2</sub>O containing TSP and analysed using a Bruker Avance III 400 MHz NMR spectrometer (Bruker-Biospin, UK). Using a randomised running order one-dimensional <sup>1</sup>H NMR spectra were acquired (128 scans), phased and manually baseline corrected using ACDlabs (version 11.0, Toronto, Canada). Data reduction was conducted by manually binning the spectra and measuring the integral for each bin/bucket between 0.84 ppm and 8.95 ppm - a total of 143 buckets were recorded. The principal component analysis (PCA) and orthogonal partial least squares discriminant (OPLS-DA) plots correlated closely with disease status and the statistical model distinguished (R<sup>2</sup>=0.75; Q<sup>2</sup>=0.40) tissue of AD patients from age-matched controls. Relative brain levels of Myo-inositol, creatine, betaine, GABA, histidine, adenosine, NAAG, taurine and glucose were significantly altered in AD brain tissue (p<0.05). It was discovered that predictions could be further enhanced by using NMR data to train an artificial neural network (ANN). A two-layer feed-forward network was trained with scaled conjugate gradient backpropagation, and this blindly validated and tested samples with up to 99% accuracy (Receiver operating characteristic; AUC=0.99). These studies indicate important metabolite disturbances associated with the onset of AD and demonstrate considerable for both studying and classifying this disease.

**METABOLIC PROFILING OF BRAIN REGIONAL VARIATION UNDER ADULT-ONSET HYPOTHYROIDISM USING A MOUSE MODEL**

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The mammalian brain, as a spatially multiscale structure, consists of regions with different physiological and biochemical roles under normal or diseased conditions. Metabolomic analysis provides insight into the dynamic mechanisms and functional properties of specific brain areas, while it can further our understanding on the pathogenesis and onset of brain diseases. To-date, brain regional variation has been mainly studied in the context of diseases, while a limited number of mostly technical publications that compare the metabolic physiology of different brain areas refer to few metabolites.

Even if the brain has been well-established as the main target tissue of thyroid hormones, the current knowledge about the effect of adult onset hypothyroidism (AOH) on brain metabolism remains fragmented. A study of our group is the first published report investigating the metabolic profile of cerebellum under AOH in male mice in a systemic and systematic way [1]. Despite the accepted notion of brain region specific responses to AOH, these have not been extensively investigated at the metabolic level. The present study provides a holistic comparative analysis of the metabolic physiology of three brain regions, cortex, cerebellum and midbrain, in a mouse model of AOH (1% w/v KClO<sub>4</sub> in the drinking water of adult male Balb-c/J mice for 60 days) using gas chromatography-mass spectrometry (GC-MS) metabolomics.

Our results demonstrate a different metabolic response to AOH among the three brain regions, which could be justified from the observed regional variation in their physiology under normal conditions. The study is providing clues on potential reasons for the observed lighter effect of AOH on certain regions and expanding our knowledge about brain function at the molecular level. More particularly, the effect of AOH is strongest in cerebellum and midbrain as compared to cortex. Our results indicate the “leaner” metabolic profile of cortex under normal conditions as potential reason.

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**FROM EPIGENETICS TO METABOLOMICS: TARGETED AND UNTARGETED MASS SPECTROMETRY-BASED METHODS FOR EXAMINING METABOLITE DIFFERENCES ASSOCIATED WITH ALTERED STRESS RESPONSE DUE TO EARLY LIFE STRESSORS**

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Acute psychosocial stress is frequently associated with activation of the hypothalamic-pituitary adrenal (HPA) axis resulting in peaks in cortisol, adrenocorticotrophic hormone (ACTH) and glucocorticoid levels. However, the HPA axis is also involved in long-term processes such as the regulation of growth, reproduction, and metabolism. Early life stressors appear to play a special role in determining long-term HPA axis function: perinatal stress has been found to influence methylation status at CpG sites associated with transcription factor binding (nerve-growth-factor-inducible protein A) in the promoter region for the glucocorticoid receptor gene. The result is an enduring decrease in glucocorticoid receptor (GR) expression and dysregulation of the HPA axis, which depends on GRs for inhibitory feedback. Dysregulation of the HPA axis, which may also be caused by chronic stress or adverse social environments, has been associated with increased abdominal fat, loss of muscle mass, and mental health risks.

Given the profile of health risks associated with HPA dysregulation, we hypothesize that the long-term alteration of HPA axis functioning under conditions of altered GR expression may be associated with systemic changes in a broad range of metabolic pathways. To examine the potential metabolomic changes associated with this model, we applied a combination of high-sensitivity mass spectrometry (MS) techniques, including targeted DI- and LC-MS/MS and untargeted LC-MS, to perform comprehensive metabolomics analyses on serum from human participants in an early life stress-exposure cohort. The metabolomics data was examined in the context of percentage of methylation at relevant CpG sites, possible trends in HPA activity (based on measurements of representative cortisol levels in hair samples), and extensive clinical data (including measures of peri-natal and early childhood stress exposure, and psychological scales of resiliency and mental wellness).

P7-1

## A METABOLIC APPROACH TO INVESTIGATE ALLELOPATHIC INTERACTIONS OF UNICELLULAR ALGAE

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Microalgae of the marine plankton are known to chemically interact with surrounding organisms in their environment. One example for such an interaction is the communication via allelochemicals, which have the potential to influence the growth, survival and reproductive success of coexisting organisms both positively and negatively. Allelopathy plays a tremendous role in shaping plankton dynamics and it is thus of great interest to understand underlying principles and identify involved infochemicals. Initial evidence suggests that cells can respond to the presence of competitors with a metabolic response, but in most cases it is still unknown, which chemical compounds are key players in these processes. Such dynamic processes are ideally suited for a comparative metabolomics approach with the aim to identify the role of hitherto uncharacterized infochemicals.

GC/Tof-MS based metabolic profiling of the two interacting diatoms *Skeletonema costatum* and *Thalassiosira weissflogii* reveals a dynamic response of the cells to the presence of the partners compared to unialgal cultures. Using the stimulatory effect of *Skeletonema costatum* on *Thalassiosira weissflogii*, samples were taken at distinct time points during the algal growth. Those time points correlated with the growth stimulating effect. By comparing metabolic profiles of both algal species in unialgal cultures as well as in co-cultivation with the interaction partner, distinct metabolic changes in both species due to co-cultivation could be visualized. This simplified approach enables us to elucidate the complexity of algal interaction in a small-scale set up and further studies will involve verification of the concepts in the ecological context of the sea.

In this poster we introduce release and uptake dynamics of chemical compounds between unicellular algae. We substantiate named changes in both the endo- and exometabolome, present first propositions of involved chemical substances and by using  $^{13}\text{C}$  labelled algae, will introduce hypothesis of chemical flux between the communicating diatoms *S. costatum* and *T. weissflogii*.

## PATTERNS OF CHEMICAL DIVERSITY ON SPONGE ASSOCIATED MARINE BACTERIA

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**Background:** Marine sponges are major components of benthic communities. In terms of biomass they are the richest sources of secondary metabolite production, with the potential to influence both benthic and pelagic systems. Currently there is a dearth of knowledge relating to microbial-sponge associations and interactions and thus the functions and biochemistries of sponge-associated bacteria are increasingly important research topics. For many sponge-associated symbionts the information available to classify their biochemical/biological difference is via their 16S rRNA sequences and the secondary metabolite production profile remains elusive.

**Objectives:** To investigate the (bio)chemical profiles of *Hyattella intestinalis*, *Dercitus xanthus* and *Cinachyrella australiensis* sponge-associated marine bacteria *Salinispora arenicola* and "*Salinispora pacifica*", collected from the Great Barrier Reef (GBR) off the north east coast of Australia.

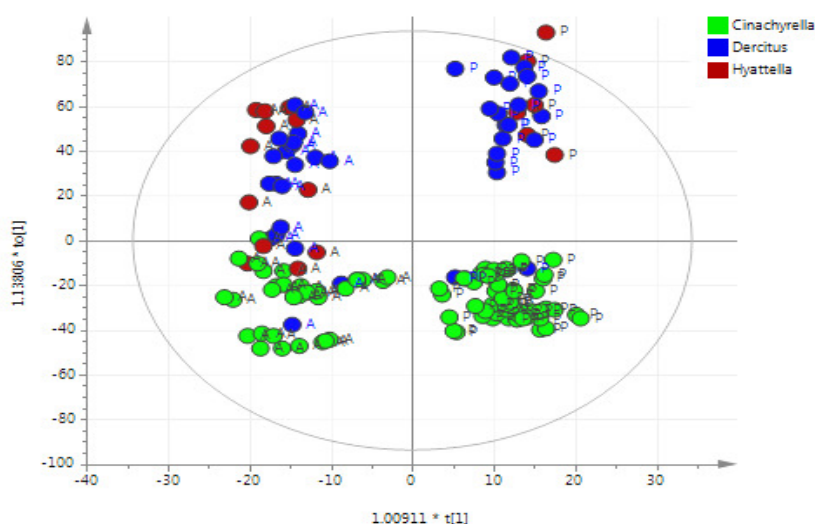
**Methods:** Microbial extracts were prepared by solvent (ethyl acetate) extraction of bacterial cell mass, followed by solvent evaporation and reconstitution. Ultra High Performance Liquid Chromatography-Quadrupole Time of Flight Mass Spectrometry (UHPLC-QToF-MS) was used to acquire metabolic profiles and subsequent multivariate data analysis used to delineate the underlying (bio)chemical diversity.

**Results:** Sponge-dependent chemical diversity was observed in two bacterial species: *S. arenicola* and "*S. pacifica*" (Fig. 1). Additionally, we have discovered and confirmed the identity of 1- deoxynojirimycin, for the first time, from these bacterial species.

**Discussion:** This metabolomic analysis of the sponge-associated bacteria *S. arenicola* and "*S. pacifica*" demonstrates the complex chemical and dynamic sponge-dependent metabolite profiles, and in turn provides opportunities for future studies of the role of secondary metabolite production in sponge-microbe interactions.

Figure 1: Metabolic profiling distinguishes bacterial samples collected from three sponges: *Hyattella intestinalis*, *Dercitus xanthus* and *Cinachyrella australiensis*: OPLS-DA plot of the predictive component (x-axis) and orthogonal component (y-axis) from a model of supervised classification of the two bacterial species *S. arenicola* and *S. pacifica*. The bacterial

classes (A – arenicola; P – pacifica) are separated in the predictive component to[1] and sub-cluster patterns for sponge-association are observed in the orthogonal component to[1]. Key: *Cinachyrella* (green), *Dercitus* (blue) and *Hyattella* (red). Each symbol represents one bacterial sample described by all detected metabolites. The ellipse represents the Hotelling's T<sup>2</sup> 95% confidence interval for the multi-dimensional data.





**HIGH-THROUGHPUT NMR- AND LC-MS/MS-BASED METABOLOMICS: INSIGHT OF THE RESPONSES OF VARIOUS ORGANS AFTER NAPHTHALENE INTERVENTION**

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IARC classified environmental pollutant, naphthalene as group 2B, a possible carcinogen to human. Current study, we aimed to elucidate the mechanisms of naphthalene-induced injuries on the target organ, lungs and the detoxification organs, liver and kidneys *via* NMR- and LC-MS/MS-based metabolomics. Male ICR mice were intraperitoneally injected with vehicle (olive oil), low dose (100mg/kg) and high dose (200mg/kg) of naphthalene. After 48 h, the organs were extracted and prepared for NMR, and LC-MS/MS analysis following multivariable analysis. High-throughput data suggesting the lung's energy metabolisms were perturbed in naphthalene treated groups and reduction of glycerophosphocholine was suspected to be related to cellular rejuvenation after injuries. In liver, antioxidative stress related metabolites were upregulated to overcome the oxidative stress during naphthalene biotransformation and detoxifications. Glutathione, an important antioxidant shielding the cellular component from reactive oxygen species was found to be increased in kidney of the naphthalene treated mice. Due to less sensitivity of the NMR, LC-MS/MS was applied to analyze the polar lipids including ceramides and phosphatidylcholine (PCs). Sphingolipid including ceramide and ceramide-1-phosphate play critical role in inter- and intracellular signalling. Besides, sphingomyelins (SM) were found to be upregulated in all of the naphthalene exposed animal tissues. Among the PCs, we found that diacyl-GPC and 1-O-alk-1-enyl-2-acyl-GPC were the major PCs perturbed in the naphthalene treated samples. Nevertheless, the functionality of these polar lipids species requires additional experiments for conformations. To conclude, NMR- or LC-MS/MS- based metabolomics enabled the characterizations of naphthalene induced injuries in various organs.

**METABOLOMIC STUDY OF *CAENORHABDITIS ELEGANS* FOR THE TOXICITY EVALUATION OF SUB-LETHAL RESPONSES TO TITANIUM DIOXIDE NANOPARTICLES USING GAS CHROMATOGRAPHY - MASS SPECTROMETRY COMBINED WITH PATTERN RECOGNITION APPROACH AND INGENUITY PATHWAY ANALYSIS**

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The increasing use of nanotechnology in our daily life have unintended effects, that can have adverse impact on human health, environment and ecosystems. Understanding the effect of toxicity and the mechanism of action of these engineered nanoparticles is still a challenging task. Metabolomics, the study of small chemical entities that are chemically transformed during metabolism, provides a unique opportunity to find out bio markers, which leads to identification of cellular pathways and their biological mechanisms. With emerging technologies of mass spectrometry, thousands of metabolites can now be analysed from minimum amount of biological material. In the present study gas chromatography - mass spectrometry based untargeted metabolomic profiling approach was utilized to study the toxicity of sub-lethal concentrations of titanium di oxide (TiO<sub>2</sub>) nanoparticles in well known soil nematode *Caenorhabditis elegans*. Pattern recognition analysis (PCA and PLS-DA) and ingenuity pathway analysis were performed to analyzing global metabomics, in order to characterize the biochemical perturbations and their potential mechanisms of TiO<sub>2</sub> nanoparticles toxicity. Potential marker metabolites, contributing to the significant differences in metabolomic profiles of TiO<sub>2</sub> nanoparticles induced *C. elegans* from healthy controls were identified, with 15 metabolites showing the best combined classification performance. The biological pathways responsible for the TiO<sub>2</sub> nanoparticles were identified, among them mainly TCA cycle and arachidonic acid metabolism, glyoxalate dicarobxylate metabolism were mainly effected. The present study demonstrates that metabolomics can be employed as a tool to understand the potential toxicity of nanoparticles in terms of organism-environment interactions and for assessing the organism function at molecular level.

## NMR-BASED METABOLOMICS TO STUDY NAPHTHALENE TOXICITY IN A TOLERANT MOUSE MODEL

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Naphthalene (NA) which widely spread in the environmental is a common polycyclic aromatic hydrocarbon. Several studies indicated that single dose exposure of naphthalene injured mouse Clara cell, a bronchiolar epithelial cell. However, when mice are administered repeated exposures of naphthalene, the susceptible Clara cell become refractory to injury. In this study, we intended to investigate the mechanisms of naphthalene toxicity in various mouse tissue among injured, tolerant and the control mice using  $^1\text{H}$  NMR-based metabolomics. Male ICR mice were administered seven repeated injections (ip) of NA (0, 200 mg/kg/day) in olive oil and gave a challenge dose (300 mg/kg/day) at eighth day. Bronchoalveolar lavage fluid (BALF) and both hydrophilic and hydrophobic extracts from the lung, liver and kidney were analysed by  $^1\text{H}$  NMR followed by multivariate analysis. The results showed that the tolerant mice are different from single exposure mice (Fig 1). Numerous amino acid and energy-related metabolites are important in response to naphthalene toxicities. In conclusion, the tolerant animals provide a good model to investigate the mechanism of naphthalene induces toxicities using  $^1\text{H}$  NMR - based metabolomics.

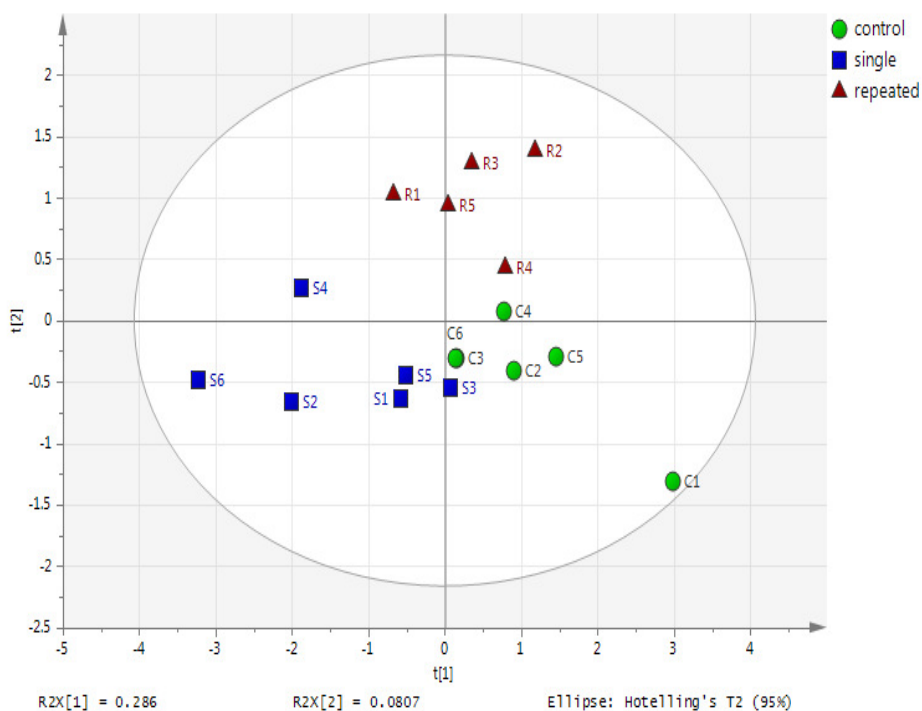


Figure 1: PLSDA scores plot from hydrophilic metabolites in the BALF. Control is represented the control group. Single and repeated were administered seven repeated injections (ip) of NA (0, 200 mg/kg/day) in olive oil and gave a challenge dose (300 mg/kg/day).

### Acknowledgements:

The NMR spectra were obtained at the Core Facility for Protein Structural Analysis supported by National Core Facility Program for Biotechnology. The research was supported by National Health Research Institutes (NHRI-EX100-9915EC, NHRI EX101-9915EC, NHRI-EX102-9915EC) in Taiwan

**GLYCEROPHOSPHOCHOLINE PROFILING OF THE CORAL *SERIATOPORA CALIENDRUM* IN RESPONSE TO COPPER-INDUCED OXIDATIVE STRESS**

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In loss process of coral symbionts, the cells experience a variety of membrane deformation according to the mechanisms involved. For diagnosing coral bleaching, it is useful to characterize the membrane lipids modulation. Glycerophosphocholine profiling in the coral of exposure to copper was examined in this study. The results were correlated with cellular accommodation in necrotic process, and suggested that dose-dependent exocytosis of symbionts share with cell necrosis in the lipids metabolism in copper-induced coral bleaching.

## DISCRIMINATING THE ACUTE TOXICITIES OF ETHION AND BIFENOX IN RAT LIVER BY NMR-BASED METABOLOMICS

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Metabolomics has been proved to possess substantial potential to discriminate different toxicities and elucidate the molecular mechanisms responding to xenobiotics. Compared with lots of drug researches, there are fewer metabolic studies on the toxicities of variant pesticides. Classifying different pesticides is important because of their complexity and persistence in the environment. In this study, we intend to discriminate the metabolic responses of two pesticides using NMR-based metabolomics. We collected the liver tissues of rats administrated with series doses of two classes of pesticides (ethion: organophosphate insecticide, and bifenoxy: diphenyl-ether herbicide) via ip. Unique metabolic signature in the rat liver were analysed by  $^1\text{H}$  and J-resolved NMR followed by multivariate analysis. Results showed a classifying pattern between two pesticides and doses in PLS-DA model (Fig 1). Numerous amino acids and energy-related metabolites were correlated with effects of classes and doses of the pesticides. This is a proof-of-concept study to demonstrate that metabolomic effects reflect mode of toxic action.

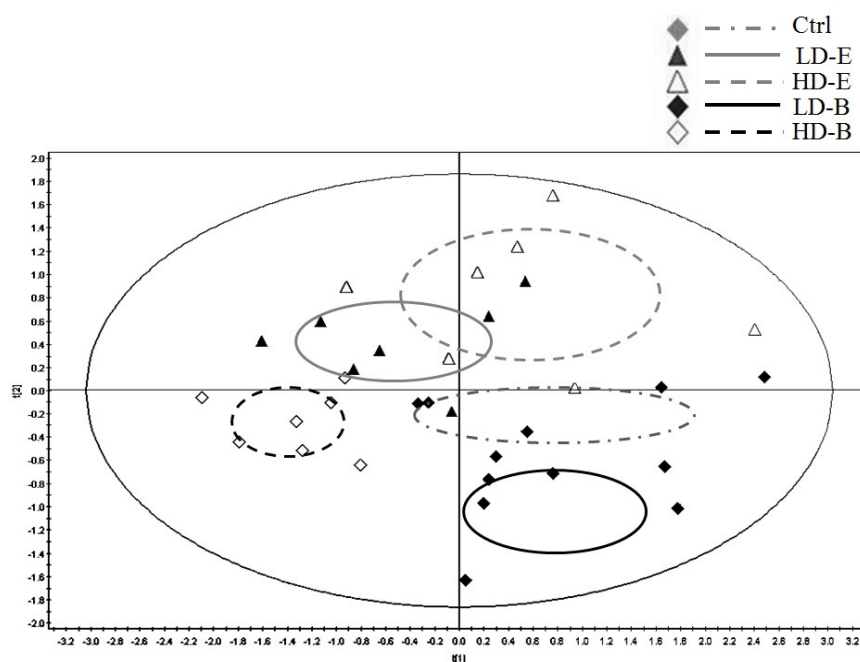


Figure 1: PLS-DA scores plot from the hydrophilic metabolites in the liver of rats treated with high or low doses (HD or LD) of Ethion (E) or Bifenoxy (B).

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The NMR spectra were obtained at the Core Facility for Protein Structural Analysis supported by National Core Facility Program for Biotechnology. The research was supported by National Science Council (NSC 99-2314-B-002-127-MY3) in Taiwan.

**THE ROLE OF MASS SPECTROMETRY IN THE METABOLOMIC STUDY OF TOXICOLOGICAL EFFECTS OF METAL TOXICITY IN LABORATORY MICE. EXPERIMENTS EXPOSURE TO As, Cd AND Hg UNDER CONTROLLED CONDITIONS**

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Metals have a central role in biological systems, regulating and participating in numerous cellular processes, as well as presenting toxic or deleterious effects on the metabolism. Hence, the study of metal-induced changes in cellular metabolic pathways is crucial for understanding the biological responses to these elements in environmental exposure. In this field, the study of biomarkers may be of great interest in environmental stress as an alert to serious and irreversible damage occurring in the environment. However, its use requires a thorough knowledge of biological processes that cause them. For these reasons, the use of *-omics* techniques has been suggested for this purpose.<sup>1</sup> Among the most recent *-omics*, metabolomics is based on the complete study of metabolites involved in different metabolic processes of organisms<sup>2</sup>. On the other hand, metallomics which uses metals or metalloids, present in one third of biomolecules in cells, as heteroatomic markers or tags to track these molecules in complex biological matrices<sup>3</sup>. In these approaches it is fundamental the use of high sensitivity detectors such as mass spectrometry<sup>4-5</sup>.

In this work, a metallomic approach based on size-exclusion chromatography (SEC) coupled to inductively coupled plasma-mass spectrometry (ICP-MS) detection is combined with anion or cation exchange chromatography to achieve a better understanding of the function, detoxification processes and regulation of metals in biological systems under controlled exposure to the most important toxic metals such as arsenic, cadmium and mercury. On the other hand, intended to get as much metabolic information as possible, several organs and biological fluids taken from laboratory mouse *Mus musculus* have been studied, after being exposed to arsenic, cadmium and mercury for different period of time. The analysis was carried out by mass spectrometry of high-resolution by direct infusion (DI-ESI-QqQ-TOF-MS). Statistical analysis of results allowed us to compare the different metabolic profiles, establishing those metabolites that are altered in the presence of these contaminants. Finally, these altered metabolites were quantified by gas chromatography-mass spectrometry (GC-MS).

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**CROSS-PLATFORM METABOLIC PROFILING OF THE AQUATIC MODEL ORGANISM  
*LYMNAEA STAGNALIS* FOR NEUROTOXICITY ASSESSMENT**

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Metabolic profiling is a powerful approach in the environmental field for revealing metabolic changes caused by chemical stressors such as neurotoxicants.

The freshwater pond snail, *Lymnaea Stagnalis* has been the interest for several studies on molecular and behavioral neurobiology showing its successfully employment as model organism.

This aquatic species can be employed to assess neurotoxicity in the environment and therefore an untargeted metabolomic approach for characterizing *L. Stagnalis* tissues has been carried out. To investigate the neuronal metabolism a targeted approach focusing on the main neurotransmitters their metabolites, and precursors has been developed.

The main goal of untargeted profiling is to analyze as many small endogenous metabolites as possible in an organism. We investigate metabolic profiles, including the neurotransmitters, in the central nervous system, albumen gland, and digestive gland of *L. Stagnalis*. Therefore, it is necessary to exploit the capabilities of sample preparation and analysis to this purpose. Concerning sample preparation, a fast and automated strategy has been adopted based on beads beating tissue disruption method and a simultaneous extraction of the hydrophilic and lipophilic fractions with a biphasic chloroform/methanol/water mixture. In order to obtain a broad picture of the metabolome two complementary techniques have been employed: liquid chromatography (LC) and gas chromatography (GC) coupled to mass spectrometry. To further increase the metabolome coverage, besides the reverse phase LC, hydrophilic interaction liquid chromatography (HILIC) focusing more on small polar metabolites has been applied as well.

Although in literature is common to find specific platform oriented metabolic profiling strategy, the different analytical platforms applied in this study have been proven the capability of gaining a more organism comprehensive picture. This multiplatform approach is a starting point for future global metabolic profiling applications for studying neurotoxicity.

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P7-10

**UNDER THE SEA --- METABOLOMIC RESPONSE OF THE SEAGRASS *HALOPHILA OVALIS* TO NICKEL INDUCED ABIOTIC STRESS**

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Seagrasses are the only known ecological group of fully submerged marine flowering plants. They are most commonly found along coastal areas and are home to a multitude of marine organisms, which in terms of biodiversity make them at par with mangroves and corals. Despite their ecological significance, very little is known about these unique group of plants, especially their response to abiotic stress. In small amounts, nickel is an essential component of many cofactors necessary for plant development, however at high concentrations it can become toxic. High concentrations of nickel can usually be found near nickel related industries such as mining and smelting, whose effluent directly flows to coastal areas. Here we utilize GC-MS based metabolomics to gain insight into the response of seagrasses to nickel induced stress. We subjected seagrass samples to increasing concentrations of nickel: 50, 200, 500 and 1000 ppm. Results reveal that some amino acids such as valine, serine, threonine and alanine decreased linearly with increasing concentration. In contrast, proline showed a reverse response by increasing with increased nickel concentration. Furthermore, metabolites such as amphetamine and pyruvic acid were present in the control and remained at similarly low concentrations regardless of concentration. This could imply that the pathway related to these metabolites may be first to experience nickel induced damage. This work provides useful insight on how angiosperms respond to dissolved nickel induced stress in a fully saline and submerged environment.



**STUDY ON THE TOLERANCE OF STONEFLY LARVAE AT THERMAL EXTREMES BY FT-ICR MS AND NMR BASED METABOLOMICS**

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In many ectotherms thermal limits are assumed to arise from a mismatch between O<sub>2</sub> supply and demand. Higher levels of O<sub>2</sub> in the environment are predicted to enhance heat tolerance, while reductions in O<sub>2</sub> are expected to reduce thermal limits. While this may not be the general mechanism in terrestrial arthropods, aquatic insect larvae should be more prone to oxygen limitation. Here we test the hypothesis of increased anaerobic metabolism and lower energy status at thermal extremes. Five groups of stonefly larvae were treated with varying temperature and oxygen pressures: hypoxia, normoxia and hyperoxia at their thermal limits, hyperoxia at the thermal limit of hypoxia, and an acclimatized control. Metabolomic analysis was performed using high-throughput direct infusion FT-ICR mass spectrometry and 1-D and 2-D J-resolved NMR, which served as complementary techniques with different strengths, but gave very similar results. Data analyses showed changes indicating that the concept of oxygen limitation applies to stonefly larvae under normoxia and is even more pronounced under hypoxia, but less under hyperoxic conditions. Oxygen limitation is especially indicated by an increase in metabolites in the anaerobic metabolism pathway and changes in the TCA cycle, and a decrease in energy status, which eventually limits survival of heat stress. While there are several possible mechanisms to explain the findings, this study provides broad support for the hypothesis of oxygen limitation.

Ref: Verberk *et al.* (2013). Anaerobic Metabolism at Thermal Extremes: A Metabolomic Test of the Oxygen Limitation Hypothesis in an Aquatic Insect. *Integr. Comp. Biol.*, epub. (doi:10.1093/icb/ict015).

## **SURVIVING STARVATION: LIPIDOMIC ANALYSIS OF ALGAE UNDER NUTRIENT DEPRIVATION**

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The unicellular green picoalga *Ostreococcus tauri* is the most primitive known free-living eukaryote. *O. tauri* holds a key position at base of the green lineage of plants, while its very small genome and simple organelle structure make it a very interesting model phytoplankton. In this study global lipidomic strategies have been employed to define the lipid response of *O. tauri* to nutrient deprivation. The algae were cultured in either low nitrogen or low phosphorous environments for 48 hours prior to harvest. Algal lipids were solvent extracted and analysed by liquid chromatography-mass spectrometry (LC-MS) in positive and negative ion modes. The lipidomic profiling was able to identify a range of algal lipids including molecular species of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), 1,2-diacylglycerol-3-O-4'-(N,N,N-trimethyl)-homoserine (DGTS) and phosphatidylcholine. The data sets were also processed and subjected to multivariate data analysis. Principal component analysis (PCA) of the positive ion data revealed the greatest metabolic disturbances, effectively segregating the nitrogen and phosphorous depleted algae from algae cultured in complete media. The PCA loadings plot revealed an elevation of triglycerides in algae grown in nitrogen-limiting conditions, whilst betaine lipids were increased when the algae were deprived of phosphorous. Understanding the mechanisms underpinning these phenotypic responses at the lipid level will provide further insights into the molecular basis of adaptive processes in algae.

**IDENTIFICATION OF METABOLOMIC BIOMARKERS INDICATIVE FOR CO-EXPOSURE OF HUMAN CELLS TO PAHs AND HEAVY METALS**

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Up-to-date risk assessment strategies are predominantly based on single compound exposure scenarios which are only valid for compound mixtures if the underlying mechanisms of toxicity reveal additive. However, they do not account for synergistic or antagonistic mechanisms possibly occurring within compound mixtures. Therefore, the identification of biomarker sets derived from co-exposure scenarios in combination with toxicity data is needed to assess the health risks resulting from multi-component exposures.

Recent investigations indicate that heavy metals like cadmium (Cd) result in synergistic effects with a variety of polycyclic aromatic hydrocarbons (PAHs) including the mutagenic and carcinogenic benzo[a]pyrene (BP). Based on these results, we treated human mammary carcinoma MCF-7 cells with low doses of BP and Cd to identify metabolic biomarkers indicative for this co-exposure scenario. To this end, we combined a targeted quantitative analysis of 163 endogenous metabolites by FIA-MS/MS with an untargeted metabolomics approach applying LC-qTOF-MS. MCF-7 cells were treated either with BP alone, BP together with Cd, or with solvent. Our results from the targeted approach show a set of 18 metabolites that could differentiate between these three treatment regimens. In the untargeted approach a set of 21 compounds is sufficient to obtain the same separation. Our results indicate a synergistic effect of BP and Cd in MCF-7 cells upon co-exposure to these toxicants.

## CLOUD METABOLOMICS - RESPONSE TO COLD SHOCK IN BACTERIA ORIGINATING FROM CLOUDS

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The presence of microorganisms in the atmosphere was demonstrated a long time ago. Only recently the detection of metabolic activity in clouds (Sattler et al., 2001) raised questions about their implication in the physico-chemical processes occurring in these environments. The first results revealed that microorganisms are contributing to the transformation of organic compounds in cloud water (Vaitilingom et al., 2013); they also could be responsible for the formation of ice crystals (Attard et al., 2012; Joly et al., 2013), which leads to precipitations (Lohmann & Feichter, 2005). However, these environments are harsh for microorganisms: low temperatures, high UV radiations and oxidants, freeze-thaw cycles, lack of nutrients and highly dynamic conditions. Hence, it is thought that bacteria living in clouds have specific adaptations for surviving and successfully disseminating by air means. In this context, we aim at documenting those potential physiological particularities through -omics approaches, for complementing previous results.

In this study we investigated the response of *Pseudomonas syringae*, a species commonly found alive in clouds (Vaitilingom et al., 2012), to temperature downshifts. After extraction of polar (and slightly non-polar) compounds, we analyzed metabolic profile of cells exposed to low temperature through LC/MS and NMR using Metabolic Profiler® facility. Multivariate statistical tools highlighted numerous elements (116 ions and 216 buckets) for relevant contribution. From this, we identified biomarkers involved in cold stress metabolism, such as trehalose, glutathione and amino acids.

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## SCREENING OF ENVIRONMENTAL POLLUTANTS IN WATER BY GCxGC-TOFMS

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The introduction of environmental legislation, such as the EU Water Framework Directive (WFD), has enhanced the requirement for confident identification of contaminants in water. Unlike traditional 'grab' sampling, passive sampling can provide a more representative view of contamination in watercourses by monitoring over an extended period of time, as well as pre-concentrating any trace analytes.

Complex environmental samples are often difficult to analyse via conventional gas chromatographic methods, due to co-eluting compounds and trace level unknowns. Two-dimensional gas chromatography is an enhanced separation technique which, when combined with time-of-flight mass spectrometry (GCxGC-TOFMS), can provide highly sensitive detection and accurate mass spectral identification of trace analytes. The combination of passive sampling with GCxGC-TOFMS delivers a method suitable for investigative work, such as screening for emerging contaminants, in addition to target-focused studies.

The main benefit of GCxGC-TOFMS - the increased separation of complex mixtures – may also be the main weakness, as it generates extreme quantities of data which must be processed and simplified into meaningful statistics. This study investigates a variety of data-mining tools to simplify the screening of environmental pollutants (such as PAHs, PCBs and pesticides) in watercourses by GCxGC-TOFMS.

**<sup>1</sup>H-NMR BASED PROFILING OF ORGANIC COMPONENTS IN LEACHATE FROM ANIMAL CARCASS DISPOSAL SITE OVER TIME**

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Leachate, is generated by decomposition of animal carcass, has many environmental, sanitary and food safety hazards. However, there is a lack of research on characteristic of leachate. In this study, we performed <sup>1</sup>H-NMR based profiling of leachate from different animal species (pig and cattle) and two types of soil (sandy soil and sandy loam soil) followed by multivariate data analysis. Principal component analysis (PCA) from NMR data is showed similar pattern between species and soil types. The organic components, including organic acids and phenols were identified and the levels of their compounds were increased over time. Exceptionally, the methylamine level in leachate from pig carcass at 18 weeks after burial was relatively higher than cattle carcass, and leachate from cattle carcass in sandy soil at one week after burial has distinguishing components (ethanol, formate, alanine, N-methyldantion and taurine) in contrast with sandy loam soil. This study demonstrates that organic components in leachate from animal carcass can be quantitatively evaluated and characterized over time using NMR based profiling approach.

**P7-17**

## **HUMAN RELEVANT DOSE OF ENDOCRINE DISRUPTING CHEMICALS EFFECT ON THE RAT PLASMA METABOLOME**

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Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone homeostasis. So far the main concern has been on their effect on reproduction and development. The present study has been conducted to evaluate the effect of EDCs on the rat plasma metabolome. Using chemical separation of plasma metabolites and liquid chromatography combined with high resolution mass spectrometry it is possible to elucidate the changes in the plasma metabolome resulting from EDCs.

The analysis was conducted on four groups of animals.

- 1) A control group
- 2) A group given human relevant dose of perfluorononanoic acid (PFNA)
- 3) A group given human relevant dose of PFNA in combination with a cocktail of 12 EDCs given in a human relevant concentration
- 4) A group given a cocktail of the same 12 EDCs

Using chemometrics analysis the impact of EDC's on the plasma metabolome was examined.

The largest effect was observed on group 3 where it was possible to detect changes in the plasma metabolite composition.

The cocktail of EDCs had an effect on the plasma metabolome. It was shown that metabolites in the lipid synthesis as well as certain hormones were different in the four groups. The main difference was a result of a cocktail effect and not a syngeneic effect of PFNA in combination with the cocktail.

**“BOTTOM’S UP” – UNDERSTANDING GLOBAL METABOLIC REGULATION THROUGH METABOLOMIC CHARACTERISATION OF ANIMAL SYSTEMS**

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Our central research aim is to understand global metabolic regulation in a variety of biological contexts. Here we present results from several projects that highlight different aspects of this central puzzle and demonstrate the usefulness of NMR-based metabolomics in characterising biological systems.

1) Phosphine (PH<sub>3</sub>) is a small redox-active gas that is used to protect global grain reserves, which are threatened by the emergence of PH<sub>3</sub> resistance in pest insects. However, little is known about the toxic action of PH<sub>3</sub> or the resistance mechanisms. We characterised these mechanisms in *C. elegans*. Determining the sources of metabolic variation between PH<sub>3</sub> resistant and susceptible strains identified the metabolic pathways affected by PH<sub>3</sub>. Combination of metabolomic and genetic data identified dihydrolipoamide dehydrogenase (DLD) as the enzyme responsible for PH<sub>3</sub> resistance. Polymorphisms responsible for genetic resistance cluster around the redox-active catalytic disulfide or the dimerisation interface of DLD in insects and nematodes. DLD is a core metabolic enzyme, central to metabolic regulation, and a new class of resistance factor for a redox-active metabolic toxin. It participates in four key steps of core metabolism, which are affected differently by PH<sub>3</sub> in mutant and wild-type animals. This study is an exceptional case in which a combination of systems biology methods has identified a single genetic cause of phenotype change that can subsequently be studied with classical methods.

2) The callipyge mutation in sheep causes muscle hypertrophy. To investigate the biological mechanism, we characterised plasma samples and showed a clear developmental effect, while gender did not influence metabolic profiles.

3) Overnutrition/Obesity: Increasing evidence indicates that maternal obesity or overnutrition during pregnancy can influence long-term energy balance in offspring. We investigated these effects in an ovine model of periconception overnutrition of fetuses at 140 days of development - which were transferred as embryos from donor into control recipient ewes. The results indicated several metabolic pathways involved in responses of donor ewes and fetuses to periconceptional overnutrition.

Reference: Schlipalius et al. *Science* 338, 807-810 (2012).



**FROM AIRBORNE EXPOSURE TO BIOLOGICAL EFFECTS: MONITORING METABOLIC RESPONSES TO CERIUM OXIDE NANOPARTICLES IN LUNG EPITHELIAL CELLS USING UNTARGETED MASS SPECTROMETRY-BASED METABOLOMICS**

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Engineered nanomaterials, defined as structures having at least one dimension in the 1-100 nm size range, are increasingly being used in a wide range of consumer products. This development has raised concerns over the potentially toxic effects of nanomaterials to the environment and human health. To date, there is insufficient information available to allow a thorough risk assessment of nanomaterials because their modes of action within cells are not understood. Omics approaches, such as metabolomics, enable an unbiased assessment of the molecular responses of cells to nanomaterials, which ultimately could help to improve risk assessment and the regulation of NPs.

Cerium oxide nanoparticles (CeO<sub>2</sub> NPs) are increasingly being used to increase fuel efficiency in internal combustion engines. The resulting environmental exposure has raised concerns about the impact of CeO<sub>2</sub> NPs on human health via inhalation.

Here we report a mechanistic investigation into the uptake and mode of action of CeO<sub>2</sub> NPs in lung cells (A549 and BEAS-2B). We have employed a variety of microscopy imaging techniques to assess uptake, and untargeted mass spectrometry-based metabolomics to measure any metabolic changes caused by the CeO<sub>2</sub> NPs. Electron microscopy demonstrated the presence of intracellular nanoparticles within membrane bound endosomes of A549 cells. Remarkably, even at relatively high concentrations (1 mg/ml), CeO<sub>2</sub> exposure does not affect cell growth and viability. Preliminary direct infusion FT-ICR mass spectrometry studies have shown no significant metabolic differences between NP-treated and untreated A549 cells, which supports the finding of no cytotoxicity. Comparative studies on BEAS-2B cells are on-going.

**ADAPTATION OF THE PHOTOHETEROTROPHIC MARINE BACTERIUM *DINOROSEOBACTER SHIBAE* DFL12<sup>T</sup> TO CHANGING LIGHT REGIMES: A SYSTEMS BIOLOGY APPROACH**

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Background: *Dinoroseobacter shibae*, a member of the globally abundant marine *Roseo-bacter* clade, is able to perform aerobic anoxygenic photosynthesis (AAP). Recently published studies investigated the transcriptional regulation of photosynthesis genes and stress response of the organism upon light, but little is known about the metabolic adaptation.

Methods: Continuous cultivations with 3l salt water medium were performed in a chemostat with a tunable light source (40-120  $\mu\text{Em}^{-2}\text{s}^{-1}$ ). Samples for metabolome (GC-MS), proteome (2D DIGE, MALDI-TOF-MS) and transcriptome (next-generation sequencing) analyses were taken within two consecutive day and night cycles.

Results & Discussion: *D. shibae* reached steady-state after approx. 40 hours of continuous cultivation at a dilution rate of  $0.1 \text{ h}^{-1}$ . With 2 g/l succinate as carbon source the cultures reached optical densities (600 nm) of 1.5 – 1.6 at an oxygen saturation of 40%. During light phases, an increase of biomass and a decrease of the Bacteriochlorophyll  $a$ -concentration (Bchl  $a$ ) were observed, whereas in dark phases a decrease of biomass, and, after 6 hours, a production of Bchl  $a$  was detected. The accession of the oxygen saturation in the medium together with the small decline of the  $\text{CO}_2$ -content in the off-gas during light periods is in agreement with the assumption that the organism switches from heterotrophic to photoheterotrophic metabolism. Metabolome analyses showed an accumulation of TCA intermediates (small increase of succinate, fumarate and 2-oxoglutarate and a high increase of citrate and cis-aconitate), simultaneously TCA-cycle gene transcription is downregulated. Furthermore, a continuous increase of the biosynthesis of fatty acids and amino acids during the light phase was detected, which is also in agreement with published transcriptome data (Tomasch et al., 2011, ISME Journal, 1-12). Further details of our study will be presented on the conference.

**METABOLIC AND PROTEOMIC ADAPTATIONS OF *PHAEOBACTER INHIBENS* DSM 17395 TO VARIOUS NUTRIENT CONDITIONS IN MARINE ENVIRONMENTS**

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Background: Heterotrophic bacteria in marine environments are challenged by complex nutrient conditions, as dissolved organic matter comprises high diversity and low concentrations of individual compounds. Additionally, the nutrient content varies seasonal, due to bloom and collapse of phytoplankton populations. How the metabolism of marine bacteria can overcome such conditions is largely unexplored.

Methods: We cultivated *Phaeobacter inhibens* DSM 17395, a model organism for the widespread marine alphaproteobacterial *Roseobacter* clade, with different nutrient compositions (glucose minimal medium, rich medium and a casamino acid mixture). Single amino acids were applied to reassess amino acid degradation pathways, that were unclear or incomplete in regard to the original genome annotation [1]. For each experiment, we conducted detailed metabolome (GC/MS) and proteome analyses (2D-DIGE, MALDI-TOF, shotgun approach via nanoLC/MS) of cells and culture supernatant.

Results and Discussion: In each case, we detected more than 90 different intracellular metabolites and more than 1,500 proteins, which overall covered the whole central metabolism. We detected distinct responses of *P. inhibens* DSM 17395 to specific nutrient conditions and determined capabilities and limitations of its metabolism. For example, when grown on rich medium, *P. inhibens* DSM 17395 imports almost all available substrates, especially amino acids, and utilizes them simultaneously. Accordingly, abundance of corresponding catabolic enzymes increased significantly. Further results of our studies will be presented at the conference.

References: [1] Thole et al. (2012), *Phaeobacter gallaeciensis* genomes from globally opposite locations reveal high similarity of adaptations to surface life. *ISME J.*, 6(12): 2229–2244.

**EVALUATION OF PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*) HEALTH DURING A SUPERINTENSIVE AQUACULTURE GROWOUT USING NMR-BASED METABOLOMICS**

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NMR-based metabolomics offers the aquaculture industry technological advances that may economically improve production and lessen environmental impact. Recent developments have helped high density shrimp aquaculture overcome problems of disease susceptibility and water quality issues from waste products with indoor, superintensive, aquaculture systems that permit year-round production by operating as essentially closed systems that promote beneficial microbial communities (biofloc). The resulting biofloc can assimilate and detoxify wastes, provide nutrition for the farmed organisms resulting in improved growth, and aid in reducing disease initiated from external sources. We employed NMR-based metabolomic techniques to assess shrimp health during a full growout cycle from the nursery phase through harvest as a diagnostic tool for detecting stressors in the culture environment. Aberrant shrimp metabolomes were detected from a spike in total ammonia nitrogen in the nursery, from a reduced feeding period that was a consequence of surface scum build-up in the raceway, and from the stocking transition from the nursery to the growout raceway. The biochemical changes in the shrimp that were induced by the stressors were essential for survival and included nitrogen detoxification and energy conservation mechanisms. Inosine and trehalose may be general biomarkers of stress in *Litopenaeus vannamei*. This study proves NMR-based metabolomics to be a useful tool for the aquaculture industry by imparting physiological insight into common environmental stresses that may limit growth or better explain reduced survival and production.

## INVESTIGATING THE MOLECULAR RESPONSE OF *DAPHNIA MAGNA* TO SILVER NANOPARTICLES USING A MULTI-OMICS APPROACH

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The nanotechnology industry has expanded rapidly over the past decade. The most common nanomaterials in use today are silver nanoparticles (AgNPs), which are exploited for their antimicrobial properties. The release of AgNPs into the aquatic environment from antibacterial clothing has been documented along with evidence of toxicity to aquatic organisms. As a consequence, investigation into the potential threat of AgNPs to aquatic ecosystems is now essential. In particular, the molecular mechanism of AgNP-induced toxicity is still unknown, though the current hypothesis is that toxicity arises through release of Ag<sup>+</sup> ions. Here we employed a multi-omics approach to characterise the potential molecular toxicity of AgNPs and to determine if they induce similar metabolic and transcriptional responses as dissolved Ag<sup>+</sup> ions.

*Daphnia magna* neonates were exposed to increasing concentrations of 10 nm PVP-stabilised AgNPs and Ag<sup>+</sup> ions (from AgNO<sub>3</sub>), for 24 hours. After exposure, all samples were processed for direct infusion FT-ICR mass spectrometry and Agilent micro-array analysis. Through principal components analysis (PCA) we have detected significant, concentration dependant metabolic and transcriptional changes in response to AgNP exposure. Similar changes are observed, but to a lesser extent, in response to high Ag<sup>+</sup> exposures at the transcriptional level only. Additionally, univariate analyses have revealed that 166 metabolic features and 148 genes changed significantly following exposure to high dose AgNP; subsequent metabolic pathway analysis suggests a perturbation to the biosynthesis of coenzyme A and energy production. Analysis of microarray data is ongoing. Our preliminary conclusions are that at the concentrations studied, AgNP and Ag<sup>+</sup> ions share similar modes of action, but the AgNPs show a considerably enhanced response. We believe this relates to the degree of silver uptake; AgNPs may be ingested and then dissolve within the *Daphnia* gut, resulting in a high, localised, internal concentration of Ag<sup>+</sup> which causes damage. Exposure to Ag<sup>+</sup> alone is not expected to result in such a high concentration within gut. Measurements of silver uptake are currently underway

## A METABOLOMICS APPROACH FOR ANALYSING MODE-OF-ACTION OF TRICLOSAN IN MICROALGAE

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Bioactive compounds like pharmaceuticals and personal care products are frequently detected in the environment rising the question of potential effects in non-target organisms. The bactericide triclosan (TCS), a commonly used personal care product with many fields of application, has been identified being one of the most important emerging toxic substances in aquatic systems (von der Ohe et al., 2012) and showed severe effects on microalgae in the range of environmental concentrations. While the mode-of-action of TCS in bacteria was identified (namely the inhibition of the Enoyl-ACP-reductase during fatty acid elongation, McMurry et al., 1998) the mode-of-action of this substance in microalgae is still unclear.

A metabolomics approach was used to identify affected metabolic pathways in a unicellular chlorophyte (*Scenedesmus vacuolatus*). A synchronised culture of *S. vacuolatus* was exposed with different concentrations of TCS (range from 0.002 µmol/l to 0.071 µmol/l) for 14 hours to derive concentration-dependent changes in metabolites and to be able to compare them to phenotypic observations (inhibition of cell growth and photosynthetic activity). After harvest of algal cultures and derivatisation of hydrophilic and lipophilic extracts, metabolites were analysed using GC-MS and subsequently multivariate statistics. Metabolites from the hydrophilic phase showed comparable sensitivity as phenotypic observations (EC50 algal growth: 0.02 µmol L<sup>-1</sup>). In contrast lipophilic metabolites of the green algae were affected significantly at the lowest tested concentration. Identification of lipophilic metabolites with the NIST and Golm library for GC-MS data revealed many fatty acids (e.g. hexadecanoic acid, oleic acid, octadecanoic acid) changing at low concentrations. These findings will be discussed in the perspective of the connectivity of responsive pathways to the known mode-of-action of the toxicant in bacteria.

**TRANSCRIPTOMIC AND METABOLOMIC ANALYSES OF MOLECULAR RESPONSES OF HUMAN CELL LINES TO A COMMONLY USED BROMINATED FLAME RETARDANT: HEXABROMOCYCLODODECANE**

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Flame retardants (FRs), which are chemical compounds used to retard the spreading of fire so as to prolong escape time, have been added for decades to various consumer materials to meet fire safety standards. As one of the most widely used brominated flame retardants, hexabromocyclododecane (HBCD) has been detected in various locations, including indoor environments. Human exposure via a variety of sources including indoor dust, air, food and direct contact with consumer products has been evidenced by the presence of HBCD in breast milk, adipose tissue and blood. Therefore, there is an increasing need to investigate the potentially toxic effects and the underlying molecular mechanisms of HBCD. In the present study, A549 (adenocarcinoma human alveolar basal epithelial) cells and HepG2/C3A (human hepatoma) cells were used as in vitro models to investigate molecular responses to HBCD, using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) based metabolomics and Agilent oligo-nucleotides microarray-based transcriptomics. Preliminary results show that few changes were found at the transcriptional or metabolic levels after A549 cells were exposed to HBCD at four concentrations (2 nM, 20 nM, 200 nM, and 2 µM) and control for 24 h. Similar results were found in HepG2/C3A cells exposed to HBCD at 4 µM for 24 h when compared to controls. A noticeable modulation of gene expression in more than 700 genes occurred, however, following treatment with 10 µM dimethylbenzanthracene as a positive control. Measurement of HBCD itself in HepG2/C3A cell pellets after exposure showed that approximately 17% amount of HBCD had entered the cells. Therefore, in view of the EC<sub>50</sub> values of the cytotoxicity of HBCD to A549 and HepG2/C3A cells for 24 h (27.4 µM and 62.9 µM, respectively) in the presence of serum, it might be concluded that there is limited adverse effects of HBCD exposure to these cell types at concentrations as high as ca. 8% of the EC<sub>50</sub> values in acute exposure.

**UNDERSTANDING LIPID METABOLISM IN THE MICRO-ALGA *CHLAMYDOMONAS* FOR BIODIESEL PRODUCTION**

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In order to improve the economic cost of microalgal biofuels we need to understand how changes in the availability of nutrients to the algae can influence the triggers that underpin triacylglyceride (TAG) metabolism and production. The rationale of this research was to study the effect of carbon supply (acetate or CO<sub>2</sub>) on TAG production and membrane autophagy in *Chlamydomonas reinhardtii* (wild type and the starch-less mutant strain *sta6*) grown under low N availability. Our hypothesis that acetate, being directly used as a precursor for *de novo* acetyl-CoA synthesis, would increase the initial rate of TAG production was correct. We also measured relatively less cellular autophagy in cells supplied with acetate, but this was only observed in wild type and not in *sta6* cells as expected. We also found that in response to decreased N, 1; increased TAG per unit dry weight does not relate to increased TAG production per culture, with the wild type cells producing more TAG per culture than the *sta6* cultures, 2; the growth rate differs between the wild type and *sta6* cells, with the latter dying in diurnal conditions and 3; chlorophyll and carotenoids concentrations are decreased in wild type cells but in *sta6* cells only the carotenoids concentrations are increased. These measurements showing the importance of carbon supply and strain selection are of economic importance, where biofuel industries would prefer to use wild type strains growing in gaseous CO<sub>2</sub> in autotrophic growth environments rather than one which requires an expensive exogenous supply of organic carbon.



**EFFECTS OF IONIC SILVER ON THE GREEN ALGAE *CHLAMYDOMONAS REINHARDTII***

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Xenobiotics are accumulating in the environment as a result of anthropogenic activity. Among the numerous data dealing with the effects of metals in algae, the molecular response of the unicellular alga *C. reinhardtii* to silver exposure still remains mostly unclear. Recent evidence suggests that reactive oxygen species induction by heavy metals negatively affects the physiological homeostasis of algae, ultimately leading to inhibition of photosynthesis and growth. Although the silver ion ( $\text{Ag}^+$ ) has long been known to display toxicity to a broad spectrum of bacteria, it is also among the most toxic metals for various other aquatic organisms. While transcriptomic and proteomic approaches are widely used to investigate stress response to metal toxicity, environmental metabolomic studies have to date only rarely been carried out with algae.

As the metabolome represents the ultimate response of an organism to genetic and environmental changes, the aim of this work is to assess silver toxicity at the metabolic level of the photosynthetic alga *C. reinhardtii* using high-resolution mass spectrometry. For this, cells are exposed to 500 nM  $\text{AgNO}_3$  over a period of 5 and 24h. A biphasic extraction method compatible with high-throughput mass spectrometry<sup>1</sup> was performed to evaluate the intracellular metabolites for the algal cultures. Preliminary results show that the metabolite profiles of biological replicates are reproducible and that the analytical procedure is adequate for examining the response of algae to silver. Principal component analysis of the GC-MS data revealed that the  $\text{AgNO}_3$  exposed algae compared to control cells can be clustered in clearly distinguishable groups based on *m/z*, retention time and chromatographic alignment. These results suggest that changes in the metabolome reflect the microalgae capability to activate the defence mechanisms against silver.

References:

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**METABOLOMIC ANALYSIS OF HONEY BY MASS SPECTROMETRY: CHEMICAL CHARACTERIZATION AND DETECTION OF POLLUTANTS**

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Honey usually refers to the nature and well-being. However, more and more bee colonies are being decimated around the world and pesticides can be considered as a part of this problem. The aim of this study is to analyze 91 pollutants (56 pesticides and 35 antibiotics) likely to be found in the honey. Traditionally, this type of analysis is performed on triple quadrupole mass spectrometer in MRM mode. This specific and sensitive method does not give more spectral information from the sample. Our approach focuses on a metabolomic analysis with acquisition of chemical fingerprint (with a lot of supplementary information) by LC/FTMS while maintaining specificity (exact mass, retention time and isotopic pattern).

76 honeys were extracted using an acetonitrile liquid-liquid extraction. Samples were analyzed by LC-ESI-Orbitrap alternately in positive and negative ionization mode. We used Quanbrowser to determine the presence of contaminants and R to perform an XCMS analysis (to create the variable peaktable) and multivariate statistics (PCA, predictive model, to discover the discriminate variables). To be sure of the repeatability of the results, experiments have been reproduced 3 times for each honey.

The results for the contaminants analysis showed that 74 of 76 honeys are polluted by at least one molecule (an average of five molecules by honey). Three pesticides were found in most honey samples (carbendazim, amitraz and chlorfenvinphos). For example, amitraz is a pesticide used in the treatment of beehives against varroa mites (endemic to Asia). Among the 23 honeys from organic farming analyzed, it appears that the number of xenobiotics is less abundant. We note the presence in this subgroup of both non-polluted honey from Mexico and Australia, low agricultural region which could explain their non-contamination.

The added value of this work lies in the acquisition of a global chemical fingerprint for the achievement of multivariate statistics on all signals generated during the analysis. Statistics performed on R showed a discrimination of honey according to their floral origin. A predictive model was also made and highlights a small number of signals characteristic of each floral origin. Fragmentation experiments are being elucidated the structures of these molecules.

**USE OF LC-Orbitrap-FTMS AND MZmine SOFTWARE FOR IDENTIFICATION OF NEW COMPOUNDS FROM BRAZILIAN RED PROPOLIS: APPLICATION IN A FINGERPRINTING ASSAY OF PROPOLIS**

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Red propolis from North East Brazil has been studied for its biological activity against parasites, microorganisms, viruses and for its cytotoxicity. The composition of the propolis varies with geographical region and seasonality. The objective of this study was to apply modern methods of screening including LC-Orbitrap-FTMS in combination with data extraction using MZmine software for fingerprinting assay of red propolis and identification of new compounds of this natural product. Propolis was collected in cities of Marechal Deodoro (Propolis A and Propolis B), Penedo (Propolis C), State of Alagoas-Brazil and in the city of Igarassu (Propolis D and E), State of Pernambuco-Brazil during the month of July/2012. Propolis (1.00g) was subjected to extraction with ethanol. Crude extracts (0.10g) were weighed and solubilised in 2mL of ethanol and diluted to a concentration of 1.00mg/mL. A volume of 10 $\mu$ L was injected into an LC-LTQOrbitrap-FTMS and acquisition was carried out in negative ion mode in the range of 100 to 1200 m/z. The data were analysed using multivariate statistics (CVA, PCA and HCA) in Simca-P. LC-FTMS analysis combined with MZmine data extraction and database searching demonstrated presence of 1,500 compounds in the crude extract of propolis including isoflavonoids, flavans and isoflavans, chalcones, phenolic acids, pterocarpanes, terpenes, propolones and guttiferones. Correlation of variation analysis showed clusters for phenolic acids/chalcones, isoflavonoids/isoflavans/pterocarpanes, propolones/guttiferones in specific retention time ranges. New compounds (isoflavonoid, flavonoid, terpenoid and flavonosides coupled to p-coumaryl groups) not yet identified in Brazilian red propolis were detected using CVA and histograms of mass peaks. PCA and HCA were useful for evaluating the similarities in the chemical profile between Propolis A, B and C. Propolis E presented differences in chemical profile while propolis D had an intermediate chemical profile. A high frequency of propolones, isoflavonosides and terpenes /terpenosides in propolis E could explain these differences. LC-FTMS and MZ-Mine software are extremely useful for identifying different classes of compounds in order evaluate the chemical profile of red propolis and assess intra-specific variability of these raw materials.

**MOUSE MODEL OF FETAL GROWTH RESTRICTION SHOWS AN ALTERED SERUM METABOLIC PROFILE WHICH IS REVERSIBLE FOLLOWING TREATMENT WITH SILDENAFIL CITRATE**

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**Introduction:** Fetal Growth Restriction (FGR) is defined as a fetus that fails to reach its genetic growth potential. Smaller size at birth is strongly associated with longer-term health consequences, such as an increased risk of developing heart disease and hypertension later in life. Animal studies and limited *in vivo* human studies suggest that the phosphodiesterase-5 inhibitor Sildenafil citrate (Viagra) might be a possible therapeutic option for FGR. In order to further investigate this potential, we looked for mechanism(s) of action responsible for the Sildenafil-induced rescue of fetal growth via aberration of the global metabolic profile in mice.

**Methods:** Catechol-O-methyltransferase knockout mice (COMT<sup>-/-</sup>) showed many signs of FGR in previous studies. COMT<sup>-/-</sup> and wild type C57BL6/J (WT) mice were randomly assigned to either Sildenafil (0.2 mg/ml) or placebo group from gestational day 12.5-18.5. On the 18.5 day of gestation dams were sacrificed and blood samples were collected by cardiac puncture (set on ice to clot; centrifuged at 4°C, 5min, 5000rpm). Serum was snap-frozen and stored at -80°C until further analysis. We applied a targeted quantitative metabolomics approach using a combination of DI-MS (Absolute/IDQ™ Kit) with a reverse-phase LC-MS/MS Kit (BIOCRATES Life Sciences AG, Austria) on ABI 4000 Q-Trap (Applied Biosystems/MDS Sciex, CA) MS. 180 metabolites were identified and quantified.

**Results:** Kruskal-Wallis test showed a significant difference in 18 metabolites between COMT<sup>-/-</sup> and WT mice (P<0.05). Sildenafil treatment reversed 13 of the altered metabolites from the acylcarnitines, glycerophospholipids, sphingolipids and bigenic amines class in the COMT<sup>-/-</sup> group.

**Conclusions:** Metabolic profiling can provide insights into pathogenesis of FGR as well as potential therapeutic mechanisms. Acylcarnitines facilitate entry of long-chain fatty acids (LC-FAs) via the Carnitine Shuttle. LC-FAs act as important fuels for many tissues by β-oxidation. A lower concentration of acylcarnitines and higher of lipids in the COMT<sup>-/-</sup> mice might suggest an imbalance in the delivery of these important nutrients to the fetus, possibly resulting in the growth restriction. Sildenafil treatment was able to significantly restore the metabolic balance, improving fetal development.

**P8-3**

**THE USE OF LC-MS PROFILING AND BIOASSAY IN THE ISOLATION AND IDENTIFICATION OF ANTI-TRYPANOSOMAL DITERPENES FROM LIBYAN PROPOLIS**

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Propolis is collected by bees from plants as a defensive substance in response environmental pressures which include a range of microorganisms and parasites. The ethanolic extracts of Libyan propolis were profiled by using LC-FTMS. The samples were then fractionated by using open and flash column chromatography and the fractions were tested for activity against *Trypanosoma brucei*. Metabolite profiling using MZmine and data base searching assisted, along with bioassay, in the isolation of active compounds.

**DROSOPHILA, METABOLOMICS AND INSECTICIDE ACTION**

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Invertebrate pests pose a threat to food security, human health and destroy property resulting in an urgent need for effective pesticides. To prevent resistance from rendering the concentrations of active ingredients in pesticides ineffective, synergists can be added. However, pest populations are increasingly insensitive to existing synergists, and medical evidence is accumulating that commonly used synergists are detrimental to human health. The development of new synergists focusing on existing targets is subject to diminishing yields making the discovery of new ones a priority. Metabolomics can identify perturbations in metabolism even if there is no observable phenotype, enabling observed bottlenecks and redundancies in stress related pathways to be exploited in synergist development. Metabolomic analysis of the response to permethrin treatment in *Drosophila* was used to identify candidate pathways and genes that altered on permethrin treatment. In principle, these could reflect either nonspecific toxicity, or be the consequences of direct attempts to metabolise and respond to the xenobiotic. One such pathway was that of tryptophan metabolism, not previously identified in the context of insecticide response. Components of the tryptophan metabolism pathway were knocked down with RNA interference in *Drosophila*, and survival upon topical application of the insecticide permethrin was scored for RNAi vs. parental fly lines. Data was processed using probit analysis followed by ANOVA and the Litchfield & Wilcoxon methods of statistical analysis. The well studied pathway of tryptophan metabolism was found to have a previously unidentified role in permethrin resistance and it is not merely the result of increased concentrations of the amino acid tryptophan.

**FUNDAMENTAL PROPERTIES OF HUMAN BLOOD AS SAMPLES FOR APPROPRIATE LIPID BIOMARKER EXPLORATION**

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[Purpose] Blood is the frequently utilized biofluid in biomarker discovery because it is data-rich and its collection is minimally invasive. Although lipid metabolites in blood are expected to be biomarker candidates, their fundamental properties have not been clarified. In this study, we aimed 1) to elucidate age and gender differences in lipid metabolites levels, 2) to determine whether plasma or serum is suitable matrix, and 3) to examine freeze-thaw effect on lipid metabolites stability. [Methods] We performed a lipidomic analysis using LC-MS(/MS) for fasting plasma and serum samples (15 subjects/group) from 4 groups consisting of young and elderly subjects of both genders. In addition, 10 freeze-thaw cycles were applied to the samples before a lipidomic analysis. [Results and Discussion] Levels of many sphingomyelin species were significantly higher in females than in males irrespective of matrices (plasma and serum) and age. As for age-related differences, many triacylglycerols showed significantly higher levels in the elderly female compared with the young female in both matrices. On the other hand, many lysophosphatidylcholines and diacylglycerols were present at higher levels in serum than in plasma, suggesting that coagulation process influences these lipid metabolites levels in serum. Most lipid metabolites are decreased by the repeated freeze-thaw cycles with some exceptions. [Conclusion] Our results suggest that gender- and age-differences were observed in some lipid species from human blood. Furthermore, plasma might be more suitable matrix for measuring lipid metabolites related to coagulation. The fundamental information on blood should be important for ensuring proper biomarker discovery and its qualification.

**CHEMICAL PROFILING OF AFRICAN PROPOLIS BY LC-UV-ELSD, HIGH RESOLUTION LC-MS AND GC-MS AND CORRELATION TO BIOLOGICAL ACTIVITY**

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The chemical composition of propolis has been well studied worldwide apart from for African propolis. Propolis represents an attractive drug discovery resource since it can be harvested without damaging plants and has already been selected for its biological activity by the bee. In order to investigate chemical properties of African propolis the ethanol extracts of 46 samples collected from 10 African countries were analysed by liquid chromatography (LC) coupled with different detection techniques including evaporative light scattering detector (ELSD), ultraviolet and visible (UV) detector and high resolution mass spectrometer (HRMS). The LC-ELSD and UV data were put into heatmap format to facilitate the visualisation of the difference between samples. The heatmaps illustrated the highly variable and complex compositions among the samples without geographic limitation which could be confirmed by a Principal Component Analysis (PCA) of the data generated by LC-HRMS. The major constituents in some selected samples were putatively identified by comparing their PDA spectra, accurate masses and fragments generated by MS/MS with the reference to the literature. The samples were also tested for biological activity against *Trypanosoma brucei*. By applying this dereplication strategy it is possible that the novel biologically active compounds can be discovered at the earliest stage before any purification work is started. Knowledge of the chromatographic properties of target compounds at the outset also assists in planning preparative scale isolation strategies.



P8-7

## **A METABOLOMICS AND GENETIC POLYMORPHISM TO ASSESS PHARMACOKINETIC VARIABILITY IN HEALTHY VOLUNTEERS**

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Drug X is under clinical development for the treatment of inflammatory diseases. In a clinical pharmacokinetic study, decreased plasma concentration profiles and rapid elimination were found in some subjects. Adverse events characteristics were different in higher plasma concentration group. The aim of this study was to investigate the possible cause of pharmacokinetic variability through metabolomic approach and to confirm effect of genetic polymorphism of metabolic enzymes.

A single dose pharmacokinetic study was performed in healthy volunteers. Plasma samples were collected at 0, 2, 6, 24 and 72 h post-dose to investigate the metabolites. The volunteers' plasma metabolites were analyzed by quadrupole–time-of-flight mass spectrometry (QTOF MS). The peak areas of metabolite were compared between rapidly eliminated plasma concentration group and slowly eliminated plasma concentration group to find significant metabolites. And, TaqMan Copy Number Assays RT-PCR was used to determine the Glutathione S-transferase Mu 1 copy number variations (GSTM1 CNVs). To assess the effect of polymorphism on pharmacokinetics, GSTM1 genotypes were classified zero copy and one copy.

Of eight subjects who received study drug, 7 metabolite-cysteine glycine conjugation, cystein conjugation, ketone to alcohol, deethylation, hydroxylation+glucuronide, methylation and oxidative defluorination were found. Of the seven metabolites, cystein glycine conjugation and cytein conjugation were detected in slowly eliminated and high plasma concentration group, however not confirmed in rapidly eliminated group. Subjects in slowly eliminated group had zero copy of GSTM1 and subjects in other group had one copy of GSTM1.

Our results showed the lower systemic exposure in some subjects with one copy of GSTM1, which suggest GSTM1 play an important role in the drug metabolism. Metabolomics approach may have potential to be successfully applied to find a candidate metabolizing enzyme which may have clinical significance.

## THE USE OF A METABOLOMICS APPROACH TO INVESTIGATE TROGLITAZONE TOXICITY IN A CELL CULTURE SYSTEM

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Troglitazone is an anti-diabetic drug introduced in the late 1990s, representing the first of the thiazolidinedione (TZD) class of drugs to be marketed. The TZDs reduce blood glucose levels by enhancing insulin sensitivity through activation of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ). However, troglitazone was removed from the market due to increased reports of hepatotoxicity.

The purpose of this study was to demonstrate that a metabolomics approach could be used to investigate the mechanism(s) of troglitazone toxicity. An *in vitro* cell culture system using HuH-7 cells (a hepatic-like cell line) and liquid chromatography coupled to mass spectrometry (LC-MS) were employed to identify metabolites correlated with troglitazone hepatotoxicity.

A MTT assay showed that troglitazone negatively affected the viability of HuH-7 cells, with an IC<sub>50</sub> value of  $25.9 \pm 1.8 \mu\text{M}$ . A LC-MS metabolomics approach showed that troglitazone, at a concentration of less than  $10 \mu\text{M}$ , decreased the levels of intracellular glutamate, malate, ATP and reduced glutathione (GSH). The results suggest that troglitazone probably affects the tricarboxylic acid (TCA) cycle and the phosphorylation of ADP and AMP to ATP, thus having a negative effect on the energy status of the cells. Furthermore, levels of GSH were decreased, which may have implications in terms of oxidative stress and cell survival.

Links between different metabolic pathways and implications for cell toxicity will be discussed and the feasibility of using a metabolomics approach to investigate mechanisms of toxicity will be addressed.

**METABOLOMICS STUDIES OF ENDOPHYTIC METABOLITES FROM MALAYSIAN MANGROVE PLANT IN THE SEARCH FOR NEW POTENTIAL ANTIBIOTICS**

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Currently, endophytic fungi have been explored not just for their ecological functions but for their secondary metabolites as a new source of these pharmacologically active natural products. Accordingly, many structurally unique and biologically active compounds have been obtained from the cultures of endophytic fungi. The fungal strains were isolated from leaves; *Aspergillus aculeatus* and stem; *Lasiodiplodia theobromae* of the mangrove plant *Avicennia lanata* collected from the East coast of Peninsular Malaysia in Terengganu Province, Malaysia. The fungi were taxonomically identified according to their morphological characteristics as well as by DNA amplification and sequencing of the ITS region. Prior to this study, metabolomics has been applied to identify and optimize the production of bioactive secondary metabolites in both fungi at different growth stages and culture media. Metabolomic studies were afforded by both high resolution mass spectrometry and NMR spectroscopy. Metabolomic profiling data was processed by utilizing the quantitative expression analysis software Mzmine 2.10 coupled with the Antimarin database for dereplication studies. SIMCA P+ 13.0 was used to prove that the optimization models were statistically sound. Respective fungi were then later scaled up either in rice-solid and liquid culture media. Crude extracts were fractionated using several high-throughput chromatographic techniques and subjected to bioactivity-guided isolation work for anti-trypanosomal active metabolomes. Structure elucidation of isolated secondary metabolites was achieved using 2D-NMR and HRESI-MS.

## IDENTIFICATION OF BIOMARKERS FOR NON-STEROID ANTI-INFLAMMATORY DRUGS INDUCED GASTRIC ULCER BY CE-MS BASED METABOLOMIC ANALYSIS

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Non-steroid anti-inflammatory drugs (NSAIDs) are among the most frequently prescribed drugs currently available. The most frequently reported serious side effects associated with NSAIDs are gastric mucosal ulceration and gastric hemorrhage. And given that NSAIDs may mask the pain associated with gastric ulceration, patients may delay seeking medical attention, which can subsequently delay ulcer healing. Presently, these side-effects are only detectable by endoscopy, and there is an unmet medical need to identify a non-invasive means of assessing gastric injury associated with the use of NSAIDs.

In this study, we applied metabolomic approach based on CE-time of flight (TOF)-MS profiles of endogenous metabolites to identify new biomarkers of NSAID-induced gastric injury in stomach tissue extracts from rats. We then determined the correlation of concentrations of these new biomarkers in tissue extracts with that of serum concentrations.

Administration of NSAIDs at doses that induced gastric injury in rats was associated with decreases in levels of citrate, cis-aconitate, succinate, O-acetyl carnitine, 3-hydroxy butanoic acid, proline, and hydroxyproline in stomach tissue extracts compared with tissue extracts collected from vehicle-treated control animals. Plotting these metabolite changes on metabolic pathway maps demonstrated that two events were associated with NSAID-induced gastric injury: (1) depression of mitochondrial function, and (2) hyperactivity of collagenase in the stomach. In addition, decreases in levels of cis-aconitate, O-acetyl carnitine, 3-hydroxybutanoic acid, proline and hydroxyproline in stomach tissue extracts were significantly correlated with similar changes in the serum levels of these compounds.

These results strongly suggest that NSAID-induced changes in levels of these endogenous metabolites in the stomach are monitorable in the serum, and accordingly making them favorable candidates for biomarkers of gastric injury in serum.<sup>1</sup>

1. Takeuchi K et al. (2013) J. Proteome Res. 12: 1399

## **METABOLOMIC BIOMARKERS FOR EARLY DIAGNOSIS OF ENDOMETRIOSIS USING <sup>1</sup>H-NMR**

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Metabonomics is concerned with the measurement of low molecular weight metabolites, which are important indicators of various diseased states. The collective measurement of the entire metabolome relies on the use of methods with high sensitivity, high resolution and wide dynamic range. NMR is a powerful method for the analysis of biofluids. Because of the information density of NMR spectra, data are analyzed by various chemometric multivariate statistical analyses. The peak position and line width changes are often handled by “spectral binning” or “spectral bucketing”.

Endometriosis is the proliferation of endometrial glands and stroma outside the uterus leading to dysmenorrhoea, dyspareunia, dysuria, abdominal pain and infertility. The pathogenesis of endometriosis remains unclear. The diagnostic value of most of the biomarkers identified till date by various methods remains unclear.

Our present study focuses on the identification of predictive biomarkers in serum for the early diagnosis of endometriosis in a minimally invasive manner using <sup>1</sup>H-NMR based metabonomics. PLS-DA modeling of bins obtained from spectra of serum samples discriminated endometriosis patients from controls with sensitivity and specificity levels of about 80% and 90% respectively. Compared with those from controls, serum samples from endometriosis patients showed increased levels of lactate, 3-hydroxybutyrate, alanine, leucine, valine, threonine, lysine, glycerophosphatidylcholine, succinic acid and 2-hydroxybutyrate as well as decreased levels of lipids, glucose, isoleucine and arginine. Our work offers valuable information for non-invasive diagnosis of the disease and may be of potential benefit to understand the pathogenesis of endometriosis.

**P9-2**

**METABOLITE PROFILING OF LIPID AND NUCLEOTIDE METABOLISMS IN A MARINE BACTERIUM DEGRADING AND METABOLIZING SEAWEED COMPONENTS**

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Seaweeds are recently emerging for producing biofuels and industrial chemicals. A marine bacterium degrading and metabolizing seaweeds, was isolated from a coast side. Biochemical pathways of metabolizing seaweed components are hardly known. We have investigated the metabolite profiling of the isolated bacterium on the components of seaweeds concentrating on the metabolisms of lipids and nucleotides. These results will lead us to the better understanding of marine bacteria metabolizing seaweeds in the ocean environment.

**COMBINING NON-TARGETED AND METABOLIC PATHWAY DRIVEN TARGETED METABOLOMICS BASED ON THE SAME LC-QTOF DATA SET – EXEMPLIFIED ON COFFEE METABOLOMICS**

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As stated in a recent review article [1], modern Metabolomics has quickly grown in the past 10 years and was in particular quickly adopted by biologists.

Biologists often think in terms of biological pathways and try to interpret results derived from Transcriptomics, Proteomics or Metabolomics experiments along these lines. In contrast scientists in Metabolomics research often do not want to risk missing a significant change in metabolite abundance when concentrating the analysis and data evaluation on known compounds only. Modern full scan high resolution QTOF instruments provide the advantage that both researcher's requests can be answered using the same dataset.

Here we present a proof of concept study based on a coffee metabolomics experiment. The acquired LC-MS data was initially evaluated using an untargeted workflow. This untargeted approach pointed to some metabolites as being characteristic for particular coffee cultivars. From those, nicotinic acid was identified and a targeted screening list was automatically generated using compounds present in characteristic metabolic pathways (in this case nicotinic acid metabolism). The list was subsequently extended by metabolites described to be present in coffee before.

A novel software tool readily allowed creating this target compound list from the metabolic pathway. It also enabled to quickly screen for the presence of the compounds in the same high resolution full scan data files used for the untargeted workflow. Compounds with significant changes between cultivars were tentatively identified taking into account accurate mass and isotopic pattern information. This statistical evaluation guided the purchase of reference standards for final confirmation.

In summary a novel workflow for combined non targeted and pathway driven targeted metabolomics based on the same data high resolution QTOF data files will be presented.

[1] Sumner and Hall 2013, *Metabolomics* 9:258–264

**HIGHLY ACCURATE CHEMICAL FORMULA PREDICTION TOOL UTILIZING HIGH-RESOLUTION MASS SPECTRA, MS/MS FRAGMENTATION, HEURISTIC RULES, AND ISOTOPE PATTERN MATCHING**

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Mass spectrometry is commonly applied to qualitatively and quantitatively profile small molecules, such as metabolites or lipids. Modern mass spectrometers provide accurate measurements of mass-to-charge ratios of ions, with errors as low as 1 ppm. Even such high mass accuracy, however, is not sufficient to determine the unique chemical formula of each ion, and additional algorithms are necessary. Mass spectrometry vendors provide their own software modules for chemical formula prediction, the capabilities of which vary by vendor. We developed a universal software tool for predicting chemical formulas from high-resolution mass spectrometry data. The tool is based on the use of a combination of heuristic techniques, including MS/MS fragmentation analysis and isotope pattern matching. The performance of the tool was evaluated using a real metabolomic dataset obtained with the Orbitrap MS detector. The true formula was correctly determined as the highest-ranking candidate for 79% of the tested compounds. For compounds smaller than 250 Da, the true formula was always predicted correctly and, in 67% of the cases, as the only candidate conforming to the heuristic rules. On average, the heuristic algorithms reduced the search space for potential formula candidates 40-fold. The novel isotope pattern-scoring algorithm outperformed a previously published method in 64% of the tested Orbitrap spectra. Great emphasis was put on user-friendly operation of the tool, which is now freely available as part of the open-source MZmine 2 framework and its source code can be accessed within the MZmine 2 source code repository.

Reference: Pluskal T., Uehara T., Yanagida M., Highly Accurate Chemical Formula Prediction Tool Utilizing High-Resolution Mass Spectra, MS/MS Fragmentation, Heuristic Rules, and Isotope Pattern Matching. *Anal Chem* (2012), **84**(10):4396-403



## RECONSTRUCTION OF INSULIN SIGNAL FLOW IN GLOBAL PHOSPHORYLATION AND METABOLIC NETWORKS

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Hormones regulate homeostasis by transmitting signals through global molecular networks that include protein phosphorylation and metabolites. However, where and when the signal of a hormone flows through a global network and regulates homeostasis has yet to be explored. Here we show signal flows of insulin, an important hormone for metabolic homeostasis. We reconstructed the static signal flow of insulin based on time-series phosphoproteome and metabolome data together with multiple databases and found where an insulin signal flowed through a global network; the insulin signal flowed through signaling pathways that involved 26 protein kinases, 76 phosphorylated metabolic enzymes, and 80 allosteric effectors, resulting in quantitative changes in 97 metabolites. We analyzed the dynamic signal flow using kinetic modeling together with model selection and model reduction, and found when specific phosphorylation and allosteric regulation selectively control temporal patterns of metabolites. Thus, we demonstrate a global landscape for the signal flow of insulin, which reveals the large-scale mechanism of metabolic homeostasis.

**GLOBAL METABOLOMIC ANALYSIS OF MIXTURES USING <sup>13</sup>C-DETECTED NMR TECHNIQUES**

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Typically in a global metabolomics study, metabolic mixtures are collected and characterized by <sup>1</sup>H NMR and/or LC-MS. Usually with NMR-based metabolomics, 1D and 2D <sup>1</sup>H-detected methods are used to analyze the global response of a biological system to perturbation/stress. Though <sup>1</sup>H-detected methods are extremely useful, 1D <sup>1</sup>H NMR of mixtures can suffer from heavy peak overlap due to low spectral dispersion (~12 ppm) as well as problems with signals that arise from solvents such as water. The limited use of <sup>13</sup>C-detected methods stem from the difficulty in acquiring adequate spectra due to the low sensitivity of carbon. Our lab, in collaboration with FSU and Agilent Technologies has designed a 1.5 mm high temperature superconducting probe that significantly increases the signal to noise ratio of the <sup>13</sup>C nucleus. The advantages of <sup>13</sup>C NMR techniques over traditional <sup>1</sup>H NMR include the carbon spectral width, which is much larger (>200 ppm) and therefore affords greater spectral dispersion. 2D techniques such as <sup>13</sup>C-<sup>13</sup>C 2D-INADEQUATE, which to our knowledge, has never been performed in a metabolomics study due to low sensitivity of carbon, allows for direct assessment of carbon-carbon connectivity. Here we present an approach to <sup>13</sup>C metabolomics using natural abundance and enriched <sup>13</sup>C-detected NMR, which has traditionally been used almost exclusively with <sup>1</sup>H-detected NMR and MS. In conjunction with well established statistical techniques such as STOCSY and SHY, we demonstrate the use of 1D <sup>13</sup>C NMR to conduct a global metabolomic analysis of natural abundance <sup>13</sup>C synthetic mixtures and apply this method to the serum of mice afflicted with muscular dystrophy. In addition, we are also developing a method for analyzing 1D <sup>13</sup>C and 2D-INADEQUATE data using the <sup>13</sup>C-enriched exo- and endometabolomes of *C. elegans* under stressful conditions.

## **INTEGRATED PROTEOMIC AND METABOLIC PROFILING OF GILTHEAD SEABREAM LIVER TO TRACK INTERACTIONS BETWEEN DIETARY FACTORS AND SEASONAL TEMPERATURE VARIATIONS**

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Gilthead seabream (*Sparus aurata*) is a fish species sometimes affected by a metabolic syndrome, known as the "winter disease", which has a significant economic impact. It is caused, among other factors, by the thermal variations that occur during colder months and there are signs that improved nutritional status can mitigate the effects of this thermal stress. For this reason, a trial was undertaken where the effect of a fortified diet on the impact of thermal stress was assessed through metabolomic and proteomic analysis of gilthead seabream hepatic tissue.

Four groups of 25 adult gilthead seabream were reared for 8 months, being fed either with a control diet (CTRL, commercial formulation) or with a diet dubbed "Winter Feed" (WF, fortified formulation containing a higher proportion of marine-derived ingredients, as well as phagostimulants, marine phospholipids, antioxidant vitamins, taurine and soy lecithin). Fish were sampled at two time-points (at the end of winter and at the beginning of summer), with liver tissue being taken for FTIR spectroscopy and DIGE analysis.

Results have shown that seasonal temperature variations constitute a metabolic challenge for gilthead seabream, with hepatic carbohydrate stores being consumed over the course of the inter-sampling period. Regarding the WF diet, results point towards a positive effect in terms of performance and improved nutritional status. This diet seems to have a mitigating effect regarding the seasonal challenge, not only in terms of carbohydrate depletion, but also in terms of the observed accumulation of lipids in the later sampling. This suggests this diet is indeed a good candidate for a "golden standard" diet against which to compare alternate (possibly more cost-effective) formulations.

**IONS FUSION OF HIGH-RESOLUTION LC-MS-BASED METABOLOMICS DATA TO DISCOVER MORE RELIABLE BIOMARKERS**

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Discovery of biomarker to diagnose disease and other wide applications is one of the main research points in metabolomics [1]. Most conventional studies employed all ion features obtained from data quality improvement step as input for chemometric optimization and modelling [2]. This may generate false positive findings since it ignores to consider the collinearity among features and equal chance of each metabolite to be selected as potential biomarker. In addition, too many metabolite features burdens the computation and effectiveness of chemometrics.

A systematic approach for fusion of fragmentation ions with derivation of common molecule is developed to generate 'one-peak-one-feature' metabolomics data. This guarantees each molecule equally selected as potential biomarker and may largely enhance the chance to obtain reliable findings with no employment of redundant ion information. The principles for ions fusion are on the basis of low mass variation in contrast to theoretical calculation measured by high-resolution mass spectrometer such as LTQ orbitrap, and high correlation of ion pairs derived from the same molecule. The mass characteristics of isotope distribution, neutral loss and adduct ions are simultaneously applied to inspect each ion extracted in the range of a pre-defined retention time window. The correlation coefficient is computed with the corresponding intensities of each two ions amongst all experimental samples.

A metabolomics data for the investigation of liver cancer and cirrhosis was utilized as an example to deliver the strategy. It includes 68, 29 and 30 samples with cancer and cirrhosis, and healthy controls. It is found that the proposed approach has good performance to fuse related metabolic features and further biomarker discovery. The average error rate for test (ERT) and validation (ERV) respectively attain to 18.90% and 34.67% with genetic algorithm - partial least square (GA-PLS) for variable selection [3] and random forest (RF) method [4] for classification using the first 15 most important ion features. Moreover, it can be extensively applied in other 'omics' studies with high-resolution mass spectroscopy as a tool to detect small or large molecules.

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**MetaboHUB : A FRENCH INFRASTRUCTURE DEDICATED TO METABOLOMICS AND FLUXOMICS**

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MetaboHUB project aims at creating a national infrastructure that will place France among the European leaders for advanced research services in metabolomics and fluxomics. MetaboHUB will provide tools and services to academic research and industrial partners in the fields of nutrition, health, agriculture, environment and biotechnology. MetaboHUB will be established by implementing and up-grading 4 existing facilities (Bordeaux, Paris-Saclay, Toulouse and Clermont-Ferrand) into a unique infrastructure sharing common regulations and complementary metabolomics and fluxomics tools. Besides providing state-of-the-art services and support to national and international projects, a major ambition of MetaboHUB is to develop innovative tools and methods to address critical biological questions. MetaboHUB will provide custom solutions for (i) high-throughput, quantitative technologies for biochemical phenotyping of large sets of samples and for systems biology through standardization and combination of state-of-art technologies, (ii) identification of metabolites in human biofluids, plants, microorganisms and animal cell extracts, through the implementation and maintenance of centralized and open spectral repositories for metabolome annotations, (iii) large-scale flux profiling and sub-cellular fluxomics through integration of analytical data from multiple analytical devices, (iv) access to high-impact services to the national scientific community and industrial actors and, (v) attracting and training a new generation of scientists and users through the promotion of metabolomics in education and the organization of training courses. The tools developed will be made available to the metabolomics community. These include tools for experimental design, dedicated bioinformatics and biostatistics software modules for data processing and integration with other 'omics' technology, biological networks reconstruction and disease modelling within a systems biology framework.

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**ERVA METHOD FOR <sup>1</sup>H-NMR METABOLOMICS DATA: A NOVEL BINNING STRATEGY HIGHLIGHTING CHEMICAL INFORMATION**

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Spectra processing is crucial in metabolomics approaches, especially for proton NMR metabolomic profiling, since each processing step may impact the following steps. Among the different processing steps, data reduction (binning or bucketing) strongly impacts subsequent statistical data analysis and potential biomarker discovery. Based on a recently published work, we propose an improved method of data reduction, called ERVA which stands for Extraction of Relevant Variables for Analysis. This new method, by providing buckets centred on resonance peaks and rid of any non-significant signal, helps to recover the chemical fingerprints of metabolites. Moreover, we take advantage of the concentration variability of each compound from a series of samples of a complex mixture, to highlight chemical information. This is performed by linking the buckets into clusters based on significant correlations, thus bringing a helpful support for compound identification. As a proof of concept, this new method has been applied to a tomato <sup>1</sup>H-NMR dataset to test its ability to recover fruit extract composition.

**ANALYSIS OF LONGITUDINAL METABOLOMIC DATA FROM ENDOCRINE DISRUPTION STUDIES: THE A-SCA METHOD**

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Metabolomics are increasingly being used in the field of toxicology. Experimental designs involving the study of dynamic changes in the metabolome raise new methodological challenges in the field of data analysis, regarding long term and perinatal *in vivo* studies. Multivariate analysis of variance (MANOVA), often used to analyze experimental data, is not always appropriate for metabolomics, especially when sample size is much smaller than the total number of variables, which prevents the testing of underlying hypotheses (normality, homoscedasticity). Multivariate methods, such as Principal Component Analysis (PCA) or Partial Least Squares-Discriminant Analysis (PLS-DA), often used to analyze metabolomic data, do not take into account data's temporal structure, resulting in a loss of information when used alone. In this study, we applied a method combining ANOVA and PCA: A-SCA (Anova-Simultaneous Component Analysis) taking into account the experimental design, as well as the relationship between variables, to allow data modelisation. Data were first separated into blocks corresponding to the different sources of variation (experimental design factors). Then PCA was independently applied on each block, and permutations test was used to evaluate the significance of model parameters. This method was applied to the study of the effects of low doses of bisphenol A (BPA) on global metabolism in SD rats exposed during the perinatal period (NIEHS project #5RC2ES018822). Pregnant rats were exposed to DMSO (vehicle-control), 0.25, 2.5, 25 or 250 ng BPA/kg BW/day. Serum samples of the F1 generation were collected on days 21, 50, 90, 140 and 200 of the experiment, and submitted to <sup>1</sup>H NMR spectroscopy. Using the A-SCA method, time effects were demonstrated based on the 5 time-points. The metabolism of glucose, lactate and fatty acids were modified over time. An in-house MatLab function was written to explore the time window for which [Time × BPA dose] interactions were significant. This longitudinal analysis demonstrated a dynamic evolution of serum metabolites between PND21 et PND90, which was significantly different depending on the BPA dose.

**DEVELOPMENT OF THE GOLM METABOLOME DATABASE TOWARDS 'BIG DATA'**

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We report an update of the Golm Metabolome Database (GMD, <http://gmd.mpimp-golm.mpg.de/>), a database harbouring primarily GC-MS datasets in plants. In our developments, we focused on the agile integration of multiple metabolomic gas chromatography (GC) –mass spectrometry (MS) data sets, paving the way towards 'Big Data' applications including searching and data mining across multiple GC-MS based metabolite profiling data sets.

For the data integration of heterogeneous and multi-structured metabolite profiling experiments, we interfaced the GMD to the eXtensible Experimental Mark-up Language (XEML, <http://xeml.codeplex.com/>) describing experimental designs and results in machine readable Meta Data and referencing ontology terms.

Combining the scalable XEML based experimental Meta Data with large scale metabolite profiling data; the GMD web site now provides several statistical visualizations for the explorative metabolome analysis. Further developments will target the semantic-based data pre-processing for cross-OMICS data mining approaches and straightforward integration of GMD datasets with other data resources such as MetaboLights (<http://www.ebi.ac.uk/metabolights/>).



**UNTARGETED METABOLOMICS FOR TOXICITY PROFILING OF HUMAN KIDNEY CELLS USING HPLC-ESI-MS AND DIRECT-INFUSION Nano-ESI-MS**

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Clinical Testing of drugs is very cost intensive and time consuming. Therefore there is a strong need for efficient screening methods at an early stage, which provide answers in fast, reliable and economic manner. For that reason a comprehensive metabolomics approach was chosen, which reflects the influence of external stimuli on cultured cells very well. The aim of this study was to screen nephrotoxic drugs such as chloroacetaldehyde and ifosfamide to observe the impact of drug treatment on metabolite abundance and to identify the affected metabolic pathways.

In these studies, three different types of treatment were employed (untreated, low dose and high dose) for three biological replicates at three different time points (Day 1, 3, 14). Metabolites were extracted from the whole cell lysates with cold methanol containing an internal standard (deutero-alanine). For toxicity profiling two different approaches were chosen, namely reversed-phase HPLC-ESI-MS and direct-infusion nano-ESI-MS. Both measurements were performed on an Exactive Orbitrap mass spectrometer both in positive and negative ionisation mode, the chromatographic separation was done with an Accela UHPLC system on a polar endcapped C18 phase (Hypersil Gold aQ C18, 2.1x100 mm, 1.9 µm). For data evaluation, tailor-made bioinformatics workflows with open-source software including OpenMS and mzMine were applied. Data evaluation involved different univariate and multivariate statistical approaches to determine differentially regulated metabolites. These metabolites were subsequently identified with accurate mass search in the human metabolite database (HMDB) and by MS<sup>2</sup> fragmentation.

Metabolic changes were observed upon treatment even at low-dose exposure; however more differential metabolites were found in samples treated with the high dose of the respective nephrotoxic compound. Principal component analysis (PCA) showed clear difference between the three doses applied. Pairwise comparison revealed that most metabolites that were up- or down-regulated at low doses also changed in abundance in the same direction at high doses. Furthermore, the results of both analytical methods were well consistent and provided statistically significant data.

## GRAPE ISOPRENOIDS: AUTOMATED DATA ANALYSIS IN MORE THAN 500 SAMPLES

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Plant isoprenoids, which include metabolites like chlorophylls, carotenoids, tocopherols, among many others, have important functions *in planta*, with specific compounds being involved in photosynthesis, photoprotection, as hormone precursors, or in attraction of pollinators. In terms of human interest, they contribute not only to fruit appeal, but have also health beneficial properties (vitamins, antioxidants), making these compounds the target of many breeding efforts.

Carotenoids in particular are of interest in the case of grape since they are the precursors of norisoprenoids, aromatic compounds with importance in wine making.

The current study is part of a system-wide project focusing on the untargeted metabolomic analysis of grape.

In this study the isoprenoid profiling of more than 500 grape samples was done using HPLC-DAD. Hundreds of 3-dimensional data sets (Retention time x Wavelength x Response) were generated, showing the presence of multiple compounds with different UV-Vis spectra. Traditionally these data would be analysed by integrating peak areas at the wavelength of maximum absorption for each individual compound and chromatogram, an error-prone and cumbersome process. Further complicating factors include retention time shifts and coeluting compounds.

By using an automated pipeline based on Multivariate Curve Resolution (MCR) we were able to process all files simultaneously and to provide estimates of pure spectra, elution profiles, and accurate estimations of peak area for each of the analysed metabolites. Furthermore retention time correction and clustering allowed for the automated population of a data matrix containing peak areas for each detected compound (known and unknown) in each grape sample.

Preliminary results of the isoprenoid profiling in grapes will be presented.

Wehrens, R., Carvalho, E., Masuero, D., Juan, A. & Martens, S. High-throughput carotenoid profiling using multivariate curve resolution. *Analytical and Bioanalytical Chemistry* (in press) doi:10.1007/s00216-012-6555-9

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**MetAnnoDB, A DATABASE OF HUMAN METABOLITE ANNOTATIONS BASED ON ACCURATE MASS AND MS/MS SPECTRA**

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Metabolomics datasets often contain a large number of unidentified metabolites, hindering the fully exploitation of acquired data in epidemiological studies. High resolution mass spectrometers with MS/MS capability may identify a large number of metabolites in the analyzed samples. We have developed MetAnnoDB, a database of annotated ions in human plasma and urine samples that were acquired using a UPLC-high resolution quadrupole time of flight (QToF) mass spectrometer. The database contains 2,000 MS/MS spectra with accurate masses of precursor and product ions. More than five hundred of these spectra have been annotated by matching to the MS/MS spectra of standard compounds as stored in HMDB/Massbank/Metlin databases and by matching to predicted MS/MS spectra. Annotated metabolites include amino acids, nucleotides, hormones, lipids, aromatic compounds and various conjugated metabolites. The annotated ions can be used in a streamlined workflow for generating metabolomic datasets using a targeted ion search approach in the MZmine software. MetAnnoDB is cloud-compatible and can be easily transferred to a cloud-infrastructure using the replication function of underlying Apache CouchDB, a NoSQL database technology. Required R and JavaScript codes for the automatic calculation of new MetAnnoDB instances from high resolution mass spectrometers and the MS/MS spectra can be downloaded from the database website at [www.metannodb.org](http://www.metannodb.org). The database would assist in the comprehensive annotation of compounds in metabolomic datasets acquired using high resolution mass spectrometry.

**DIFFERENTIATION OF ANALYTICAL AND BIOLOGICAL VARIABILITY IN METABOLOMICS USING STANDARD REFERENCE MATERIALS AND MULTIVARIATE STATISTICS**

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NIST has long developed and provided reference materials to assist others in making reliable measurements. The NIST Standard Reference Materials (SRMs) include several complex biological materials, especially human plasma and urine. These SRMs are particularly useful to distinguish between biological variability and analytical variability in metabolomics experiments.

Current projects have accumulated a considerable amount of data from the standards for human plasma and urine on different liquid chromatography/mass spectrometry (LC-MS) platforms and in-house software has been developed for chromatography alignment, spectral similarity, peak deconvolution, and molecular feature extraction [1]. We have found that several factors influence the reproducibility of the global profiling of biological fluids. For example, several low abundance compounds co-elute and produce signals of similar intensities, so in untargeted experiments these ions are selected for fragmentation almost randomly. Also, sensitivity and scan speed can vary substantially from one instrument to another and make the comparison across platforms very difficult. To overcome (or minimize) this problem, we are generating material-oriented libraries that include consensus spectra of all components detectable by MS (identified and unidentified.)

A particular example dealing with the reproducibility of metabolite profiling will be discussed in detail. SRMs of urine from smokers and non-smokers were used to compare the analytical and biological variability of the extracted profiles of the nicotine pathway metabolites and also the global profiling of the material. We discuss a standard protocol to evaluate the analytical variability associated with LC-MS measurements using SRMs, principal component analysis, partial least squares, and other multivariate statistics [2].

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**MetaboLights: A CROSS-SPECIES REPOSITORY FOR METABOLOMICS EXPERIMENTS AND DERIVED INFORMATION**

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MetaboLights is a database for metabolomics experiments and the associated metadata. It is the first comprehensive, cross-species, cross-platform/technique database which combines curated reference data of pure metabolites, curated information about their occurrence and concentration in species, organs, tissues and cell types under various conditions with data characterizing the experiment which lead to these findings and allows ready cross-referencing between experiments. Protocols documenting how metabolomics experiments were conducted are also available.

Like all other EBI resources, the MetaboLights database is completely open to the public, including open access to the data. Data are made available in publicly accepted open standards compliance with MIBBI (The Minimum Information for Biological and Biomedical Investigations). The software is open source and adheres to the promotion of open source file formats, such as mzML and nmrML. EBI makes an institutional commitment for a long-term support of the resource and the archived data.

MetaboLights is not intended to replace specialist resources for Metabolomics. Rather, it will build on prior art and collaborate. We are dedicated to close collaboration with all major parties involved in the creation of this prior art, such as the Metabolomics Society, Metabomeeting and the Metabolomics Standards Initiative (MSI). MetaboLights aim to agree on formal data sharing agreements with major resources such as the Human Metabolome Database, the Golm Metabolome Database and the Rikken Metabolomics Platform. Currently we house selection of experimental raw data and their associated metadata for different platforms such as NMR, GC-MS and LC-MS.

MetaboLights use the ISA Software Suite as it's main submission channel.

**A SYSTEMATIC APPROACH TO OBTAIN VALIDATED PLS MODELS FOR PREDICTING LIPOPROTEIN SUBCLASSES FROM SERUM NMR SPECTRA**

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Lipoprotein particle distributions (LPD) are used in the assessment of risk of cardiovascular disease, insulin intolerance and diabetes and for directing pharmaceutical, lifestyle, and dietary interventions. Here we established PLS models for estimation of cholesterol and triglyceride concentrations in lipoprotein subclasses from diffusion edited <sup>1</sup>H NMR spectra of fasting serum from apparently healthy humans. The PLS models were calibrated on HPLC derived lipoprotein subclasses and validated with an external dataset. In addition to total VLDL, LDL, and HDL lipids, the lipoprotein profiles resulted in significant models for 13 subclasses. These included 5 VLDLs (particle size 64-31.3 nm), 4 LDLs (particle size 28.6-20.7 nm), and 4 HDLs (particle size 13.5-9.8 nm). Triglyceride concentrations were well predicted in the VLDL ( $0.82 < Q^2 < 0.92$ ) and HDL ( $0.69 < Q^2 < 0.79$ ) subclasses. Better models were found for the larger LDL08 and LDL09 subclasses than for the smaller LDL10 and LDL11 particles. The best models for cholesterol concentrations were obtained for the HDL subclasses ( $0.68 < Q^2 < 0.96$ ). The  $Q^2$  -values of the models predicting the cholesterol concentrations in VLDL and LDL subclasses ranged between 0.26 and 0.80. The potential of the PLS-LPD model for the assessment of dietary effects was evaluated by comparing the LPD of 52 subjects before and after a 4-week treatment with dietary supplements that were hypothesized to change blood lipids. Significant ( $p < 0.001$ ) reductions in triglycerides in all VLDL subclasses and in cholesterol in the majority of the VLDL and LDL subclasses as well as increases in cholesterol in the two large HDL subclasses were observed. The lipoprotein subclass resolution in the predicted lipoprotein profiles of fasting serum also proved to be suitable for modeling lipoprotein turnover.

## GENOME SCALE METABOLIC RECONSTRUCTION OF T BRUCEI USING GENOMIC INFORMATION AND COMMUNITY-BASED ANNOTATION

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*Trypanosma brucei* is the causative agent of African trypanosomiasis, which if left untreated, can be fatal. The current treatment regimes for trypanosomiasis have many undesirable side-effects. In this study, we plan to develop a genome-scale metabolic network model which can be used to study, suggest new drug targets.

Metabolic network genome based reconstruction consists in developing a mathematical model of the organism metabolism which will allow integration and interpretation of transcriptomics, proteomics and metabolomics data. In order to avoid false positives from automated reconstruction techniques that rely on non organism-specific databases, we have used a method which looks for orthologs in proteomes of *T.brucei* and *L.major* obtained from TritypDB database. This list of candidate genes was used to build an initial draft of *T.brucei* from the manually curated *L.major* iAC560 model. Similarly orthologs of *T.brucei* proteins in *E.coli* were identified and components from the extensively curated iJO1366 model were used to build an *E.coli* based *T.brucei* model. In parallel we support a community based manual annotation of the *T.brucei* through the Trypanocyc project including the developmental stage specific information on reactions and pathways. These models have been merged to produce a compartmentalized model of *T.brucei* capable of running Flux balance analysis and other network analyses. MetExplore server was used in the interpretation of *T. brucei* metabolomics data.

The final aim of this project is to provide a model to the community capable of accurately predicting effects of network modifications such as gene deletions, addition of drugs into the system or medium perturbation.

**THE RETENTION TIME ALIGNMENT FOR NON-TARGETED LC/MS ANALYSIS USING KERNEL DENSITY ESTIMATION WITH NOVEL BANDWIDTH ESTIMATOR**

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Goal: Align the retention time to identify compounds and increase reliability of detected compounds.

Observation: Retention time in LC-MS data contains unknown scale deviation, which makes compounds with similar m/z indistinguishable. The statistical approach would be a good solution.

Method: Kernel density estimation based on the spectrum similarity is used to align with m/z and retention time information. Parameters estimation method is also included.

Result: This approach shows better bandwidth estimation than traditional ones.



## **METABOLOMICS TECHNOLOGY VALIDATED QUALITY MARKERS FOR BIOBANK PLASMA SAMPLES**

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Background: Research in the healthcare area like identification and validation of new diagnostic biomarkers, drug target discovery and treatment monitoring approaches often starts with the analysis of existing biobank samples. The quality of these biobank samples can be impaired by various pre-analytical sample processing steps that will confound the analytical results and decrease the value of research if not identified and addressed properly. Metabolite profiling is a well-suited technology to support the identification of technical biomarkers for the quality assessment of biobank samples due to its high sensitivity plus the broad coverage of physiological and chemical processes.

Materials and methods: Human EDTA plasma samples obtained after applying defined pre-analytical confounding factors were subjected to mass-spectrometry based metabolomics including selected targeted platforms MxP™ Broad Profiling, MxP™ Eicosanoids, MxP™ Catecholamines and MxP™ Lipids.

Results: Metabolomics data sets were analyzed by simple and mixed-effect linear models. Various pre-analytical processes resulted in significant and reproducible changes of the human plasma metabolome. Several metabolites suited as Quality Markers were identified and validated in independent data sets after Bonferroni-Holm correction of the false-positive rate with p-values being <0.001.

Conclusions: The plasma metabolome is influenced by the pre-analytical phase. Reproducible and meaningful biomarker research demands standardized protocols for sample handling (quality assurance) as well as a quality control of samples. High-level result interpretation of metabolomics studies requires framework studies to understand the impact of the pre-analytical phase on the results and to elucidate the underlying physiological and chemical mechanisms. A quality control service for EDTA plasma is currently being developed.

**BN SERVER: A WEB-BASED SERVICE FOR LC/TOFMS-BASED METABOLOMICS DATA NORMALIZATION AND STATISTICAL ANALYSIS**

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BN server is implemented as an integrated web-based platform to normalize and analysis the liquid chromatography/time-of-flight mass spectrometry-based metabolomics data. It is designed for the scientists with little or without background in programming and statistics. After users uploading the metabolomics data to BN server, BN server will automatically remove the batch and injection order effects, perform the preliminary hypothesis test and principal component analysis.

**A HIGH-THROUGHPUT AND ROBUST QUANTUM MECHANICAL TOTAL LINE SHAPE FITTING APPROACH FOR QUANTITATIVE PROFILING OF THE SERUM METABOLOME**

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NMR spectroscopy is a very powerful technique for quantitative analysis of biological samples. However, the accurate quantitative analysis using conventional signal integration of proton NMR (1H-NMR) signals is hampered by the signal overlap. To overcome this a Quantum Mechanical Total Line Shape (QMTLS) fitting model was implemented for quantitative profiling of 43 metabolites in ultra filtrated serum samples covering a large concentration range. Each metabolite was described in the model by a set of spectral parameters such as chemical shift, couplings, and line width. These parameters were optimized for each metabolite in each sample by iteratively minimizing the difference between the calculated and the experimental spectrum. With the proposed procedure 90% to 98% of the signal intensities of the selected regions in the experimental spectrum were explained by the calculated spectrum. The model was validated against straightforward signal integration for metabolites with isolated signals and standard additions including also metabolites with overlapping signals. The biological applicability of the QMTLS model was demonstrated in a human intervention study involving 37 human volunteers that underwent exercise challenges with subsequent longitudinal read-outs of metabolic homeostasis perturbations in the serum metabolome. The QMTLS model is suitable for a high-throughput analysis as it can be performed in automated manner for analysing large sets of samples.

## ASSESSING THE REPEATABILITY AND STATISTICAL ADVANTAGES OF HOMONUCLEAR 2D-NMR SPECTRA: A CLUSTERING APPROACH

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NMR techniques are widely used together with multivariate analysis approaches in order to characterize perturbations in metabolic pathways occurring during biological processes.

A large amount of recent scientific and statistical works are available concerning 1D spectra (principally <sup>1</sup>H-NMR spectra). More recently, two-dimensional NMR spectroscopy techniques have been investigated: homonuclear (COSY,...) and heteronuclear ones (HSQC,...). It is commonly accepted by users (biologists, pharmacologists) that the recent introduction of 2D-NMR methods represents a huge qualitative gap for metabolomics investigations in terms of metabolites and biomarkers identifications. Indeed, it seems obvious that additional dimension means more predictive power. But, until now, no statistical study clearly proved this assumption. Therefore, a fundamental question is "Is supplementary information equivalent to relevant and crucial information ?".

In order to extend the statistical properties and tools developed for 1D spectroscopy to the new challenges raised by 2D spectra, a rigorous study of the repeatability of 2D-NMR spectra is needed as a prerequisite. In the context of first homonuclear COSY experiments (spectra of complex biological media), we will present a methodology based on accurate multivariate clustering tools. Numerical quality indexes and graphical clustering results will be shown, obtained via binary vectors of positions, via recoded intensity vectors and through different levels of spectral resolution. A second objective is to compare these 2D results with corresponding 1D results (<sup>1</sup>H-NMR) obtained in the same conditions. Our preliminary results seem already promising: COSY appears to be a statistically robust tool and, furthermore, additional information appears to be relevant.

## ADAPTATION OF RANK PRODUCT STATISTICS FOR METABOLOMICS STUDIES

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Metabolomics aims at understanding biology by comprehensive metabolite profiling (the simultaneous measurement of as many low molecular weight compounds as possible in a biological sample). Very often, lists of extracted features or metabolites have to be compared between different conditions, e.g. two different treatments or cell lines, based on very few and noisy replicates. Classical statistical methods (e.g. t-tests) have been shown to be unreliable in this setting.

In this work we present an adaptation of the rank products [1] statistical test for metabolomics data sets, introducing a necessary modification of the original algorithm for handling unpaired data. Rank products method produce robust differential expression estimates for data sets with a small number of replicates (2 as a minimum) superior to standard parametric methods. We will also demonstrate the improved estimate of rank product  $p$ -values [2] based on the exact probability distribution instead of using a computationally expensive permutation approach.

Our results are illustrated the in a variety of metabolomics studies and compared to commonly used statistical methods. New software will be available in the Bioconductor project [3] and integrated with mzMatch [4] and IDEOM [5].

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## EXTRACTION OF METABOLOMICS DATA TO DISCLOSE MINOR PATHWAYS OF ORGANIC ACID METABOLISM IN ISOVALERIC ACIDEMIA

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Isovaleric acidemia (IVA) is an autosomal recessive inborn error of leucine metabolism caused by a deficiency of isovaleryl-CoA dehydrogenase, which catalyses the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA. Using a metabolomics approach we have previously reported a comprehensive metabolite profile for untreated IVA patients, based on significant changes in their urinary organic acid excretion relative to controls (Dercksen et al<sup>1</sup>). A current paradigm in untargeted metabolomics assumes that many known and novel observations of metabolic changes may be discovered through quantitative metabolomics. We therefore formulated the following hypothesis with regard to IVA: *“Information on minor metabolic pathways, indicative of a finer categorization of perturbations in IVA, might also be encapsulated in the data matrix used to identify the primary markers of IVA.”*

To test this hypothesis, we performed data extraction and multivariate as well as univariate analyses on the original matrix used for the investigation of IVA cases but excluding the diagnostic biomarkers from that matrix. This approach disclosed information on a number of novel markers of minor metabolic perturbations present in IVA, namely: lactic acid ( $p=0.001$ ), 3-methyladipic acid ( $p=0.036$ ); 2-methyl-3-hydroxybutyric acid ( $p=0.001$ ); succinic acid ( $p=0.035$ ), methylcitric acid ( $p=0.015$ ) and citramalic acid ( $p=0.017$ ). The information from these new markers, as well as the metabolic perturbations which we propose from them, indicate the effect of IVA on intermediary metabolic homeostasis with a potential of further assistance to physicians to identify high-risk IVA patients.

<sup>1</sup> Dercksen, M. Koekemoer, G. Duran, M. Wanders, R.J.A. Mienie, L.J. & Reinecke, C.J. (2013) Organic acid profile of isovaleric acidemia: A comprehensive metabolomics approach. *Metabolomics*, DOI 10.1007/s11306-013-0501-5

**METABOLOMICS CHARACTERISATION OF ANGELICA SPECIES BY COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY COUPLED WITH TIME-OF-FLIGHT MASS SPECTROMETRY (GCxGC-TOFMS)**

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Due to the difference of GCxGC-TOFMS data among three types of Angelica samples, Japan, Hualien and ChungHua, is not obvious, we propose a method to improve the performance of classification. 2D chromatograms were first aligned and the supervised learning method was used for identifying major components and relative quantities of each species in 75 Angelica samples. Major components of each species, which is used to separate three type of Angelica, were identified in our approach.

## MRMPROBS: DATA ASSESSMENT AND METABOLITE IDENTIFICATION TOOL FOR LARGE-SCALE MRM-BASED WIDELY TARGETED METABOLOMICS

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We developed a new software program, MRMPROBS, for widely targeted metabolomics by using the large-scale multiple reaction monitoring (MRM) mode. The strategy became increasingly popular for the simultaneous analysis of up to several hundred metabolites at high sensitivity, selectivity, and quantitative capability.

In contrast to technological improvements, software development for the data analysis of MRM transitions lags behind in metabolomics: data assessment usually relies on manual evaluation due to the lack of automated probabilistic measures. Manual verification without probabilistic criteria is not only laborious, but often subjective, erroneous, and even irreproducible. Therefore, objective evaluation is needed to minimize misinterpretations of biological issues.

To satisfy these requirements we developed a probabilistic scoring scheme for large-scale MRM-based widely targeted metabolomics and implemented our MRMPROBS software program. It evaluates the metabolite peaks by posterior probability, defined as the odds ratio by means of a newly optimized multivariate logistic regression model, and visualizes large-scale MRM data sets with user-friendly graphical user interfaces to allow data curation and statistical analyses. Specifically, the probability of a peak is calculated from five machine-independent variables, i.e. peak intensity and retention time-, ratio-, shape-, and co-elution similarity without decoy transitions and label compounds. The posterior probability enabled us to filter not only noise peaks but also peaks from isomeric metabolites even without label compounds. Moreover, our objective criterion, by means of probability, facilitates the meaningful and useful assessment of identification results.

For a demonstration, we conducted a widely targeted metabolome analysis (152 metabolites) of propagating *Saccharomyces cerevisiae* measured at 15 time-points by gas- and liquid chromatography coupled to triple quadrupole mass spectrometry. We expect MRMPROBS to be a useful and practical tool for the automated, objective, and consistent evaluation in widely targeted metabolomics. Our program is freely available at <http://prime.psc.riken.jp/>.



**COMPARATIVE ANALYSIS OF SAMPLE PREPARATION METHODS FOR LC-ESI-qToF-MS-driven PLASMA FINGERPRINTING. LESS IS MORE WHEN HANDLING THE COMPLEXITY OF BLOOD FLUIDS METABOLOME**

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Sample preparation is still one of the challenging steps of the metabolomic workflow. Although phospholipids in blood fluids are known to cause ion suppression phenomena, thus masking biological variation, their removal from samples prior to untargeted analysis is generally not considered.

The present study applied a LC-ESI-qToF-MS-driven metabolomic workflow and multiple chemometric techniques to evaluate the efficiency of five plasma sample preparation methods (i.e. a novel hybrid method combining solvent extraction and SPE-mediated phospholipids removal, a membrane-based solvent-free technique, and three more “traditional” extractions with organic solvents). Pooled plasma samples collected during a dietary intervention trial (a cocoa acute intake study) were partly analyzed as raw samples and partly spiked with distinct concentrations of a 16-metabolite mix (1-5 µg/mL) and subjected to the five different preparation procedures.

The combination of solvent extraction and selective removal of phospholipid species revealed to be the most suitable method to handle the complexity of the plasma metabolome, in terms of biomatrix effects minimization, reproducibility, metabolite coverage, differentiation of raw *versus* spiked samples, and detection of real-life diet-dependent acute plasma metabolomic changes. Typical markers of cocoa consumption were detected (purine alkaloids, phase II metabolite of cocoa polyphenols), together with unexpected metabolome modifications (decrease of plasma acylcarnitines, bile acids and glycine conjugates).

Results suggested that there is no reason not to sacrifice information on lipid species such as phospholipids (specific object of interest of lipidomics) in order to gain information on non-lipid metabolome.

## A NEW LIPID SOFTWARE WORKFLOW FOR PROCESSING ORBITRAP-BASED GLOBAL LIPIDOMICS DATA IN TRANSLATIONAL AND SYSTEMS BIOLOGY RESEARCH

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**Introduction:** Application of lipidomics to disease phenotype analysis is a growing area in medical research. Identification of unique biomarkers to distinguish healthy humans compared to those with a disease can have an impact on the early detection of diseases and personalized medicine. Identification of lipids requires sophisticated software with an extensive database. We present an evaluation of a new workflow for Orbitrap-based mass spectrometers for lipidomics analyses using model systems including wt vs. knockout for Co-Q production in yeast and Zucker fatty rat serum, a model for type II diabetes. Lipid Search software was compared to manual annotation of the yeast dataset.

**Methods:** Profiling of mitochondrial lipids, isolated from wild type yeast (*S. cerevisiae*) and a knockout strain that lacking CoQ production were performed by LCMS using a Q Exactive Orbitrap mass spectrometer. LCMS and direct infusion analysis of ZDF rat serum lipids was performed at 140K resolution in positive and negative MS and 70K in MSMS using a Q-Exactive. The HPLC gradient was 60:40 Acetonitrile/Water to 90:10 IPA/Acetonitrile (0.1% formic acid, 10mM ammonium formate) in 25min using an Ascentis Express C18 column (Supelco, 2.1x150mm, 2.7 $\mu$ m) operated at 260 $\mu$ L/min and 50°C. Data was analyzed with Lipid Search software (MKI) to identify the lipids present in each sample.

**Preliminary Data:** Analysis of WT vs. KO yeast served as a benchmarking study to determine the merits of the software workflows. Lipids were tentatively identified by MW search of HMDB using ChemSpider. MS/MS data were analyzed manually to assign the lipid species giving 148 lipids identified. The new workflow described here uses Lipid Search (MKI) software for lipid identification through a database search of the accurate masses of precursors and the fragment ions predicted for each potential adduct form of the lipids in the database (>10<sup>6</sup> entries). Relative quantitation and statistical analysis is provided along with a graphical summary of the chromatographic and spectral data for each lipid result. Each identification is ranked by mass tolerance, match to theoretical fragmentation and predicted retention time. The results display provides the integrated chromatogram of the precursor and MS/MS spectrum annotated with fragment ions used to make the identification. The new workflow also aligns and merges the LC-MS data obtained on different samples to obtain a relative quantitative comparison of the sample set, allowing statistical comparison between the groups compared to control. The identified lipids were quantified in each sample to provide a relative comparison of the lipids in WT vs. KO yeast, and in lean vs. obese ZDF rats. Lipid Search software provided a fully automated identification of 256 lipids in yeast from 514 different MS-MS spectra compared to the 148 lipids identified manually. The improved identification workflow dramatically reduces the time spent on identification of lipids. In addition, the >10<sup>6</sup> entry lipid database coupled with unique spectral matching and scoring algorithms ensure that the most likely lipids are matched. In addition, spectra containing 2 different species are properly matched with both lipids being annotated correctly.

## FULLY AUTOMATED NMR SPECTRAL PROFILING FOR METABOLOMICS - BAYESIL

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In NMR-based metabolomics, spectral profiling or spectral deconvolution is frequently used to identify and quantify metabolites from 1D <sup>1</sup>H NMR spectra. The process involves fitting individual reference NMR spectra of pure compounds to the NMR spectrum of a biofluid mixture. Key to this process is the availability of a spectral library and appropriate software to help with the spectral fitting. Currently there are a number of software packages that support semi-automated spectral profiling, with or without compound quantification. However, all of these packages either require manual fitting or manual spectral processing – both of which require a substantial amount of time and both of which can lead to inconsistent or incorrect results. Ideally what is needed is a software system that automatically performs both spectral processing (i.e. Fourier transformation, phasing, solvent filtering, referencing, baseline correction, reference line shape convolution) and spectral fitting. Furthermore, such a system should be able to analyze complex mixtures (>50 compounds) accurately (>98% correct) and yield compound concentration data that is within 10% of the known concentrations. Here we wish to describe such a system, called BAYESIL. It uses a variety of novel phasing and baseline correction methods to automatically process 1D NMR spectra. It also uses probabilistic graphical models in conjunction with stochastic optimization to rapidly perform very accurate spectral deconvolution. Based on extensive testing with defined mixtures and real biological samples BAYESIL consistently performs with sensitivity and specificity greater than 98% for compound identification in mixtures with up to 60 different compounds. It also determines metabolite concentrations (down to 10 μM) within 10% of the known or expert-measured concentrations. In other words, BAYESIL operates at a level that meets or exceeds the performance of the most highly trained human experts. BAYESIL appears to be the first system that supports fully automated and fully quantitative NMR-based metabolomics. This may open the door to routine applications of NMR in a clinical setting. A detailed description of the software, its algorithms, its spectral databases and its testing performance will be presented.

**ESTABLISH TARGETED LIPIDOMICS WORKFLOW USING TIPick WITH ADJUSTED PARAMETERS ON DENOISE AND PEAK PICKING STAGES**

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Targeted lipidomics analysis aims at identified known lipid species from cells. TIPick is a denoising/peak picking algorithm developed to extract true ion signals from LC/MS data. To establish workflow for targeted lipidomics analysis, parameters of TIPick are adjusted for lipidomics use. Extracted ion chromatograms of 237 lipid standards are used to examine noise level and detect peaks using TIPick. We have successfully established workflow for targeted lipidomics by adjusting suitable parameters of TIPick.

## **A FULLY AUTOMATED UNTARGETED LIPID DATA PROCESSING METHOD USING MZmine COMPARED TO AGILENT'S RECURSIVE ANALYSIS**

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Data analysis is one of the many important steps in a study involving untargeted metabolomics. While targeted data processing methods are well established, untargeted methods of extracting LC-MS data are have many potential pitfalls.

Here we analyze and discuss untargeted data processing from three popular software programs, MZmine, Agilent Mass Profiler Professional, and XCMS. Data was collected from lipidomic plasma extracts on an Agilent accurate mass Q-TOF 6530 using a Waters CSH C18 column and exported to mzdata.xml format. This data was then processed on both Agilent's qualitative analysis using the Agilent Mass Profiler Professional recursive analysis method and an in house workflow developed for MZmine. Both workflows were compared by assessing common problems such as duplicate peaks, missing values, ease of use, processing parameters, and false positives. Corresponding XCMS results for the same data set were investigated in the same manner.

Annotations were based on accurate mass and MS/MS fragment search using Lipid Blast. We explored ways to minimize problems for parameter settings in our final workflow. The biggest difference between the methods was the number of missing values. The Agilent recursive analysis workflow resulted in a good data set with fewer parameter adjustments but it yielded many missing values and showed poor back filling. MZmine processing methods were much harder to set up but gave very few missing values for metabolite intensities. MZmine was capable of running in batch mode for hundreds of chromatograms which enabled a one-button data processing method, from raw data files to a full peak lists. We provide a download for the fully functional MZmine method along with an annotation database file. This download is currently used to process data from large cohort studies with more than 1,000 samples per month but can also serve as good starting point for users who implement own data processing methods.

**DENOISE METHOD TO REMOVE SCAN-BASE NOISE AND AUTOMATIC DRAIN ALGORITHM THRESHOLD DETERMINING PRE-PROCESSING METHOD APPLY ON GC-TOFMS**

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Remove noise-like intensity to improve the result of identification and decrease data size. Noise level depends on scan in our GC-TOFMS data. Drain algorithm is commonly used for picking region with compounds co-eluted, however threshold tuning is difficult. Evaluate threshold in each scan by regression model. Threshold of drain algorithm determined by the number of cluster automatically. We find the number of identified compounds increase especially in higher match factor threshold.

## REST ARCHITECTURE FOR MASSBANK DATABASE SERVICE

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The rapid development of metabolomics has resulted in a significant increase in the number of *online database services* that store, manage, and analyze metabolomic datasets. These services are usually accessible through *Web Application Programming Interfaces (web APIs)*. Publicly-available databases with web APIs (Golm Metabolome Database, MassBank, and METLIN) support programmed access by client applications for searching, annotating, and visualizing data. Using web APIs, multiple services can also be integrated for use in data-mining and analysis tools, web indexers, and cross-domain services such as genomics and proteomics databases.

Designing a database service that supports web APIs poses several challenges regarding long-term evolvability and scalability. This poster proposes a *Representational State Transfer (REST)* approach for the *community-web service* MassBank. In place of the previous star network topology for all operations, the service uses different topologies. To support this, the network is organized into three levels of nodes: a *master node* that orchestrates critical operations through a star topology, *major nodes* (data hubs) whose contents are regularly crawled, integrated (e.g., integrated standard spectra), and shared through a partially-connected topology, and *minor nodes* representing transient users. Metabolomic data (e.g., mass spectra) are shared by all nodes through a fully-connected topology. We encourage users to actively participate in the community by submitting metabolomic data and to automatically receive integrated results. We introduce the next version of the MassBank database service, which implements the RESTful architecture for this distributed service as a reference implementation.

## **BiPACE - A GENERIC RETENTION TIME ALIGNMENT ALGORITHM FOR GAS-CHROMATOGRAPHY MASS-SPECTROMETRY DATA**

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One- and two-dimensional gas-chromatography mass-spectrometry (GC-MS, GCxGC-MS) are important techniques for the separation and analysis of complex biological samples. Especially GCxGC-MS is used in the field of metabolomics due to its increased peak capacity compared to one-dimensional GC-MS, yielding improved chromatographic separation of chemically closely related analytes. However, this also increases the size of the resulting datasets drastically, hampering manual data analysis workflows. One recurring problem in this domain that we address with BiPACE and its variants is the automated matching and association of peaks and their associated mass spectra across a large number of samples.

BiPACE is a generic algorithm for retention time alignment of multiple datasets from one and two-dimensional chromatography, coupled to FID, MS, or in principle, arbitrary detectors. It is based on a composable similarity function that is used to compare features pairwise from different datasets and complemented by a k-partite clique finding and merging phase that constructs a multiple alignment based on the pairwise similarities. The alignment is reported by the algorithm in tabular output format, where each row represents features contained in the corresponding clique.

We demonstrate BiPACE's ability to support various similarity functions for retention time and mass spectral similarity on a set of publicly available GCxGC-MS datasets and compare BiPACE's performance to the previously published algorithms mSPA and SWPA. We further evaluate the performance of BiPACE using symmetric and asymmetric retention time similarity functions, where the latter allow for a better modeling of the tailing behaviour of chromatographic peaks. Additionally, we provide an overview of the software framework developed for the evaluation, and how to integrate new algorithms into it.

BiPACE is freely available as a part of the Open Source software framework Maltcms.

BiPACE is freely available in the Open Source software framework Maltcms.



## **MAMBO-MS: A WEB-BASED GC-MS AND LC-MS MASS SPECTRAL LIBRARY MANAGEMENT SYSTEM**

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There is an increasing demand for curated mass spectral databases to facilitate the identification of both known and uncharacterized metabolites detected in GC/MS and LC/MS experiments. A number of mass spectral databases have been developed, including freely available libraries such as HMDB, METLIN, GMD and MassBank and commercial libraries such as the NIST11 mass spectral library and the Wiley registry of mass spectral data. While much work has gone into developing the content stored by these databases, less investment has been made in providing the research community with a database framework that can be downloaded and used directly to store mass spectral records, without having the need to build such databases from scratch.

We present MAMBO-MS, a downloadable and installable framework for a web-based mass spectral library management system for GC-MS and LC-MS data. MAMBO-MS comprises a Lab Management System through which one or more labs are able to access the system, the User Management System, which defines different user groups and privileges to users of the labs and a Data Management System that defines the metadata of the system along with how the different mass spectral libraries are stored, curated and accessed. MAMBO-MS can thus be used by multiple labs to store and share GC-MS and LC-MS mass spectral libraries.

With the increased generation of metabolomics data and the recognition that a large number of metabolites are yet to be identified, stored and shared through metabolite mass spectral databases, a solution such as MAMBO-MS is invaluable to the research community. MAMBO-MS is freely available under the GNU GPL v2 licence and can be accessed from <http://code.google.com/mambo-ms/>

**NOVEL SOFTWARE SOLUTIONS FOR LIQUID CHROMATOGRAPHY-HIGH RESOLUTION MS (LC-HRMS) METABOLITE PROFILING OF LEGUMES UNDER DROUGHT AND FUNGAL INFECTION CONDITIONS**

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The EU FP7 five-year project ABSTRESS ([www.abstress.eu](http://www.abstress.eu)), currently in its first year, is set to revolutionise the way in which new plant varieties are produced. Currently the yield of legume crops is severely undermined by both drought and fungal infection (*Fusarium oxysporum*). *Fusarium* is a soil borne pathogen that causes a disease which wilts infected plants. The damage it causes is compounded during drought conditions. The prevalence of this economically devastating fungal disease is predicted to increase due to climate change.

This work describes the optimised methods currently being explored to accurately obtain and interpret metabolite profiles of biotic and abiotic stressed vs non stressed legume plants. Particular emphasis is placed on the critical step of data processing and interpretation.

Samples from stressed and control plants were collected from plants exposed to both drought and *fusarium* infection (singularly) as well as samples from combined stresses and control plant. Extracts were analysed on a Thermo Exactive LC-HRMS.

A range of compounds potentially associated with *fusarium* infection and drought stress have been identified from leaf and root samples of stressed legume plants using the Progeneis Comet software. The correlation analysis feature in the software offers a dendrogram that groups significant compounds to how similar their abundance profiles are. This allows an evaluation of the relationship between these compounds under certain stresses. This information combined with concomitant NMR metabolite information and genomic / transcriptomics studies sheds light into the molecular / physiological pathways involved in a legume plants reaction to biotic and/or abiotic stress.

The work describes PCA and correlation analysis for this experiment and gives further details of compounds discovered using this innovative software.

## EVALUATION AND CORRECTION OF BATCH VARIATION IN METABOLOMIC WORKFLOWS USING DIRECT INFUSION MASS SPECTROMETRY (DIMS)

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Untargeted metabolomics experiments using direct infusion mass spectrometry (DIMS) can measure many hundreds of samples in a single experiment. Minimising and correcting for analytical variation is of particular concern in large scale, multibatch studies. Here, we describe the results of a purpose-designed study analysing mammalian heart tissue constructed to characterise and correct the intra and inter-batch analytical variation. Heart tissue from two species was analysed over seven days as an eight-batch DIMS metabolomics study using nanoelectrospray (nESI) Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry. Initially, the intrinsic analytical variation of the study was measured to assess our existing workflows and their suitability for multi-batch studies. Assessment of the inter-batch variation was undertaken using criteria that included the median relative standard deviation (RSD) of both quality control (QC) samples and biological replicate samples in addition to an evaluation of our ability to discriminate true biological differences from other sources of variation. To reduce the analytical variability, a workflow was implemented including total-ion-current filtering, QC-robust spline batch correction and spectral cleaning. The findings objectively demonstrate that implementation of this workflow reduces analytical variation and increases the percentage of significantly different mass features. Analysing samples across seven days and eight different batches we report an overall analytical precision of 15.9% median RSD. This compares favourably to FDA guidelines which specify an RSD of <20% for biomarker studies. We conclude therefore that our workflows are suitable for the analysis of large-scale, high throughput studies of this nature.

**FUSION OF METABOLOMICS DATA FROM DIFFERENT PLATFORMS USING PLATFORM SPECIFIC MEASUREMENT ERRORS**

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In metabolomics, biological samples are measured on different platforms to cover a broad range of metabolites. The statistical analysis of these multiple sets of metabolomics data corresponding to the same samples requires special data analysis methods often referred to as data fusion methods. A problem of fusion of different platforms of metabolomics data is that each platform has its own characteristics in terms of measurement errors. At the one hand we have measurement errors at different intensity levels: measurements errors are proportional to intensity level at large intensity levels, but constant at low intensity levels. On the other hand we have platform-dependent multivariate errors as well.

Maximum-Likelihood Fusion method (Maximum-Likelihood version of PCA) was developed to manage such problems. This method consists of a step in which measurement error structure is derived from replicate metabolomics data and the error structure is used as an input for ML PCA to obtain the principal components model. We study different methods to obtain the measurement error structure, such that they can be used in the fusion method. In this work we will compare the new data fusion approach with standard methods that ignore the measurement error differences.

**HYPOTHESIS-FREE IDENTIFICATION OF GENETICALLY INFLUENCED NMR SPECTRAL FEATURES AND THEIR INTERPRETATION USING MASS SPECTROMETRY DERIVED METABOLIC TRAITS**

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Metabolic profiling of blood serum and plasma from cohort studies is a major tool in the discovery of new disease-relevant biomarkers and metabolic individuality in the general population. Nuclear magnetic resonance (NMR) spectrometry is a platform often used in metabolomics studies since it provides robust readouts of many metabolic parameters in a single experiment.

We present a hypothesis-free approach to identify genetically influenced NMR features using plasma samples of 1,757 individuals from the KORA F4 cohort together with 655,658 genetic variants. We show that ratios between NMR signals at two different spectral positions display comparable associations to genetic polymorphisms as mass spectrometry (MS) derived metabolic features.

Furthermore, we characterize NMR traits by correlating the raw spectra with the concentrations of over 460 metabolites obtained from two distinct MS based platforms in the same blood samples. Conversely, the resulting correlation spectra could be used to annotate metabolites of unknown chemical identity measured on MS platforms. This kind of bidirectional knowledge transfer might provide new ways for the harmonization of metabolic data collected on different but complementary platforms.

**UNTARGETED METABOLOMICS OF *FUSARIUM GRAMINEARUM*: COMPARISON OF STABLE ISOTOPIC LABELLING ASSISTED AND CONVENTIONAL DATA PROCESSING STRATEGIES**

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**Introduction:** We present MetExtract a novel software tool for the processing of LC-HRMS data derived from stable isotopic labelling (SIL)-assisted experiments. The software and data processing strategy is exemplified with culture samples of the plant pathogenic fungus *Fusarium graminearum*. Additionally, we compare our SIL-based method with a traditional labelling-free strategy.

**Methods:** We cultivated the plant pathogen *Fusarium graminearum* wild type (PH1) and its knock-out mutant (tri5) in parallel on <sup>12</sup>C and uniformly <sup>13</sup>C labelled glucose and subsequently measured the culture filtrates with an LTQ Orbitrap XL. Raw LC-HRMS chromatograms of mixtures of <sup>12</sup>C and <sup>13</sup>C supernatants were processed with MetExtract while those from <sup>12</sup>C supernatants were processed with XCMS and CAMERA. Comparison of the extracted features and their assigned feature groups (MetExtract vs. XCMS and CAMERA) was mainly performed qualitatively. For selected putative metabolites also chromatographic peak picking and integration were compared which -for both approaches- were derived from similar wavelet algorithms. Moreover, uni- and multivariate data analysis was performed for both strategies to find differences between the genotypes.

**Results and Discussion:** XCMS extracted about 5 to 6 times the number of features (including different isotopologues) than MetExtract, which only considers monoisotopic ions. An overlay of the features revealed that most of the MetExtract features were found by XCMS as well, however, a minor number was found by MetExtract only. The majority of features found only by XCMS revealed themselves as non-sample related material or low intense features. Feature group comparison revealed similar groups between XCMS and MetExtract. However, more features within a group were found with XCMS. Manual inspection of automatically integrated features showed a high correlation between the peak areas calculated by MetExtract and XCMS. Multivariate analysis (HCA, PCA) clearly showed that both approaches were suited to distinguish between the two fungal strains.

## NOVEL STABLE ISOTOPIC LABELLING-ASSISTED WORKFLOWS FOR IMPROVED LC-HRMS BASED METABOLOMICS OF FUNGI AN PLANTS

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Due to the large proportion of non-metabolite related signals of electrospray ionisation -liquid chromatography - mass spectrometry (ESI-LC-MS), automated feature detection and annotation of metabolites are still major challenges of untargeted metabolomics approaches. Moreover, matrix effects in the ESI limit the reliability of relative quantification of metabolites. In vivo isotopic labelling offers powerful techniques to conquer these obstacles as it enables to elucidate the global biochemical composition of biological samples, to study the metabolisation of labelled tracers or to improve quantification by internal standardisation.

In this study we present novel LC-high resolution (HR) MS based, stable isotopic labelling-assisted workflows for the untargeted profiling of biological samples involving the measurement of mixtures of <sup>13</sup>C labelled and non-labelled biological samples or tracer metabolites. MetExtract and FragExtract, novel data processing tools for global metabolome characterisation, tracer metabolisation and MS/MS spectrum evaluation will be presented. In a first step the recently developed MetExtract software evaluates mass spectra by hierarchical clustering of MS signals which are recognized from labelling specific patterns and assigns the number of carbon atoms to each of the extracted ion signals from mass increments between corresponding isotopic peaks. Subsequently, MetExtract performs chromatographic peak picking using a wavelet implementation, predicts feature groups to convolute different adducts and in-source fragments of the same metabolite using Pearson correlation coefficients, and performs chromatographic alignment with a polynomial time warping algorithm. The resulting data matrix does not only form a reliable basis for metabolite annotation and further statistical analysis but is also excellently suited for evaluating the performance (e.g. sample extraction, precision, matrix effects) of the complete analytical workflow.

A detailed description of the data processing concepts will be given and their performance and limitations are discussed. The complete workflows are exemplified with selected biological studies on the characterisation of the metabolome of wheat, and the untargeted profiling of mycotoxins in wheat as well as phenylalanine in grapevine.

**FRAGEXTRACT: A NOVEL SOFTWARE TOOL FOR MS/MS SPECTRUM EVALUATION FOR STRUCTURE ELUCIDATION IN METABOLOMICS RESEARCH**

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Today structure elucidation of unknown compounds in metabolomics experiments is still a major bottleneck in metabolomics studies. For definitive compound identification, tandem mass spectra (MS/MS) need to be recorded and compared against mass spectral databases or MS/MS spectra of authentic reference standards obtained under the same experimental conditions. However these tasks are frequently hampered by a lack of both authentic standards and MS/MS reference spectra. Nonetheless one elegant approach, which can be used to facilitate MS/MS spectrum interpretation, is full in vivo stable isotopic labelling (SIL) of whole organisms. Those are cultured in parallel on media containing either  $^{12}\text{C}$  or  $^{13}\text{C}$  glucose as sole carbon source.

Here we present FragExtract, a novel algorithm for processing liquid chromatography high-resolution tandem mass spectrometry (LC-HR-MS/MS) data of SIL-assisted experiments where MS/MS spectra of native and corresponding  $^{13}\text{C}$  labelled compounds are acquired in the same analytical run. In the presented algorithm the mass shift between  $^{12}\text{C}$  and  $^{13}\text{C}$  is used to find pairs of corresponding fragment signals in the MS/MS spectra of native and labelled substances and to calculate their number of carbon atoms. This way, artefacts and noise-related signals can be filtered out efficiently. Additionally, FragExtract provides meaningful suggestions for elemental formulas of the fragment ions based on the calculated number of carbon atoms, which reduces the proposed elemental formulas. To visually browse through the results FragExtract offers a user-friendly graphical interface.

The performance of the algorithm was verified using mixtures of native and labelled substances that were spiked at different concentration levels into culture filtrates of the filamentous fungus *Fusarium graminearum*. Although the target analytes were measured in complex biological matrices it did not alter FragExtract's ability to properly filter out unspecific signals. Furthermore, FragExtract was used successfully to evaluate MS/MS spectra and to annotate metabolites in samples of *Fusarium graminearum*. The presented software FragExtract shows high potential to assist in structural elucidation and annotation of (unknown) compounds. The combination of FragExtract's results with MS/MS spectral comparison in the future will further benefit the usage of the presented software.



**UNDERSTANDING THE COMPLEX INTERACTIONS INSIDE THE HOST-MICROBIOME-METABOLOME AXIS IN INFLAMMATORY BOWEL DISEASES VIA (ULTRA)HIGH RESOLUTION SPECTROMETRIC TECHNIQUES**

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Various human diseases can be described on the level of the organism's metabolism. Currently more attention has been paid to the identification of the relationship between the metabolism and the gut microbiota of the host. The microbial community can affect humans' health in a positive and negative way depending on its functional composition. Although the literature describes differences in the microbial composition of healthy and non-healthy subjects, only little is known about the corresponding metabolomic changes expressing their function.

Our goal is to setup metabolite maps via non-targeted and targeted analytical approaches and to correlate them to the nutritional, clinical, microbiological data. We analysed fecal samples from a human trial including three groups (control group, Crohn's disease patients, and ulcerative colitis patients) for their microbial and metabolomics composition. The individuals received an iron therapy either orally or intravenously administered over a time period of three months. Fecal samples were taken before and after the three months of the therapy.

Fourier-Transform Ion Cyclotron Resonance Mass-Spectrometry (FT-ICR-MS) was used as a first screening tool in the non-targeted metabolomics. After analysing the signal output and revealing significant masses that contribute to the difference between groups, some of these masses were annotated and mapped onto their metabolic pathways. Further annotation and, possibly, identification of metabolites will be followed by using bioinformatical tools, (Ultra)High Performance Liquid Chromatography coupled with Time-of-Flight Mass-Spectrometry ((U)HPLC-ToF-MS), and Nuclear Magnetic Resonance (NMR) spectroscopy. Finally we will make a correlation between the nutritional, clinical and microbial data sets.

**WORKFLOWS FOR THE CREATION OF LIBRARIES OF RECURRING MASS SPECTRA (GC/MS, LC/MS/MS) FROM BIOLOGICAL MATERIAL INCLUDING STANDARD REFERENCE MATERIALS**

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A major problem in metabolomics is the large number of unidentified components encountered. Such complex mixtures may be characterized by frequently recurring components in terms of their chromatographic retention and mass spectral characteristics. In order to provide searchable libraries of such recurring components, workflows are being developed to process very large numbers of data files and to provide high quality spectra with well characterized retention parameters.

In GC/MS, the developed tools for processing large data sets include determination of the scatter for retention index data. In addition, the deconvolution parameters of the Automated Mass Spectral Deconvolution and Identification System (AMDIS, [1]) are used to filter out poorly resolved or incompletely resolved data. AMDIS output is further filtered by the NIST MSSEARCH algorithms [2] giving an independent measure of spectral quality.

The workflows have been applied to 7000 urine extract data files as well as 1620 essential oil samples. Libraries of identified and unidentified recurring spectra have been created and are available for download.

Problems encountered during development of the automated procedures such as retention index variations, molecular weight estimations, peak overloading and perfectly overlapping components are described and discussed with examples.

In LC/MS/MS, a similar workflow is being developed for the creation of libraries of recurring MS/MS spectra using ion trap, Orbitrap and QTOF instruments for serum, urine, cranberry, blueberry, and will be made available with related SRM data.

[1] Stein, S.E. (1999) J. Am. Soc. Mass Spectrom. 10, 770-781.

[2] NIST 11; <http://www.nist.gov/srd/nist1a.cfm>

**HUMAN PLASMA METABOLITE PROFILING: THE IMPACT OF CHROMATOGRAPHY AND DATA ACQUISITION METHODS ON METABOLITES DETECTED BY LC-MS**

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The diverse compound classes present in complex matrices such as human plasma require careful consideration when undertaking untargeted global metabolite profiling. Advances in rigorous method development and batch handling of large sample groups have led to standard operating procedures including pooled quality control samples before, during and after sample analysis with batches of approximately 120 samples. Sample preparation, separation and detection underpin comprehensive coverage of the metabolome however identification plays an equally important role with several choices of databases available with varying degrees of tandem MS data present.

In these experiments NIST standard reference material of human plasma (SRM-1950) was analysed to optimise data acquisition methods. Reversed phase chromatographic separation accommodated both rapid elution of polar compounds (amino acids, fatty acids) whilst balanced with later elution of non-polar compounds (diacylglycerophosphocholines, triacylglycerols) over 50 minutes. The mass range of data acquisition demonstrated that although typical metabolomics experiments focus on ions of  $m/z$  100-1000, many compounds fall outside of this mass range and could be missed. Data acquisition on the LCMS-IT-TOF was designed to maximise sensitivity using a wider mass range whilst simultaneously acquiring MS<sub>n</sub> data to enable metabolite identification. The process of identification considered both MS mass accuracy and confirmatory MS/MS ions present in databases. This demonstrated both the excellent coverage of compounds already present in databases but also the need for more MS/MS data available for lipid classes demonstrating the necessary need for authentic standards for biomarker confirmation.

## **METABOLIGHTS REFERENCE LAYER: A COMPREHENSIVE KNOWLEDGE BASE OF METABOLITES**

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The MetaboLights Reference Layer is a comprehensive knowledge with a Metabolite-centric view. It includes elements such as reference spectra of various types, biological and chemical reference data, protocols and cross-references to other worldwide resources.

The MetaboLights Reference Layer has comprehensive manually curated data, including chemical structures and characteristics from ChEBI, metabolic pathways, reference spectroscopy and chromatography. Furthermore there is information about reference biology, metabolites, their occurrences and concentration in the species, organs, tissues and cellular in various conditions, both in healthy and diseased. Publication references and protocols are available too.

This reference layer will enable experimentalists to get a comprehensive metabolic view of known metabolites. This is an ongoing development.

MetaboLights consists of two distinct layers: a *repository*, enabling the metabolomics community to share findings, data and protocols for any form of metabolomics study, and a *reference layer* of curated knowledge about metabolites. MetaboLights is a database for metabolomics experiments and the associated metadata. It is the first comprehensive, cross-species, cross-platform/technique database which combines curated reference data of pure metabolites, curated information about their occurrence and concentration in species, organs, tissues and cell types under various conditions with data characterizing the experiment which lead to these findings and allows ready cross-referencing between experiments. Protocols documenting how metabolomics experiments were conducted are also available.

The MetaboLights Reference Layer is not intended to replace specialist resources but is specifically designed to build on prior art and extensively collaborate with the existing databases to ensure that data are exchanged and that assimilation efforts target gaps in worldwide available knowledge.

**THE USE OF MASS DEFECT FOR UNKNOWN BIOMARKERS RECOGNITION, CLASSIFICATION AND CHEMICAL FORMULA PREDICTION WITHIN LC-MS UNTARGETED EXPERIMENTS**

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Typical LC-MS-based untargeted metabolomic experiments follow this workflow: 1) spectral data collection using high-resolution mass spectrometers, after separation of the analytes by UHPLC; 2) features alignment, extraction and putative grouping using ad-hoc algorithms (XCMS, METalign and many others); 3) putative biomarkers identification through statistical softwares; 4) biomarkers identification, confirmation and semi-quantification by injection of standards of known compounds; 5) *unknown* biomarkers annotation through assignment of a putative formula via MS-libraries search, MS/MS and MS<sup>n</sup> data mining using softwares for mass spectral interpretation and successive manual curation. In particular, when dealing with plants extracts, the unknown compounds are >80% of the extracted signals. In the last decades many bio-informaticians improved the annotation of the *unknown* signals extracted from LC-MS untargeted experiments, but the chemical structure elucidation is still a complex and error-prone process. Data requires manual checking, identification and confirmation of the putative chemical formulas is time consuming and the MS/MS data are not always univoque or even available.

In this work, using the data coming from an untargeted metabolomic experiment on grapes, we employed a further step of systematic data visualisation in order to classify the *unknown* biomarkers signal using the *mass defect ratio* (MDR) and the *retention time* (RT) to create a 2D plot. The advantage of the plot is that it gives information about the chemical nature of the *unknown* compound; in facts, these parameters are affected from the chemical formula (both MDR and RT) and the chemical structure (RT). This plot allows a clear separation of the different *unknown* biomarkers. When the appropriate reference standards of the main classes are inserted, the MDR\*RT plot gives useful information about the putative classes of the biomarkers, helping in excluding improbable formulas and structures. This MDR\*RT plot, in combination with the latest rules for formula generation (seven golden rules, isotopic mass defect and others) allows to obtain a restricted pool of suggested formulas, ranking the correct one in the first positions and suggesting the chemical class of the compound. The joint use of online chemical databases and MS/MS data gives a satisfactory tentative identification of the experimental *unknown* biomarkers.

## EVALUATING THE PREDICTION RELIABILITY OF COMMERCIALY AVAILABLE SOFTWARE TOOLS DEVELOPED FOR TANDEM MASS SPECTRAL FRAGMENTATION SIMULATION

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Tandem mass spectrometry (MS/MS) represents one central technique for compound identification in metabolomics. Fragmentation data from MS/MS experiments enable the reduction of the number of possible metabolites to a single structure. Fast and automated identification can be accomplished by library search. However, the identification of a compound in the absence of the respective reference spectra constitutes a challenging analytical task. Approaches for the simulation of the MS/MS fingerprint and identification of the potential structure are gaining more and more interest.

ACD/MS Fragmenter (ACD/Labs) and Mass Frontier (MF, HighChem) are commercially available tools that generate fragments based on cleavage rules known from the literature.

This study evaluates the prediction reliability of these software tools by querying "Wiley Registry of Tandem Mass Spectral Data, MSforID" library (WRTMD, John Wiley and Sons) against the simulated spectra of 600 compounds (435 in positive mode, 165 in negative mode). WRTMD, at the current stage of development, contains 12,122 high resolution MS/MS spectra of 1,208 compounds involved in pharmaceuticals, toxicology, and forensics.

Spectra simulated by ACD/MS Fragmenter and MF returned the correct compound in the highest ranking order at a percentage of 42% and 49%, respectively, in positive ionisation mode. In negative mode the respective percentages were 45% and 59%.

In a second set of experiments, the two sets of simulated spectra were used to establish MS/MS libraries and the experimental spectra extracted from WRTMD were used as input. Correct identifications were returned at 74% (+ve mode) and 62% (-ve mode) of cases and at 79% (+ve mode) and 50% (-ve mode) for ACD/Fragmenter and MF, respectively.

The obtained results suggest that the software tools tested are convenient for creating simulated MS/MS data. Simulated spectra usually represent specific identifiers of underlying chemical structures. The high number of correct positive identifications obtained by using simulated data as library spectra is furthermore a clear indicator that computational spectra can be used to generate mass spectral libraries. This strategy may overcome the current limitations of available libraries in terms of compound coverage which may streamline metabolomics workflows.

**FUTURE OF METABOLOMICS STANDARDS; ROLE OF COSMOS**

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An EU coordination action for developing metabolomics standards with a worldwide participation, called COordination of Standards in MetabOlogicS - COSMOS (<http://cosmos-fp7.eu>), was launched in October 2012. EBML-EBI is coordinating this consortium of 14 European partners, with the MetaboLights metabolomics repository (<http://www.ebi.ac.uk/metabolights/>) playing a central role. A key aspect of this effort is to develop efficient policies ensuring that metabolomics data is encoded in open standards, tagged with a community-agreed and complete set of metadata, supported by communally developed standards. We aim to support open source data management, dissemination of metabolomics results via open-access databases, while adhering to existing standards and encourage support by vendors and publishers. COSMOS will interface with the existing resources in other biomedical life science e-infrastructures, such as BBMRI, Elixir, EU-Openscreen, EuroBioimaging and INSTRUMENT. Our aim is to deliver the exchange formats and terminological artifacts needed to describe, exchange and query metabolomics experiments, in addition to using the ISA-Tab (<http://isatab.sourceforge.net/>) as a core for capturing the description of experiments (contextual metadata) and build additional 'layers' for the data matrices. We wish to ensure that the proposed standards are widely accepted by involving major global players in the development process. In addition we aim to develop and maintain exchange formats for raw data and processed information (identification, quantification), building on experience from standards developed within the Metabolomics Standards Initiative (MSI) and Proteomics Standards Initiative, (PSI). Additionally we are planning to collaborate on developing the missing open standard NMR Markup Language (nmrML) for capturing and disseminating Nuclear Magnetic Resonance spectroscopy data in metabolomics and GC-MS and LC-MS metabolomics results within an existing mzML file format. We aim to explore semantic web standards that facilitate linked open data (LOD) throughout the biomedical and life science realms, and demonstrate their use for metabolomics data. We would like to invite all interested parties to join this effort contributing to advancement of metabolomics standards.

## **FLEXIBILITY VERSUS STANDARDIZATION TRADE-OFF IN LC/MS DATA PRE-PROCESSING OF LIPIDOMICS EXPERIMENTS**

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The goal of a workflow for processing and analysis of LC-MS data is to standardize the procedure by which the results are generated, and to make it as user-friendly as possible. However, experience shows that the downside of standardization is often a loss of flexibility, or the ability to deal with exceptional occurrences.

The workflow for lipidomics datasets described here is based on the R-package XCMS [Smith et al., Anal. Chem. 2005, 87, 779] with some modifications and additional functionalities written in the R programming language. The scope of the pipeline ranges from improved peak finding to identification of phospholipids and isotope correction.

The peak finding step of the pipeline includes a noise peak filter and a modification which makes it possible to extract neighboring or partly overlapping peaks.

The identification step for each class of phospholipid (PL) which was present in the dataset makes use of the probability for each PL to occur and patterns in the peaks of consecutive chain lengths or number of double bonds.

For the isotope correction step, the R-package "Rdisop" was used, which calculates expected isotope percentages, based on natural atomic abundances [Boecker et al., Algorithmica 2007, 48(4), 413]. This, together with normalization of the intensities to the known quantities of IS added to each sample, enabled the quantification of PL abundance in the sample.

The output of the pipeline consists of a set of Excel files, generated at intermediate and final steps, with embedded EIC and box plots, for easy assessment of the quality of the peak groups. In addition, a set of close-up 2D plots of the peaks in m/z/RT space are automatically generated for each subsection of the dataset.

This new pipeline is at once practical enough to enable rapid and standardized processing of large datasets, and at the same time flexible enough to allow for differences in approach for different datasets.



## PROBABILISTIC ANNOTATION OF LC-MS BASED METABOLOMICS

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Liquid chromatography coupled to mass spectrometry has the broader sampling power for metabolome studies.

However, even with well established data acquisition and preprocessing steps, the subsequent compound annotation step is a complex task, where the researcher has to deal with an extensive list of possible compound to mass assignments, usually hindering the inference of systemic changes on metabolism.

Here we present the adaptation and extension of a previous probabilistic method to provide further insights in the compound annotation process. We build on well established

preprocessing steps to maximize the utilization of information from those steps and create an extensive framework to model prior knowledge as information, resulting in an analysis flux that allow a higher quality annotation. We show the feasibility of this analysis implementing the R package probmetab. The probabilistic annotation stands as a very promising approach to allow researchers to investigate biological processes instead of be limited to a small set of peaks, an automated way to achieve the annotation is essential to help the experimenter to deal with the complex multidimensional data generated by mass spectrometry. Initial validation with data available at MetaboLights project shows that we can find meaningful sub-networks in an automated fashion.

**METABOLOMIC CHARACTERIZATION OF THE RELATIONSHIP BETWEEN THE  
HYPERTHERMOPHILIC *IGNICOCCUS HOSPITALIS* AND *NANOARCHAEUM EQUITANS***

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*Nanoarchaeum equitans*, the only cultured representative of Nanoarchaeota, is dependent on direct physical contact with its host, the hyperthermophilic Crenarchaeon *Ignicoccus hospitalis*, for survival. Both organisms have severely reduced genomes and together represent the simplest symbiotic association currently known. While genomic, transcriptomic, and proteomic studies have been undertaken to understand this symbiosis, the molecules and mechanisms responsible for the interaction between the two organisms have yet to be identified. To address this, a metabolic analysis was undertaken for cultures of *I. hospitalis* alone and co-cultures of *I. hospitalis*-*N. equitans*. A combined approach using electrospray ionization mass spectrometry, gas chromatography mass spectrometry, and nuclear magnetic resonance was carried out to characterize the metabolite and lipid composition of the cultures. Over 1500 molecular features were detected in each profile, and the most abundant features were selected for further analysis. XCMS and CAMERA software were used in conjunction with the Metlin database to prioritize differentially expressed molecular features for verification of identity. This work represents the first untargeted metabolomics analysis of interaction between archaeal organisms, providing valuable information on their biology at the functional level.

## mzCloud: A KEY CONCEPTUAL SHIFT TO UNDERSTAND “WHO’S WHO” IN UNTARGETED METABOLOMICS

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Untargeted metabolomics, in combination with novel mass spectrometric techniques, allow the detection of thousands of low-concentration compounds that can provide important insights into physiologically relevant processes if the identity of the relevant components is known. However, the existing spectral libraries only cover a small fraction of the compounds found in biological samples and even so, inherent spectra reproducibility problems in high resolution LC/MS<sup>n</sup> experiments hinder identification further. To address the obstacles present in many areas of metabolomics, a key conceptual shift in spectra library technology has been devised to allow the determination of structural information of unknown components.

If an unknown compound is not represented in the traditional library, it cannot be identified by this method. Using mzCloud, partial structural information can be derived, even if the reference spectrum of the unknown is not present in the library. Taking advantage of the structural continuum and the structural conservation of eukaryotic metabolism, all possible product ion spectra of the various MS<sup>n</sup> stages of the unknown are matched against substructurally characterized product ion spectra in mzCloud library. To determine the correct identity of the fragment structures needed for the substructural characterization of precursor ions, all fragments in the mzCloud database are being predicted using advanced heuristic and a broad range of quantum chemical methods.

To harness the spectra reproducibility problem and to control the spectra matching times of rich high resolution/accuracy mass spectra, each original MS<sup>n</sup> scan is additionally represented as a filtered and recalibrated spectrum for each used collision energy separately. Spectra are filtered using novel chemically intelligent peak removal algorithms. In order to accommodate numerous methods and immensely large and heterogeneous data collections, mzCloud uses advanced database technology. Since it is impossible for a single or a few research groups to acquire the substructural fingerprints represented as spectral trees that would cover a significant portion of biologically relevant structural space, contributors from various metabolomics or natural compound fields are welcome to contribute to the freely accessible mzCloud library.

**AUTOMATED OPERATION OF MASS FRONTIER TO CONSTRUCT IN-SILICO MASS SPECTRAL MS<sup>N</sup> LIBRARIES**

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Currently the identification of small molecule metabolites observed in untargeted metabolomics studies represents a significant bottleneck in the discovery of new biochemical knowledge. In many cases multiple empirical formulae and/or putative structures are reported for a single observed metabolic feature (i.e.  $m/z$  value). Multi-stage (MS<sup>n</sup>) mass spectrometry, which is a technique to collect in-depth fragmentation data related to the metabolite's structure, is often applied to increase accuracy and specificity in metabolite annotation. However, experimental MS<sup>n</sup> mass spectral libraries currently do not adequately cover the search space for all metabolites. The generation of in-silico MS<sup>n</sup> libraries would improve the specificity of annotation of metabolites detected in metabolomics studies, in particular when experimental MS<sup>n</sup> data of authentic standards is unavailable.

Mass Frontier (HighChem) software can predict fragmentation patterns of metabolites based on an extensive set of fragmentation rules and/or mechanisms manually derived from the literature. However, this software cannot readily perform batch processing on the scale required to generate comprehensive in-silico libraries for thousands of metabolites. Here we present freely-available software to automatically control Mass Frontier to enable the generation of an MS<sup>n</sup> in-silico fragmentation library applying a high-throughput strategy. The overall pipeline includes three modules: (1) automated retrieval of chemical structures from a chemical or metabolite library, such as ChemSpider, Kyoto Encyclopedia of Genes and Genomes, or PubChem, where retrieval of compounds is based on accurate mass, empirical formula, name or identifier; (2) high-throughput multi-stage in-silico fragmentation by Mass Frontier using visual scripting (visual scripting allows the use of images to identify and control GUI components); and (3) an organizer to collect and visualise the output. For example, 2D images of all the fragments, list of exact masses and in-silico derived fragmentation mechanisms. The automation software has been validated against several groups of chemical compounds, including a top 200 list of the most purchased/prescribed drugs and a list of metabolites that occur in the phenylalanine metabolism KEGG pathway.

## A MULTIVARIATE APPROACH TO REVEAL BIOMARKER SIGNATURES IN INSTRUMENTAL PROFILES OF BODY FLUIDS

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A general recipe for interpretation and for revealing patterns of predictive biomarkers in multicomponent profiles is presented. The instrumental profiles are modeled by latent variables using partial least squares (PLS) regression. Target projection (TP) is subsequently performed to condense the features related to each y-variable onto a single latent variable. The ratio between the variance explained on the target projected latent variable and the residual variance, i.e. selectivity ratio (SR), provides a ranking of the explanatory variables according to their ability to predict the investigated y-variable. Biomarker candidates are thus easily identified. A probability measure is calculated for each explanatory variable and can be used to assess the reliability of each biomarker candidate.

Our approach thus consists of the following steps: i) Instrumental profiling of samples, ii) PLS regression/PLS discriminant analysis (PLS-DA) using repeated double cross validation together with other validation techniques, iii) Finding a single predictive latent-variable component using TP, and, iv) Construction of SR plot with flexible boundary calculated from Wilcoxon rank sum concept for selection of biomarker candidates.

The approach will be illustrated on proton NMR profiles of serum samples. Applications done in multidisciplinary collaboration with health personnel, physicians, analytical chemists, and data analysts will be shown: Changes in lipoprotein patterns in obese individuals undergoing lifestyle interventions or surgery, and, connection between lipoprotein patterns and age, weight, physical state etc. in normal individuals.

**IMPROVING QUANTITATIVE MASS SPECTROMETRY BASED METABOLOMICS**

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Determining whether the metabolome of a biological system is significantly different implies that analytical variation is limited and controlled so consecutive statistical (data) analysis “only” has to focus on the real biological variation of interest. In order to quantify the analytical variation two crucial steps in the whole metabolomics workflow can be considered; the measurement design and data pre-processing. The measurement design is of absolute importance, especially when performing untargeted metabolomics. A correct placement of replicate samples, pooled quality control (QC) samples and/or inclusion of blank samples in the whole measurement scheme are of invaluable importance and favor any subsequent data pre-processing and analysis. We demonstrate that effective use of (pooled) quality control samples allows us to select the best internal standard that can be used to correct for all sorts of unwanted analytical variation. For a larger timeframe we demonstrate that these same samples can also be used to correct for within and between analytical batch variations. To extend the timeframe even further we show that including a sub-set of samples from a previous study (transfer samples) allows quantitative integration of metabolomics data from different studies. The quality of the fusion of studies however depends heavily on the data pre-processing step which is often left untouched: feature extraction and integration. The variation in the processing of current data processing software often results in unpredictable missing values, incorrect peak assignments or integration mistakes, forces the researcher to manually curate and verify their results, or use data of lower data quality. If the search is for known peaks only the integration results can be validated by eye and manually adjusted if necessary which is a time consuming and expensive process. If the approach, however, was untargeted data processing the researcher does not know what to look for and there are no integration parameters to optimize. Therefore, we developed an untargeted feature extraction and integration method that only has to be configured by a few parameters so that no expert knowledge is necessary. Both the data pre-processing and measurement design are important steps in the whole metabolomics workflow that, if properly handled, improve the way how high quality quantitative mass spectrometry based metabolomics data is obtained.

**BUILDING A POLYPHENOL METABOLITE ION DATABASE FOR HIGH MASS ACCURACY LC-MS METABOLOMICS EXPERIMENTS**

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Assigning identity of peaks identified to be significant in LC-MS experiments, even when they are obtained at high mass accuracy, is fraught with problems due to limitations of metabolomics libraries. In a recent experiment feeding grape seed extract to rats, the major ion detected in urine with  $m/z$  467.1184 was identified in METLIN as one of four different polyphenol glucosides when in fact it was (epi)catechin glucuronide. Further examination of METLIN even after it was supplemented by data from HMDB 3.0 revealed that it contained very few polyphenols or their bacterial metabolites. This is important since many lab chow diets contain copious amounts of the soy isoflavones, a class of polyphenols that are well absorbed into the systemic circulation and hence appear in copious amounts in urine. Polyphenols and their metabolites are also present in many foods consumed by humans, particularly from fruits and vegetables. The goal of the present study therefore was to generate a database of polyphenols and their phase I and phase II metabolites. Approach A useful source of chemical information about polyphenols is <http://www.phenol-explorer.eu/compounds>. The 516 curated polyphenols were downloaded into an Excel spreadsheet. Since the molecular weights were only given to one decimal place, the molecular formulae which were also provided were used to calculate the  $m/z$  values of the positive and negative ions of each polyphenol to four decimal places. The website also contained a downloadable table for metabolites of the polyphenols. However, visual inspection revealed that many metabolites were not included; in particular, there were no sulphated metabolites. Therefore, the empirical formulae of the aglycones of polyphenol metabolites were added to the Excel file. For each polyphenol and its metabolites, the empirical formulae (and hence accurate masses of their positive and negative ions) were determined for their glucuronide (+176.0321) and sulphate (+79.9568) phase II metabolites. These ion masses were combined with their names and chemical structures to create .SDF files and were added to metabolite databases.

**IMPACT ON HUMAN METABOLITE PROFILES OF METHODOLOGICAL DECISIONS RELATING TO THE ANALYSIS OF PLASMA AND URINE**

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Background: Longitudinal metabolomics screening approaches are frequently being used in biomarker discovery, yet no standardised and validated methods exist for analysis. **AIM:** To determine the impact on human metabolite profiles of methodological decisions relating to the analysis of plasma and urine.

Methods: 20 healthy males (mean  $\pm$  SD, age:  $26.5 \pm 5.0$  yr, body mass:  $74.8 \pm 7.7$  kg, height:  $179.5 \pm 5.4$  cm) received r-HuEPO injections of  $50 \text{ IU}\cdot\text{kg}^{-1}$  body mass every two days for 4 weeks. Serial blood and urine samples were obtained 2 weeks before, during rHuEPO and 4 weeks after administration. Plasma samples from 18 subjects (n=174) and urine samples from 19 subjects (n=181) were analyzed on an Accela HPLC coupled to an Exactive Orbitrap (Thermo Fisher Scientific) in positive and negative mode. Data were normalized and subjected to multivariate statistical analysis incorporating several principal factors which include 'date order', 'participant' as well as 'phase' effect.

Results: The LC-MS method utilized exhibited good selectivity and sensitivity given the untargeted approach selected for the identification of a potential trend in plasma and urine in response to the intervention. This resulted in the identification of some 400 unique metabolites in plasma and 600 in urine. Separation of each condition was achieved after careful modelling with principal component analysis. This adapted multivariate model confirms clustering between each experimental phase as well as a potential metabolic trend.

Conclusion: These preliminary results indicate great complexity within the metabolic profiles and several statistical approaches have not so far provided a clear cut picture of the effect of what is a major metabolic intervention although there are clearly correlations between groups of metabolically linked metabolites within individuals.



**VISUAL METABOLOMICS CORRELATION ANALYSIS: A NOVEL METHODOLOGY FOR ELUCIDATING CO-REGULATION AND METABOLIC PATHWAY ABERRATIONS IN RADIATION METABOLOMICS DATASETS**

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Metabolomics, the global study of small molecules, has the potential of providing unprecedented insight into the underlying metabolic pathways behind radiation injury phenotypes. The majority of methods, however, have only explored this through basic statistical analysis of changes in excretion levels of various metabolites in biofluids such as urine and serum. These methodologies focus on examining metabolites as independent biomarkers, without any consideration for the complex correlation structure that exists between them. In ignoring this fundamental property of metabolomic data, many biologically significant patterns and mechanisms can be overlooked. As such, a new methodology, called Visual Metabolomics Correlation Analysis (ViMCA), has been developed to specifically explore this correlation structure. ViMCA calculates and analyzes Pearson's product-moment correlation coefficients (PCC) between all ions in a metabolomic dataset, and visualizes the metabolome correlation structure via heatmaps. Through these heatmaps, significant metabolite co-regulation nodes can be identified and examined for changes when comparing heatmaps from different experimental sample groups. Furthermore, ViMCA conducts statistical analysis on PCC values to look for significant shifts in correlation structures when comparing data from two experimental sets. Significant correlation shifts provide strong evidence for altered co-regulation of metabolic pathways, and can be used to identify specific biological processes relevant to radiation exposure. Urine metabolomics data from a previous study, which involved exposing 8-10 week old C57Bl/6 male wild type mice to 8 Gy of gamma rays, was analyzed using ViMCA. Urine samples were collected for 24 hours post exposure via metabolic cages. Urine was also collected from non-irradiated mice for baseline comparisons. Metabolomics data were acquired by Ultra Performance Liquid Chromatography (UPLC) coupled to time-of-flight mass spectrometry (TOFMS), and pre-processed using MarkerLynx software (Waters). ViMCA was able to identify numerous metabolic pathways relevant to radiation damage, as well as a global breakdown of baseline correlation structures, which suggests widespread dysregulation of metabolic pathways as a result of exposure.

**STATISTICAL ANALYSIS OF GCXGC-TOFMS METABOLOMICS DATA TO INVESTIGATE TRAUMATIC BRAIN INJURY**

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Metabolomics studies are useful for gaining insight to biological systems and are often part of hypothesis-driven studies in which various sample classes are compared. The metabolome can be a good representation of the phenotype as many of the downstream effects of genome and proteome changes can often be observed through metabolite changes. In this study, we investigate the metabolic changes in a mouse model related to traumatic brain injury relative to a control group. Metabolites were extracted from plasma and derivatized for GC×GC-TOFMS analysis. With GC×GC-TOFMS metabolomics studies, a large amount of data is produced that can be challenging to interpret. This instrumentation effectively turns a complex sample into complex data that contains hundreds to thousands of analytes spanning a wide range of concentrations. The use of chemometric data processing techniques can often assist in drawing meaningful information and conclusions from these complex data. We present a data analysis approach with commercially available software that allowed for the characterization and comparison of these data and samples. LECO's Statistical Compare software feature provided alignment to compile peak tables across multiple samples and a Fisher Ratio based feature selection algorithm to determine class distinguishing analytes to gain insight to traumatic brain injury.

## **A CRITICAL REVIEW OF DATA ANALYSIS METHODS AND REPORTING IN METABOLOMICS DISEASE PREDICTION STUDIES**

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The future of metabolomics as a diagnostic tool is very promising especially for early pre-symptomatic stages of disease and multifactorial disease. The number of publications in this area has grown rapidly in the last 10 years. The success of metabolomics as a diagnostic tool depends on many factors, not least of which is the ability of reviewers to assess and compare the performance of different algorithms, pre-processing techniques and validation tools. This review seeks to examine the literature on one part of the metabolomics study workflow pipeline, namely the predictive algorithm step in human serum metabolomics studies, and to assess the reporting quality of this step, to discover the algorithms that are used, to report on whether a quality control step is implemented and whether a validation steps are employed correctly.

Unfortunately the results of this review showed that studies in this area are plagued by an inconsistent approach to reporting results, that many different predictive algorithm performance measurements are reported in different studies, that validation procedures are poorly employed, if at all, and that insufficient information is provided to enable replication of the analysis.

A unified approach to write up and presentation of results is urgently required, including at a minimum enough information for the data analysis step to be replicated on another dataset. Without the ability to initially assess the performance of predictive algorithms, to understand why and how they are applied to the data or the ability to review and compare different algorithms against eachother the field of metabolomics will not progress at the speed that is deserving of a technology of such enormous potential.

**P9-64**

**PILOT METABOLOME PROFILE STUDY OF IN INBORN ERROR OF METABOLISM WITH H<sup>1</sup> NMR**

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Inborn errors of metabolism are a rare group of genetic disorders that can produce serious clinical consequences if undiagnosed and untreated. The most common metabolic disease in Iran is Phenylketonuria (PKU). Tandem mass spectrometry (LC/MS) and nuclear magnetic resonance spectroscopy (NMR) are new in neonatal screening for inborn errors of metabolism. We study the metabolome profile of PKU patient and compare with that of normal with the help of H<sup>1</sup> NMR Spectroscopy.

**AN INTEGRATED NMR AND MS BASED COMPLEMENTARY METABONOMICS APPROACH FOR NON-INVASIVE DIAGNOSIS OF ENDOMETRIOSIS**

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Endometriosis, affecting almost 10% of women in their reproductive age, is defined as the proliferation of endometrial glands and stroma outside the uterus leading to chronic abdominal pain and infertility. The current gold standard method for the diagnosis of endometriosis is laparoscopy which is an invasive procedure. Though, extensive research has resulted in the identification of several serum markers for early diagnosis of this disorder, the diagnostic value of these biomarkers remains inconclusive. Moreover, diagnosis of the disease is difficult in the early stages (Stage I-II). To address this issue, an integrated LS-MS and NMR based approach was used to identify endometriosis biomarkers on a metabolomics platform. Metabolite profiling of serum samples of women with early stages of endometriosis (n=22) as compared to controls (n=23) was investigated. CPMG 1H NMR spectra was recorded using 700 MHz NMR with 512 scans, 16384 data points and with a spectral width of 14000 Hz. PLS-DA modeling of bins obtained from CPMG spectra of serum samples of endometriosis patients and controls discriminated endometriosis patients from controls with sensitivity and specificity levels of about 80% and 90%, respectively. On spectral assignment as many as 30 metabolites could be identified in the characteristic metabolite fingerprint of serum from endometriosis patients. Increased serum levels of lactate, 3-hydroxybutyrate, alanine, leucine, valine, threonine, lysine, glycerophosphatidylcholine, succinic acid and 2-hydroxybutyrate as well as decreased levels of lipids, glucose, isoleucine and arginine was seen in endometriosis patients as compared with controls. Five samples, each from the endometriosis and controls group were subjected to MS analysis. A spectral library of 100 metabolites were created using standards in AB-Sciex 4000 Q-Trap MS/MS system. Among these, as many as 89 metabolites were detected in serum of women with endometriosis. However, only 12 metabolites were consistently detected in all cases analyzed with MS. When compared with controls, five metabolites including 3-methylphenylacetic acid, shikimate, indoleacrylic acid, uridine, 1,3-diphosphoglycerate were found to be under-expressed whereas phenylpropionic acid was found to be over-expressed in women with endometriosis as compared with controls. These preliminary findings suggest that while NMR based metabolomics is highly reproducible, mass-based metabolomics is very sensitive. It is concluded that when used together, these two approaches may prove to be a complementary platform in determining novel biomarkers for endometriosis and other disorders. Further validation of both the techniques on a larger sample size is underway.

**NEW APPROACH FOR AUTOMATED XCMS PARAMETER OPTIMIZATION**

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Untargeted metabolomics based on LC/MS relies on automated data processing, such as peak detection, peak picking, retention-time correction or annotation. The software tools available for peak detection and picking have many parameters for adjusting algorithms to data set characteristics like MS resolution, chromatographic peak geometry or background noise. None produces the best possible results with just the default parameters. Hence, a structured automatable workflow for optimizing these parameters would be advantageous.

A workflow has been developed [1] that optimizes all parameters of the subprocedures consecutively, and has been tested in XCMS [2] using a diluted series of pooled samples. However, it is now usual to periodically analyze QC samples in metabolomics LC/MS studies. These QC samples contain equal aliquots of each sample or a representative study sample. Since diluted samples are often not measured, finding other work flows based on QC sample analysis would be beneficial. Our XCMS parameter optimization is based on a sequential design of experiments (DoE) approach. Six pooled serum samples measured by LC/MS within a small metabolomics study were used to develop this approach. Since we aimed to optimize all parameters at once, we selected 'centWave' for peak picking, 'obiwarp' for retention-time correction and 'density' for grouping algorithms. A screening experiment based on a Plackett-Burman-Plan was used to identify which parameters could be important in explaining the target variables (e.g. coefficient of variation, number of outliers and C13-isotopes). The following modeling step helped to further decrease the number of necessary experiments using a fractional factorial design. The data obtained in the modeling step was fitted by a multivariate optimization approach based on desirability functions. The optimization was started with a Central-Composite design (CCD), the data from which was used to estimate a response surface model to find the optimal settings for the XCMS parameters.

With this workflow we have fully optimized our target values. For coming optimizations we could skip the screening step which would make the workflow faster. Furthermore, we may need to change the weighting of target values in the desirability function. We also plan to test the workflow on other peak-picking, retention-time correction and grouping algorithms.

## QUANTILE REGRESSION-BASED DRIFT CORRECTION APPLIED TO METABOLOMICS DATA FROM HUMAN SERUM SAMPLES

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We aim to compose a workflow to identify and compensate for bias from various sources (sample collection and preparation, HILIC-FTMS analysis). The workflow comprises data filtering and drift correction. The statistical methods to be applied for signal drift correction depend primarily on the data structure, the size of the study, the number of QC samples, and technical specificities of the analytical method. QC samples are generally used for correcting metabolomics data [Kamleh et al., 2012, Dunn et al., 2011, Kirwan et al. 2013].

We present here our quantile regression approach. The main advantage of quantile regression over other regression techniques is its flexibility for modelling data with heterogeneous conditional distributions. As variability of features is high, smoothing by a locally adaptive regression technique is required to retrieve the maximum valuable information. The modular structure of the workflow allows for different perspectives concerning batches and overall study consideration. To optimize the workflow, data are investigated to determine the number of features affected by batch influence. This leads to the decision on whether steps should be applied batch-wise or batch-wise and study-wise. The filtering steps do not just exclude non-informative data; they also detect patterns related to systematic bias. Criteria to evaluate the success of the workflow are overall lower variation in QC samples, graphical representations of drift features and multivariate modelling based on batches as class variables to determine the degree of batch overlap. Batch separation before and after data preparation is compared through multivariate modelling approaches (PCA & Random Forrest).

In the present project, metabolite fingerprints of 106 human deproteinized serum samples from diabetes patients were generated using HILIC-FTMS (LTQ Orbitrap XL). Study samples were divided into 5 different analysis batches. Peak detection and peak grouping was performed using *xcms* (R). The workflow application resulted in a feature reduction of more than 50% (from 12000 initially detected features), lower variation over the QC pool samples, and no visible batch differentiation. To consider various feasible drift patterns, a systematic analysis of the behaviour of the quantile regression approach for such data is currently under investigation.

P10-1

**METABOLIC PROFILES AND FREE RADICAL SCAVENGING ACTIVITY OF *CORDYCEPS BASSIANA* FRUITING BODIES ACCORDING TO DEVELOPMENTAL STAGES**

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The metabolic profiles of *Cordyceps bassiana* according to fruit body developmental stages were investigated using gas chromatography-mass spectrometry. We were able to detect 62 metabolites, including 48 metabolites from 70% methanol extracts and 14 metabolites from 100% n-hexane extracts. These metabolites were classified alcohols, amino acids, organic acids, phosphoric acid, purine nucleosides and bases, sugars, saturated fatty acids, unsaturated fatty acids, and fatty amide. Significant changes in metabolite levels were found according to four developmental stages. Relative levels of amino acids, purine nucleosides, and sugars were higher in development stage 3 than the other stages. Among those, amino acids, such as valine, isoleucine, lysine, histidine, glutamine, and aspartic acid, which were associated with ABC transporters and aminoacyl-tRNA biosynthesis also showed higher levels in stage 3 samples. The free radical scavenging activities, which was significantly higher in stage 3 than the other stages, showed positive correlation with purine nucleoside metabolites such as adenosine, guanosine, and inosine. These results not only showed metabolic profiles but also suggested the metabolism associated with fruiting body development stages in cultivated *C. bassiana*. In addition, samples at stage 3 were proposed as good resources containing higher levels of metabolites related with free radical scavenging activities.



P10-2

**INVESTIGATION OF THE FACTORS CAUSING CESSATION OF 1-PROPANOL PRODUCTION BY MASS SPECTROMETRY-BASED METABOLIC PROFILING**

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1-propanol is currently used as a multi-purpose solvent in a variety of industrial products such as paint, cleaner and cosmetics. Previous work has demonstrated the production of 1-propanol in *Escherichia coli* from 2-ketobutyrate derived from threonine pathway and citramalate pathway. A high producing strain had been constructed based on biochemical knowledge, physiological insight and stoichiometric-based strategy. Metabolomics technology is a powerful tool for metabolic engineering because it enables us to perform comprehensive analysis of a wide range of metabolites that may lead to the identification of metabolic compounds that are important for specific biological questions. Therefore, we can observe the accumulation of metabolites that inhibit 1-propanol production or find out key metabolites that contribute to higher production. In this study, we conducted GC/MS and LC/MS –based metabolic profiling to investigate the factors causing the cessation of 1-propanol production. We cultivated *Escherichia coli* in fed-batch culture and monitor the metabolite changes that occurred. The results of metabolomics analysis revealed phosphoenolpyruvate (PEP) and pyruvate accumulation coincided with a decrease in glycolytic metabolites. This observation was in good agreement with feedback inhibition of glycolytic metabolites caused by PEP accumulation, which finally resulted in a dramatic decrease in glucose uptake rate when production has reached a plateau. The result of this work demonstrates the usefulness of metabolomics analysis to provide explanation of a complex biological phenomenon commonly observed in metabolically engineered strains.

P10-3

**METABOLIC PROFILING REVEALS COORDINATED REGULATION OF METABOLIC PATHWAYS OF *S. CEREVISIAE* IN A CHARDONNAY WINE FERMENTATION**

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The transformation of grape juice to wine is a complex metabolic relationship between two species, *V. vinifera* and *S. cerevisiae*. Grape juice, composed primarily of water, sugar, organic acids and additional secondary metabolites, provides nutrients for the yeast resulting in the production of wine, composed primarily of water, ethanol, glycerol, organic acids and additional components. The final molecular composition, developed from the grape/yeast relationship, contributes to the flavor, aroma and mouthfeel of the wine. In this study we examined this complex relationship by identifying the exo- and endo-metabolome at three timepoints of a Chardonnay wine fermentation. We identified 227 metabolites in the exometabolome and 404 metabolites in the endometabolome, each of which was placed into metabolic pathways or families. This work provides insight into the regulation of metabolic pathways, discriminated by the stage of fermentation. Considerable metabolic variation was seen at each timepoint allowing us to describe patterns of primary and secondary metabolism during fermentation. Our results suggest that the regulation of metabolic pathways is coupled to fermentation progress. These data provide an understanding of the differential utilization and production of primary and secondary metabolites during a wine fermentation. This work provides key understanding of cell communication mechanisms, metabolic engineering and industrial biotechnological processes.

**P10-4**

**OPTIMIZING SAMPLE PREPARATION FOR METABOLOMICS COVERAGE IN ROBUSTNESS IN YEAST**

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Yeast cells are important for biotechnology, molecular biology and biochemistry. While a range of metabolomic studies have already been conducted on yeast, new methods may increase metabolome coverage and improve robustness of analysis. Using a novel homogenizer to disrupt cell walls, the GenoGrinder, we tested four quenching solvents, and performed six extraction solvents on each quenched group, including a control (unquenched) group.

Data on primary metabolism were acquired using a Leco GC-TOF-MS and an Agilent GC-CI-QTOF MS whereas complex lipids were profiled using CSH-QTOF MS/MS. Peak finding and mass spectral deconvolution was conducted by the Leco ChromaTOF software and subsequent BinBase metabolite annotations for GCTOF MS, and using mzMine2 and Agilent MassHunter Quant for CSH-QTOF MS/MS data, using LipidBlast, Metlin and MassBank for compound annotations. Both cell pellets and supernatants were analysed to test for metabolite leakage during quenching periods. Apart from statistical analysis for robustness analysis, we also investigated biochemical coverage for presence or absence of metabolites that are prone to oxidation such as cysteine and vitamins, as well as comparing biochemically viable ratios metabolites related to various pathways such as the saturated and unsaturated membrane lipids, glycolysis, TCA cycle, pentose phosphate pathway and amino acids.

Results are presented with respect to method blank controls and we propose sample preparation conditions for these types of pathways and metabolomic methods that yield optimized metabolome coverage and yielded high quantitative robustness. These methods are ready to be implemented as standard operating procedures for the West Coast Metabolomics Center at UC Davis, a fee-for-service core facility.

**SAUVIGNON BLANC METABOLOMICS: IMPACT OF TRACES OF LIPIDS IN GRAPE JUICE ON THE DEVELOPMENT OF WINE VOLATILE THIOLS**

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New Zealand Sauvignon Blanc (SB) wine is well recognized worldwide mainly for its relatively high level of volatile thiols. Volatile thiols, such as 3-mercaptohexyl acetate (3MHA), 3-mercaptohexan-1-ol (3MH) and 4-mercapto-4-methylpentan-2-one (4MMP), are extremely odorous molecules, which contribute to passion fruit, grapefruit and box tree (cats pee) aromas respectively. However, the biosynthesis of these volatile thiols during fermentation is not well understood. It was previously indicated that concentrations of 3MH and 3MHA have a low correlation to their putative precursors in grape juice. Recent studies have shown that trace amounts of some non-polar metabolites (lipids) in grape juice can significantly influence the development of volatile thiols in the wine. Thus, we profiled all lipid traces in 250 SB juices from three different harvest seasons. We observed a large diversity of lipid molecules both identified and unknown in grape juice samples that has not been reported before. Concentrations of identified and unknown lipids were compared to the concentrations of thiols in the respective fermented wines through Pearson and Spearman correlation analyses. The correlation results revealed a series of non-polar metabolites in grape juice with both strong positive and negative correlation to volatile thiols in wines. The correlation information will be used to carry out juice manipulation experiments to validate the metabolomics hypotheses using laboratory-scale wine fermentations.

**ASSESSMENT OF SAMPLING STRATEGY FOR METABOLOME ANALYSIS OF LACTOBACILLUS CASEI 431®**

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Microbial metabolomics has added value to a range of microbial research areas, such as cellular physiology and stress response determination. The reliability and suitability of sampling, sample preparation, data acquisition, data pre-processing and data analysis are prerequisites for a trustworthy biological interpretation. Sampling is especially critical in microbial metabolome analysis due to fast turn-over rates and small pool size of the intracellular metabolites of interest compared to the surrounding supernatant. Therefore, an instantaneous quenching of the metabolism is essential. One aim of quenching is for the intracellular metabolites to remain inside the cells. If metabolites were to leak from the cells, the intracellular levels will be severely underestimated and the metabolite fingerprint would be affected. The suitable quenching strategy clearly depends on the specific of interest. Therefore, we develop and validate a sampling strategy for quenching of *Lactobacillus paracasei* subsp. *paracasei* (*L. casei* 431®, Chr. Hansen) in MRS media to determine an appropriate method to approach global analysis of changes in the metabolite fingerprint during growth. *L. casei* 431® is grown in an anaerobic batch culture fermentor, and culture broth is quenched in the late-exponential phase by different quenching procedures: (1) 60 % (v/v) methanol, (2) 60 % (v/v) methanol buffered with HEPES and (3) 60 % (v/v) methanol buffered with ammonium bicarbonate. The quenching procedures will be assessed by evaluation of the ability to halt metabolic activity and preserve cell integrity. The efficiency of the inactivation of metabolic activity is evaluated by addition of C<sup>13</sup> labelled glucose to quenching solutions followed by inspection of changes in the labelling pattern of the metabolic intermediates. Leakage, as result of cell disruption, is estimated by the levels of selected primary metabolites in quenching- and washing solution as well as in cellular extract. Remaining viability of *L. casei* 431® followed quenching will be assessed by flow cytometry. The metabolite fingerprints are acquired using gas chromatography time of flight mass spectrometry (GC-TOF). Overall, it is hypothesized that the addition of buffer to the quenching solution will stabilize cells and thereby reduces metabolite leakage from the cells.

P10-7

**QUANTITATIVE DETERMINATION OF NADP<sup>+</sup> AND NADPH USING LC-MS: WORKFLOW OPTIMIZATION IN METABOLOMICS**

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Nicotinamide adenine dinucleotide phosphate (NADP) is a cofactor and redox agent involved in many anabolic reactions in all cell types and organisms. The ratio of the reduced form (NADPH) and the oxidized form (NADP<sup>+</sup>) contributes to the overall redox state of the cell that is important for many cellular functions and cell viability. An elucidation of the metabolic role of such cofactors is important for metabolic engineering and helps the refinement of metabolic models. However, for NADP<sup>+</sup> and NADPH there are currently only estimates of the intracellular levels since the accurate determination is hindered by methodological problems. Enzymatic assays are available but lack robustness and are of limited use for complex samples like cell extracts. The most serious obstacle to the quantitative analysis is the instability of NADPH under various conditions that demands for an elaborate evaluation and optimization of the sample preparation process. The use of isotopically labeled internal standards in mass spectrometry allows not only the monitoring of the sample content along the analytical workflow but also permits correcting for any alterations of the original sample content arising from the sample preparation procedure, thus making the workflow suitable for quantitative studies.

For the first time, a complete analytical workflow for the quantification of the redox cofactors NADP<sup>+</sup> and NADPH, including a thorough evaluation and validation of the process employing isotopically labeled internal standards is reported. Applied in a metabolic study in yeast, this analytical workflow using hot extraction and LC-MS/MS analysis was found appropriate and delivered data fit for biological interpretation.

**COMPARATIVE METABOLOME ANALYSIS OF JAPANESE FRESH AND AGED SAKE REVEALS THE DIFFERENCE OF ORGANIC ACID CONCENTRATIONS**

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Japanese sake, so-called rice wine, is a popular alcoholic beverage in Japan that is made of brewing rice as an ingredient and is made by performing unique "parallel brewing" with special yeasts, *Saccharomyces cerevisiae* and *Aspergillus oryzae*. Through the process of brewing, many variables such as the selection of *A. oryzae* and *S. cerevisiae* strains, quality of water and rice, and the brewing environment critically effect on the quality of final product. However, the molecular components of Japanese sake have barely been understood due to lack of appropriate measurement devices. Currently, advancement in methods such as capillary electrophoresis mass spectrometry (CE-MS) is driving the analysis for research on Japanese sake. Therefore, the purpose of this study is to explore the metabolite profile of Japanese sake, and find out the metabolite differences between Japanese fresh sake and aged sake which is well known as long-term fermented sake. CE-MS based metabolome analysis on 98 fresh sake and 19 aged sake samples revealed the increase of organic acid concentrations such as succinate and malate in the aged sake as compared to the fresh sake. In addition, the type of brewing rice had little effect on the composition of the fresh sake, but had bigger impact on that of the aged ones, suggesting that brewing time and rice type should be important for the quality of aged sake. Further metabolome analysis on the aged sake showed that organic acid and amino acid concentrations were different based on the type of brewing rice. These results indicate the increment in organic acid concentrations may relate to the maturation of Japanese sake.

P10-9

## DERIVATIZATION PROCEDURES FOR MICROBIAL METABOLIC FOOT PRINTING BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Metabolic profiling attempts to qualify and quantify as many small metabolites as possible in order to gain insight in the functioning of biological systems. GC-MS analysis covers a broad range of metabolites in microbial foot printing and is mostly used for strain screening, process development, and application testing in Chr. Hansen. However, the reliability of the analytical data strongly depends on an efficient and robust derivatization of all compounds of interest.

Silylation is one of the most used derivatization approaches in metabolic profiling<sup>1</sup> because it provides derivatization of all relevant functional groups. However derivatization efficiency is poor for some groups, as e.g. amino groups. Furthermore, excessive silylation agent causes fast degradation of GC liner and column.

A silylation procedure was optimized focusing on a compound composition representative for fermentation samples. Samples contained high amounts of glucose, lactic acid in MRS medium representing a common fermentation matrix. Derivatization efficiency and robustness was tested for organic acids, sugars, amino acids, vitamins, and nucleobases comprising a broad variety of structural patterns. Finally, the silylation procedure was compared to the well-established derivatization protocol using Methyl chloroformate. Throughout optimization the effect on liner and column performance were monitored by the breakdown of DTT/Endrin and a mix of compounds developed by Grob *et al.*<sup>2</sup>

<sup>1</sup>Koek *et al.*, *Metabolomics* (2011) 7:307-328.

<sup>2</sup>Grob *et al.*, *J. Chromatogr.* (1978) 156:1-20.



**ENHANCED PHASE II DETOXIFICATIONS CONTRIBUTES TO BENEFICIAL EFFECTS OF DIETARY RESTRICTION AS REVEALED BY MULTI-PLATFORM METABOLOMICS STUDIES**

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Dietary restriction (DR) has many beneficial effects, but the detailed metabolic mechanism remains largely unresolved. As diet is essentially related to metabolism, we investigated the metabolite profiles of urines from control and DR animals using NMR and LC-MS metabolomic approaches. Multivariate analysis presented distinctive metabolic profiles and marker signals from glucuronide and glycine conjugations pathways in the DR group. Broad profiling of the urine phase II metabolites with neutral loss scanning showed that glucuronide and glycine conjugation metabolites are generally higher in the DR group. The up-regulation of the phase II detoxifications in the DR group were confirmed by mRNA and protein expression levels of the UGT and GLYAT in actual liver tissues. Histopathology and serum biochemistry showed that the DR had beneficial effects of low serum ALT level and low glycogen granules in liver. In addition, the Nrf-2 signaling pathway was shown to be up-regulated, providing a mechanistic clue for the enhanced phase II detoxification in liver tissue. Taken together, our metabolomic and biochemical studies provide a possible metabolic perspective in understanding the complex mechanism of the beneficial effects of DR.

P10-11

## CHARACTERISATION OF THE WINE METABOLOME: LINKING SENSORY ATTRIBUTES TO GENOTYPE

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The aroma of a wine is very important to the overall quality of a wine and research into the fermentation bouquet is of great interest in the industry today. This project aims to identify new genes responsible for the production of these important aroma compounds using an overexpression library (Jones *et. al.* 2008) to simplify the ambitious task.

The *Saccharomyces cerevisiae* overexpression library used in these experiments is encoded in a ~1500 clone *E-coli* plasmid library, in a 2 micron-based *LEU2* vector (Jones *et. al.* 2008). The only restriction with this library is the *Leu2* $\Delta$  mutation required in the transformant yeast strain and the need for a fermentation media lacking leucine. This overexpression library was extracted from the host *E. coli* and transformed into a wine strain *Leu2* $\Delta$  *S. cerevisiae* yeast to be used in the fermentation studies.

Micro-fermentations were performed in 1.8 mL volumes of chemically defined grape juice media lacking leucine (CDGJM-Leu). Over the course of five time-points, the samples were tested for sugar content and then frozen until aroma analysis. Over the trial period of 19 days, 51% of the ferments completed fermentation, with 33% these finishing before the parental strain. The most important aroma compounds were analysed in samples which completed fermentation using a newly developed headspace solid-phase microextraction coupled with gas chromatography mass spectrometry (HS-SPME GC-MS) method. This data was then analysed using statistical software to give rise to important clones which will be analysed in further experiments.

P11-1

**METABOLOMIC ASSESSMENT OF THE PROTECTIVE EFFECT OF CJ-1 IN HIGH FAT DIET-INDUCED HEPATOSTEATOSIS IN MICE**

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CJ-1, a naturally abundant plant phenolic compound in vegetables and fruits, has been shown to have potent anti-oxidative and anti-obesity activity. However, the effects of CJ-1 on nonalcoholic fatty liver disease (NAFLD) are poorly understood. In this study, we first investigated the beneficial effects of CJ-1 administration on nutritional hepatosteatosis model by a more “holistic view” approach, <sup>1</sup>H NMR-based metabolomics, to proof efficacy and to obtain information that lead to understanding the mode of CJ-1 action. The male C57BL/6 mice were placed for 16 weeks on a normal chow diet, a high fat diet (HFD, 60%), and a high fat diet supplemented with CJ-1 (50 and 100 mg/kg/day, orally). The liver histopathological and serum biochemical examinations indicated that daily CJ-1 administration protects against hepatic steatosis, obesity, hypercholesterolemia, and insulin resistance in HFD-induced NAFLD mice. In addition, partial least squares discriminant analysis scores plots demonstrated that the cluster of HFD feeding mice is clearly separated from that of normal group mice, indicating that the metabolic characteristics of these two groups are distinctively different, while CJ-1-treated mice are located close to normal group mice, indicating that HFD-induced disturbed metabolic profiles was reversed by CJ-1 treatment. Our results illustrate that the hepatoprotective effect of CJ-1 in part through reversing the HFD caused disturbed metabolic pathways, including lipid metabolism, glucose metabolism (glycolysis and gluconeogenesis), amino acids metabolism, choline and gut-microbiota-associated metabolism. Taken together, this study suggested that a <sup>1</sup>H NMR-based metabolomics approach can provide the platform for natural product therapeutic evaluation. The selected metabolites could probably be the potential therapeutic biomarkers for understanding of the effect of CJ-1 in hepatosteatosis animal model.

P11-2

**HUMAN STUDY FOR OBESE RELATED METABOLOMIC ANALYSIS WITH DIET INTERVENTION BY UPLC-QTOF-MS**

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The aim of the work is to identify the key metabolites related to weight reduction in humans by studying the metabolic profiles of serums obtained from thirty four participants who have undergone the dietary intervention of taking one packet of soybean peptides after every meal for 12 weeks. For longer than thousands years, Korean people has taken the fermented soybean products such as chungkukjang and doenjang, main products of which were the peptides. Soybean peptides are made as mimics of peptides from chungkukjang and doenjang using some enzymes which were involved in soybean fermentation. Firstly we investigated effects of soybean peptides on BMI, blood composition of lipid and etc. Serums of the research participants were analyzed using UPLC-QTOF-MS and the data obtained was analyzed using PLS-DA score plot. BMI and body fat percent of the test group were found to be reduced and eleven metabolites namely betaine, benzoic acid, pyroglutamic acid, pipercolic acid, N-phenylacetamide, uric acid, L-aspartyl-L-phenylalanine, and lysophosphatidyl cholines (lysoPCs) containing C18:1, C18:2, C20:1, and C20:4, showed significant increases, whereas twenty metabolites, including L-proline, valine, L-leucine/isooleucine, hypoxanthine, glutamine, L-methionine, phenylpyruvic acid, propionylcarnitine, butyrylcarnitine, L-hexanoylcarnitine, L-octanoylcarnitine, palmitylcarnitine, linoleylcarnitine, and lysoPCs containing C14:0, PC16:0, C15:0, C16:0, C17:1, C18:0, and C22:0 levels were significantly decreased during the research period. In particular, lysoPC 16:0 with a VIP value of 12.02 is esteemed to be the most important metabolite for evaluating the differences between the two serum samples. Our result confirmed weight losing effects of soybean peptides, accompanied by favorable changes in metabolites in the blood of the subjects. Therefore, this research enables us to better understand obesity and increased the predictability of the obesity risk by studying metabolites present in the blood.

P11-3

**METABOLIC PROFILING STUDY OF CARDIOVASCULAR DISEASE USING ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY/QUADRUPOLE TIME-OF-FLIGHT MASS SPECTROMETRY**

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Cardiovascular disease (CVD) refers any disease that affects the cardiovascular system such as angina pectoris and myocardial infarction and is the most prevalent cause of death in developed nations. Although there are a number of risk factors like age, gender, high blood pressure, high serum cholesterol levels, obesity, and renal failure, the mechanisms underlying CVD are still not fully understood. Moreover, the main obstacle to the treatment of CVD in clinical practice is the asymptomatic processes, that are associated with plaque formation, develop causing silent yet progressive tissue damage. In this study, we applied ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF MS) to investigate metabolic change in serum from healthy individuals and patients with cardiovascular disease (Angina pectoris and Myocardial infarction). The Partial least squares-Discriminant Analysis (PLS-DA) model was generated from metabolic profiles and the score plot showed the significant difference between groups. The serum of patients with cardiovascular disease was especially characterized by the decreased levels of tryptophan and acyl carnitine. These results suggest that global metabolite profiling of serum from healthy individuals and patients with cardiovascular disease using UPLC/Q-TOF MS could provide new phenotypic biomarkers for CVD diagnosis and treatment.

## LIPIDOMIC ANALYSIS OF HEART TISSUES FROM A HAMSTER MODEL FOR DILATED CARDIOMYOPATHY

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**Purpose:** Dilated cardiomyopathy (DCM) is a common cause of heart failure characterized by cardiac dilation and reduced left ventricular ejection fraction, but its underlying mechanisms are still unclear. The purpose of this study is to understand the mechanistic basis for cardiac dysfunction of DCM by a lipidomic approach.

**Methods:** Myocardial tissues from left ventricles were isolated from J2N-k cardiomyopathic hamsters at the age of 4 (DCM presymptomatic phase) and 16 (the symptomatic phase) weeks, and age-matched healthy controls, J2N-n. Their lipid metabolites were extracted and measured using liquid chromatography-mass spectrometry.

**Results:** Principal component analysis showed that the overall profiles of lipid metabolites were different between J2N-k and J2N-n even at the presymptomatic phase. At the presymptomatic phase, we observed increased levels of phosphatidylcholine (PC) containing unsaturated (especially polyunsaturated) fatty acids in J2N-k myocardial tissues compared with those from J2N-n. In addition, significant decreases in phosphatidylethanolamine (PE) and ethanolamine plasmalogen levels, especially containing linoleic acid (18:2) and/or eicosapentaenoic acid (20:5), were observed in J2N-k hamsters at 16 weeks. Thus, altered PC and PE levels and their composition of fatty acids were suggested to be related with DCM pathogenesis. The levels of major triacylglycerol species significantly decreased in J2N-k compared with J2N-n tissues at 16 weeks, suggesting lowered energy production from fatty acid oxidation, thereby leading to impaired cardiac function. Besides, increased levels of 4 eicosanoids, including prostaglandin (PG) E<sub>2</sub> and 6-keto-PGF<sub>1α</sub>, in the symptomatic phase suggested activation of the protective response pathways.

**Conclusion:** These results provide mechanistic insights into DCM pathogenesis and may help identify new targets for therapeutic intervention and diagnosis.

**METABOLOMICS APPROACH IN ACUTE PULMONARY HYPERTENSION DUE TO PULMONARY EMBOLISM IN AN ANIMAL MODEL**

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Acute pulmonary hypertension (PH) due to acute pulmonary embolization (PE) is associated with high mortality. Additionally, in some patients, pulmonary artery obstruction leads to the development of chronic PH, a cause of significant morbidity and mortality. The diagnosis of PE is challenging due to unspecific clinical symptoms and the lack of specific diagnostic blood tests. Prediction of chronic PH development is currently not possible. In our study we have used animal model (*Sus scrofa*) to evaluate alterations of metabolites in dextran microspheres embolism of the pulmonary circulation. Non-targeted metabolomics approach with LC-MS and GC-MS fingerprinting has been used for the differential analysis of profiles of pig plasma samples obtained before and 1 hour after PE. Both univariate and multivariate statistical analysis have been performed on previously pre-processed data in order to find endogenous compounds that have been responsible for classification between groups. We have found statistically significant differences in the profiles of plasma in compounds mainly involved in sugars, lipids and amino acids metabolism before and after PE. Our results have shown that metabolomics could be a powerful tool for the diagnosis of acute PE and potentially to understand the mechanisms that are responsible for the progression to chronic PH and the development of new therapeutic targets.

**A METABOLOMIC ANALYSIS OF HEART TISSUE IN A HAMSTER MODEL FOR DILATED CARDIOMYOPATHY USING CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY**

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Dilated cardiomyopathy (DCM) is characterized by cardiac dilation and reduced left ventricular ejection fraction, and it is a common cause of heart failure. Nevertheless, the underlying cause of DCM is still unclear.

In order to study the mechanism of DCM, we have used capillary electrophoresis coupled with time-of-flight mass spectrometry (CE-TOFMS) for comprehensive metabolic analysis of myocardial tissues in a hamster model of DCM. The tissues were obtained from left ventricles of J2N-k cardiomyopathic hamster at the age of 4 (presymptomatic phase) and 16 (symptomatic phase) weeks old, as well as age-matched healthy counterparts (J2N-n).

Among the 180 profiled metabolites, a total of 15 and 62 metabolites were significantly different ( $p < 0.01$ , Student's *t*-test) between J2N-n and J2N-k hamsters at the age of 4 and 16 weeks, respectively. Notably, decreased levels of intermediate metabolites in glycolysis, pentose phosphate pathway and TCA cycles were observed, suggesting a decreased energy production leading to cardiac contractile dysfunction in the J2N-k hamster. In addition, increased level of ophthalmic acid at 16 week-old J2N-k hamsters indicated the augmented oxidative stress. Furthermore, the ornithine/citrulline ratio, which indicates the activity of urea cycle, was also increased in J2N-k hamsters, suggesting a net decrease in the urea cycle activity in these animals.

The metabolic perturbation identified in the current study could provide an important insight for the identification of new targets for therapeutic intervention of DCM.



P11-7

## **METABOLITES FROM STRAWBERRY AND SEA BUCKTHORN BERRY DIFFER WITH RESPECT TO EXCRETION KINETICS**

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**Background:** While the prevalence of cardiovascular disease has been decreasing in the last decade, the incidence of type 2 diabetes is still rising at a global level. Berry consumption has been shown to improve CVD surrogate markers, the cluster of which partly defines the pre-diabetic state. In order to correctly assess the association between foods and their biological effect, reliable dietary assessment methods are required. The aim of this study is to identify exposure and possibly effect markers in urine after consumption of sugar-sweetened strawberry and sea buckthorn meals through LC-QTOF-MS metabolomics.

**Methods:** A cross-over meal study has been conducted in 16 overweight men. Each subject had a randomized sequence of the three intervention meals: strawberry, sea buckthorn berry and control. Urine samples were collected on each test day, at different time points (baseline, 0-1h, 1-2h, 2-24h). Principal component analysis has been used to explore the dietary exposures. Thereafter, partial least squares discriminant analysis (PLS-DA) has been applied to discriminate the most important features for the different test meals at each time point.

**Results:** Different urinary postprandial patterns have been observed after intake of the two berry meals. Strawberry exposure gave rise to early markers, with a peak in excretion at 1-2h. In contrast, sea buckthorn intake mainly gave discriminant features in the 2-21h and pooled 24h samples, suggesting longer-term markers. However, a few potential exposure markers for sea buckthorn were also found to peak before 2h.

**Conclusion:** Potential characteristic exposure markers have been distinguished in human urine after consumption of strawberries and sea buckthorn berries. The markers have clearly distinct time-sequences of excretion. We speculate that markers observed only later than 2h might originate from microbial metabolites. Identified markers will be presented.

## A METABOLOMICS APPROACH TO GESTATIONAL DIABETES MELLITUS THROUGH GLOBAL FINGERPRINTING

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Multiplatform metabolomics analysis gives an excellent opportunity to study pathophysiological mechanisms underlying the development of many diseases, provides information about molecules that can serve as unambiguous biomarkers and allow for early prediction of disorder. We applied LC-QTOF-MS, GC-Q-MS and CE-TOF-MS to identify the clinically relevant changes in circulating metabolites in the plasma and urine samples of women with Gestational Diabetes Mellitus (GDM), which nowadays becomes one of the most common metabolic abnormalities occurring during pregnancy. Plasma and urine of 20 pregnant women with GDM and 20 women with normal glucose tolerance (after 2-h 75-g OGTT) matched according to age and week of gestation were enrolled into the study. Liquid chromatography/mass spectrometry (LC-QTOF-MS; Agilent 6550), gas chromatography/mass spectrometry (GC-Q-MS; Agilent 7890A) for plasma and capillary electrophoresis/mass spectrometry (CE-TOF-MS; Agilent 6224) for urine analysis followed by matrix data alignment, data filtration as well as univariate and multivariate statistical analysis have been applied to the study. According to LC-MS/MS analysis we confirmed 147 statistically significant compounds (98 in positive and 49 in negative mode). By GC-Q-MS analysis we determined 12 relevant compounds and 6 were identified using CE-TOF-MS. Our data indicate for possible relationship of identified compounds with disturbance of many biochemical pathways related to development of insulin resistance and impaired glucose regulation in pregnancy.

**P11-9**

**METABOLIC DIFFERENCES OF INFLAMMATORY DISEASES IN A MURINE MODEL**

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Myocarditis is a cause of acute heart failure, sudden death, and chronic dilated cardiomyopathy. Comprehensive metabolic mechanism on myocarditis is still unclear. We explored the holistic metabolite perturbations in blood plasma of rats whose the inflammation is induced by inflammation adjuvant (CAF) alone and myosin together with CAF, through NMR-based metabolomic approach. Furthermore, the influence of cachexia observed in the inflammatory rats on their plasma metabolome was compared with that in food-restricted rat model.

P11-10

**THE SULT1A1 DETOXIFICATION PROFILE IN THE SOUTH AFRICAN TSWANA POPULATION GROUP INVOLVED IN THE PURE EPIDEMIOLOGICAL STUDY**

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The Prospective Urban Rural Epidemiology (PURE) study is a large-scale epidemiological study on individuals residing in 17 low-, middle-, and high-income countries around the world. Individual data collection included lifestyle and behaviour. Biological samples for biochemical and genetic analyses, focusing aspects on heart disease and diabetes were collected<sup>1</sup>. We investigated sulphotransferase detoxification as a biochemical parameter in this study.

Using PCR-RFLP method, we established the *SULT1A1* genotype and allele frequency in a Tswana population group of 1867 individuals from PURE study, and found the *SULT1A1\*1* and *SULT1A1\*2* alleles present at a frequency of 0.68 and 0.32, respectively. Using a quantitative multiplex PCR method we estimated the *SULT1A1* gene number of copies to be between one and five copies: 0.65% having a single copy and 60.14% with three or more copies<sup>2</sup>.

We subsequently applied a standard metabolomics approach to data selected from cardiovascular parameters of the PURE group. A complete natural separation in PCA between controls and the genetically well-defined PURE cases could not be achieved. However, blood triglycerides, cholesterol and high-density cholesterol (HDL-C) had the highest effect size values and are all related to lipid metabolism and diagnostic for defects associated with disturbances related to lipid metabolism. We could also establish the effect of the polymorphisms and copy-number on the sulphonation ability in the samples analysed.

<sup>1</sup> Teo et al, (2009) Examining the societal influences on chronic non-communicable diseases in low- middle- and high-income countries. *Amer. Heart Journal*, 158, 1-7.

<sup>2</sup> Mbongwa et al (2011) Single nucleotide and copy number polymorphisms of the *SULT1A1* gene in a South African Tswana population group. *S Afr J Sci.* 107, 395-401

P11-11

## IMPACT OF METFORMIN ON HepG2, Hepa1-6 AND DIFFERENTIATED 3T3-L1 CELLS UNDER GLUCOSE AND GALACTOSE CONDITIONS

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Type 2 diabetes (T2D) is a highly complex, multifactorial metabolic disease [1] with impact on multiple organs and their metabolite profile [2]. The disorder is frequently treated with the anti-hyperglycemic drug metformin. This drug has been associated with a vast number of complex responses, its mode of action however, remains elusive [3]. To address the topic of action we focused on metabolite profile changes in human and murine cell lines (HepG2, Hepa1-6 and differentiated 3T3-L1) upon metformin treatment. We also looked at the influence of different nutrient conditions on the treatment by cultivating the cells either in glucose or in galactose medium. Applying targeted metabolomic analyses, we assessed 186 metabolites out of 5 metabolite classes (amino acids, acylcarnitines, biogenic amines, hexoses and phospho- and sphingolipids).

A strong effect of metformin on the metabolite profiles of all tested cells was revealed although not uniformly. The hepatoma cell lines HepG2 and Hepa1-6 showed a higher number of significantly regulated metabolites than the 3T3-L1 adipocytes. Acylcarnitine, sphingomyeline, phosphatidyl- and lyso-phosphatidylcholine levels displayed predominantly up-regulations. For amino acids we observed more complex responses: branched chain amino acids were up-regulated in HepG2 and 3T3-L1 but not in Hepa1-6 cells and the glucogenic amino acids asparagine, aspartic acid, and serine were significantly down-regulated, but only in HepG2 cells.

The metformin induced effects were additionally modulated by the kind of hexose used. In hepatoma cells (HepG2 and Hepa1-6) the changes in metabolite profiles were found to be more pronounced under galactose conditions, whereas with differentiated 3T3-L1 glucose had more impact.

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2. Bain JR, Stevens RD, Wenner BR, Ilkayeva O, Muoio DM, Newgard CB. Diabetes 2009, **58**:2429-2443.
3. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F. Clin. Sci. 2012, **122**:253-270.

**P11-12**

**NMR METABOLOMICS OF RODENT MODELS OF OBESITY AND LIPOATROPHY**

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Causes of both type 2 diabetes (T2DM) and severe lipotrophic diabetes are associated with insulin resistance. To its development contributes disturbed endocrine function of adipose tissue at obesity or its absence at lipodystrophy. Our project followed metabolic changes caused by insulin resistance resulting either from obesity or lipoatrophy. Several rodent models have been developed and used to investigate the pathophysiology of diabetes and its complications. An initial metabolic characterization of model under study is necessary due to a profound influence of strain background on the metabolome. We characterized 2 different models using NMR urine fingerprinting: Mouse model of diet-induced obesity (DIO) and transgenic model of A-ZIP lipoatrophic mice. Our results indicated the satisfactory distribution according to different diet or genetic background. High-fat and standard diet in DIO model was distinguished mostly due to significant changes on methylamine metabolism pathway.

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**EFFECTS OF PREBIOTICS ON INSULIN RESISTANCE AND THE GUT MICROBIOME METABOLISM: A NON-TARGETED METABOLOMICS STUDY**

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Recent evidence has shown, that many factors (e.g. pre-/probiotics, lifestyle) can modify the gut microbiome and may therefore also influence host metabolism and disease susceptibility (e.g. type 2 diabetes). A study was carried out to assess the impact of differently digestible carbohydrates (prebiotics) on the gut microbial function and its complex metabolic interactions and to evaluate the effect on the insulin resistance (IR) using a non-targeted metabolomics approach.

We focus in this presentation on the metabolomics methods used in the study. For a comprehensive overview of the host and the gut microbial metabolites, fecal samples of IR individuals were collected at three time points and prepared for analysis with Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR-MS). This technique benefits from ultra-high mass accuracy (0.2 ppm) combined with ultra-high resolution and is well suited for e.g. lipid metabolism analysis. The analysis generated highly dimensional data, containing thousands of mass signals. At this stage, different pre-processing steps were performed to improve the quality of the complex data, including filtering and annotation using MassTRIX web server to assign masses to putative metabolites. Multivariate Data Analysis (MVA) showed a clear separation in the fecal metabolite composition due to the different diets. Supervised analyses (PLS/O-PLS) were performed to retrieve significant masses, which were responsible for the differentiation between each group. In order to show the importance of the gut microbiome on the host-metabolism the putative metabolites were assigned on KEGG metabolic pathways and classified to either host or bacterial derived metabolism.

We showed here, that our FT-ICR-MS based metabolomics approach is highly suitable for investigating both host and microbial metabolism. The chosen diet affected the gut microbiome metabolism. Now we will investigate possible beneficial effects of this metabolism change on the IR of the host. Further analysis of the same samples with NMR and LC/MS are under investigation to complement and verify our structural hypothesis.

P11-14

## IDENTIFYING NOVEL PATHWAYS OF ATHEROSCLEROSIS USING AN UNBIASED METABOLOMICS APPROACH UTILIZING MULTIPLE PLATFORMS

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Myocardial infarction, stroke, and heart failure are among the leading causes of death in the United States. Identifying novel pathways and biomarkers of atherosclerosis will allow for early detection and prevention of these adverse cardiovascular outcomes. Metabolomics analysis was performed on GeneBank patients undergoing elective cardiac evaluations (subset of N>10,000 patients) with over 400 clinical phenotypes and longitudinal outcome data. Primary metabolites such as amino acids, sugars, free fatty acids, aromatics, keto acids, and hydroxyl acids were profiled using gas chromatography time-of-flight mass spectrometry (GC-TOF-MS). UPLC/MS was applied as CSH-QTOF MS/MS for complex lipids as well as HILIC-QTOF-MS/MS for hydrophilic compounds such as acylcarnitines, betaine, carnitine, TMAO and choline.

Polar metabolites were extracted using 3:3:2 ratio of isopropanol / acetonitrile / water at -20°C. Subsequently, one aliquot was used for HILIC-TOF analysis, while the other one underwent two-step tri-methyl-silyl derivatization for GC-TOF analysis. Extraction of plasma lipids was carried out using a bi-phasic solvent system of cold methanol, methyl *tert*-butyl ether and water. For all analyses, a combination of internal and external standards was used for normalization and validation of results. Using these instrumental platforms, 50 µL of plasma were sufficient to maintain high analytical precision and repeatability for species identification.

Combining these three analytical approaches allows us to identify upwards of 500 compounds, with another 400-500 potential unknowns. Multiple data processing platforms were used for annotating compounds, as well as for statistical and pathway analysis. Interpretation of results will be presented with respect to implications for cardiovascular health risks.



## APPLICATION OF METABOLOMIC ANALYSIS TO A POPULATION-BASED, PROSPECTIVE COHORT STUDY: DESIGN AND SAMPLE PREPARATION METHOD OF THE 'TSURUOKA METABOLOMIC COHORT STUDY

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[Backgrounds and Aims] Omics approach is expected to shed light on the 'black-box' mechanism of various types of non-communicable diseases such as cancer, cardio-/cerebrovascular diseases, metabolic diseases as well as that of ageing. An epidemiologic study with high throughput technologies is of value for discovering potential biomarkers for health and disease in humans. In particular, metabolomics is relevant to profile metabolic phenotype and understand the consequences of gene-environment interaction, leading to identify early pathophysiological changes of chronic diseases and personalized preventive health care. Thus, we initiated a population-based metabolome-wide cohort study in Tsuruoka, Japan. Here, we report study design and sample preparation method.

[Study design] The study base is residents or workers in Tsuruoka city aged 35-74 at baseline. A 3-year baseline survey was started in 2012 and expected to recruit 10,000 subjects who participate yearly municipal/worksite health check-up program. Standard items of clinical chemistry are examined, and both plasma and urinary metabolome are analysed at Keio with CE-MS and LC-MS after 12-h fasting. For CE-MS, 115 polar metabolites were quantified. Detailed demographic and life style information as well as subjective health condition including depression is collected through a self-administered questionnaire. Food frequency questionnaire is also applied. Follow-up survey is planned for obtaining information on incidence of major diseases, medical records, and ADL (>age 65). Plasma and urine specimens will also be obtained repeatedly during follow-up period. The study protocol was approved by the Institutional Review Board of Keio University School of Medicine, and all participants provided written informed consent. During the first year of the baseline, in total 4278 participants were agreed.

[Sample preparation] Sample preparation must be not only precise but also feasible in a field setting of an epidemiological study. Serum and plasma metabolomes with CE-MS were well correlated ( $r=0.96$ ), but, in serum, some acidic amino acids concentration increased and methionine decreased over the time. Thus, plasma was chosen for blood metabolome analysis. Blood samples are kept at 4°C and preparation is done within 6h.

醗生アミノ酸(グルタミン酸、アスパラギン酸)が増加、メチオニンが減少した。一方血漿では、氷冷下6時間まで各物質濃度は安定していた。また遠心分離後、除蛋白を開始するまでの時間経過(室温下)についても同様の結果であった。前処理済みサンプルでは凍結解凍反復によっても安定であったのに対し、前処理前のサンプルでは、凍結解凍反復によって、血清、血漿ともに醗生アミノ酸、メチオニン等が変化した。

【結論】本研究の検査の範囲では、血液検体でメタボローム解析を行う場合、4°Cで保管した血漿を、採血後6時間以内を目標に前処理し、測定まで80°Cで保管することが望ましい。引き続き、処理前血液検体長期保存や前処理後サンプル保管の安定性について検討する。

**PLASMA METABOLITES PROFILE AND LIVER DISEASES IN COMMUNITY-DWELLING ADULTS: TSURUOKA METABOLOMIC COHORT STUDY**

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**Purpose:** We started a cohort study in Tsuruoka City from 2012, and performed CE-MS metabolome profiling on the 2,136 plasma samples of Japanese. Our aim is to examine the metabolic status of healthy people and to identify early pathophysiological changes which can predict development of lifestyle-related diseases. In this paper, our special interest is to describe how daily ethanol intake affects plasma metabolome, and to determine the metabolic status associated with development of alcoholic and non-alcoholic hepatopathy.

**Methods:** **Subjects:** Subjects are 35 to 74 years-old residents in Tsuruoka City who enrolled into a longitudinal cohort study. 2,161 Japanese (average age: 63 years old) underwent medical examinations including blood and urine examinations, abdominal echography, and detailed questionnaire from April to July 2012, as a part of three-year baseline study.

**Metabolome profiling:** We performed metabolome profiling on 2136 fasting plasma samples and profiled 115 polar metabolites; amino acids, amines, carnitines, organic acids, purines and the others, by CE-MS in Institute for Advanced Bioscience, Keio University.

**Results and Discussion:** 74.3 % (755/1015) men and 25.4 % (282/1109) women drink more than once a week. Average amount of ethanol intake is 279.4 g/week in men and 85.8 g/week in women.

We examined the association between 115 metabolite concentrations and ethanol intake. We fitted O-PLS regression models and constructed a moderately valid model in men to predict ethanol intake ( $R^2=0.35$   $Q^2=0.26$ ). Arg, Gln, Glu, Lys and Thr especially had high loading scores. We also performed regression analysis with each metabolite concentration, age and BMI, these five amino acids significantly related to ethanol intake ( $P<0.001$ ).

To determine the metabolic status associated with  $\square$ -GTP increment by drinking, we fitted O-PLS models in the highest tertile of ethanol intake. Gln, Glu, and Thr had high loading scores in a valid model ( $R=0.71$ ,  $Q=0.52$ ) and significantly related to  $\square$ -GTP in regression models ( $P<0.0001$ ). These findings suggest that these metabolites have important roles in the pathogenesis of alcoholic hepatopathy and might serve as predictors. We also examine the contribution of these metabolites to development of non-alcoholic fatty liver disease.

**P11-17**

**SIGNATURE LIPID BIOMARKER ANALYSIS OF NON-ALCOHOLIC LIVER DISEASE**

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Nonalcoholic fatty-liver disease (NAFLD) is one of the most common liver disorders, but the understanding of its progression from steatosis to steatohepatitis (NASH) is limited. Our UPLC/MS-based platform allows the semi-quantitative determination of around 1000 lipids and 150 polar metabolites. We have used this platform to determine the sera metabolite profile of 467 biopsied individuals with normal liver histology or diagnosed with steatosis or NASH, obtaining a robust BMI-dependent lipidomic signature that reliably differentiates them.

P11-18

## PHARMACOMETABOLOMIC OF ASPIRIN REVEALS NEW INSIGHTS ABOUT EFFECTS OF AN OLD DRUG

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Despite aspirin's use for over a century, some mechanisms by which it exerts its multiple effects remain unclear. While aspirin is generally effective for primary and secondary prevention of cardiovascular disease, considerable variation in drug response exists. Here, we used pharmacometabolomics to establish new signatures of aspirin exposure and to identify metabolites implicated in response to aspirin antiplatelet therapy. We profiled serum samples from healthy subjects pre- and post-aspirin treatment (14 days, 81 mg/day) and established a strong signature of aspirin exposure on amine metabolites (15 compounds changed,  $p < 0.05$ ,  $q < 0.1$ ), suggesting systemic effects beyond cyclooxygenase inhibition. We correlated amine metabolite profiles to ex-vivo agonist-induced platelet aggregation, a widely used surrogate marker for aspirin response, to identify novel biomarkers that could aid in predicting those who do not respond well to treatment. In two independent populations, we identified metabolic pathways that correlated with poorer aspirin response. Individual metabolites from this pathway were investigated for follow-up functional studies using ex-vivo platelets and we confirmed that they influenced on-aspirin platelet aggregation and might help predict individuals that retain high on-aspirin platelet reactivity. By linking metabolite profiles to clinically relevant surrogate endpoints, this investigation illustrates that pharmacometabolomics can help identify new mechanisms and potential biomarkers of variation in drug response, a critical step towards personalizing drug therapy for cardiovascular disease.

P11-19

## MAPPING THE GENETIC DETERMINANTS OF TISSUE METABOTYPES PROFILED BY UPLC-MS IN CARDIOMETABOLIC DISEASES

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Cardiometabolic diseases (CMD) correspond to a cluster of conditions such as obesity, hypertension, insulin resistance leading altogether increasing the risk of developing type 2 diabetes and cardiovascular diseases, which represents a real healthcare challenge for the Western and developing world.

In order to define how genetic variation influences the metabolome and therefore to identify causal gene variants influencing metabolism leading to diabetes or hypertension, we profiled a panel of recombinant RIs rat lines derived from a genetic intercross between a normotensive BN-Lx and Spontaneously Hypertensive Rat (SHR) strains. The metabolome of ten tissues (incl. heart, aorta, soleus, adipose (peri and brown), brain, liver, spleen, adrenals, kidney), biofluids (urine, plasma) and primary cell cultures (e.g., cardiomyocytes) will be characterized by untargeted <sup>1</sup>H NMR and UPLC-MS.

Sample extraction methods were optimised to accommodate for various tissue matrices (liver, heart, brain and aorta so far) prior to untargeted profiling by <sup>1</sup>H NMR and UPLC-Q-TOF MS, leading to a comprehensive analysis of lipids and polar metabolites in each tissue. Preliminary mapping of lipid and metabolic phenotypes (*i.e.*, “metabotypes”) obtained from liver and heart onto the rat genome has been conducted. The majority of UPLC-MS heart metabotypes are genetically determined, with heritabilities (genetic variance/total variance) ranging from 0.08 to 0.7. These heritable metabotypes are currently being mapped onto the rat genome to identify genetic polymorphisms influencing their concentration. Further structural identification of these metabotypes is also in progress.

Altogether, these results show for the first time that tissue metabotypes are genetically determined and detailed analysis of metabolic variation in various tissues should reveal the tissue-specific map of genetic polymorphisms affecting metabolism.

P11-20

## PROFILING PLASMA AND TISSUE METABOLOMES OF MOUSE MODELS IN DIFFERENT STATES OF DIABETES AND OBESITY

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The development of Type II diabetes is described by a range of developmental stages and conditions often starting with obesity and reversible insulin resistance, and ultimately leading to a catabolic state with pancreatic insufficiency. It is important to precisely determine the developmental disease stage of the patient to predict complications and to finetune treatment and medication.

By analyzing mouse models in different states of diabetes and obesity we aim to identify metabolites and metabolite groups that mark these different stages. By analyzing not only plasma but also different tissues (liver, muscle, kidney, adipose tissue), we try to determine the origins of the metabolite changes observed in plasma.

The following mouse models were used:

- the highly obese but rather diabetes-resistant *ob/ob* mouse model, representing a pre- stage of diabetes in which no hyperglycemia is seen but where steatosis of the liver can be observed
- the diabetes-prone *db/db* mouse model, both in an early stage with weight gain and hyperglycemia (20 weeks of age) and in a later stage where loss of weight is observed (28 weeks of age)
- a streptozotocin-induced diabetes model, characterized by a state of catabolism, seen in type 1 diabetes and in the final stage of type 2 diabetes

For standardization, all mice were fed the same chemically-defined high carbohydrate control diet. Metabolite levels were quantified via LC-MS/MS using Biocrates Absolute IDQ p180 kit measuring 186 metabolites in 5 compound classes (acylcarnitines, amino acids, biogenic amines, hexoses and phospho- and sphingolipids) and by a semi-quantitative GC-MS analysis that also determines numerous fatty acids, sugar-phosphates and organic acids. Additionally, amino acid levels were quantified via LC-MS/MS with aTRAQ labelling. Data analysis employs the software environment R.

P11-21

## STATE-OF-THE-ART LC-MS BASED LIPIDOMICS APPLIED IN THE STUDY OF LUNG DISEASES

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In recent years, the realization that lipids not only serve as building materials of membranes and as energy providers but are also involved in biological processes such as signaling, cell-cell interactions, etc. and, moreover, are linked to diseases such as diabetes, obesity, atherosclerosis, Alzheimer, etc., has led to the emergence of the discipline of lipidomics. Lipidomics aims at the comprehensive measurement of the lipids present in a cell, tissue, biological fluid, etc. and the concomitant detection of the lipid responses to various stimuli, e.g. disease, pharmaceutical treatment, genetic modification. This holistic approach, simultaneously measuring hundreds of compounds, is revolutionary since it allows to reveal differences between conditions without a priori knowledge.

The present contribution reports on the application of a powerful lipidomics method combining high resolution reversed-phase liquid chromatography (RPLC) with high resolution quadrupole time-of-flight mass spectrometry (Q-TOF) in the study of lung diseases. Induced lung sputum, although thought to be prone to dilution effects due to effectiveness of induction and individual variability, is shown to be a promising matrix for monitoring lung disease. Using our lipidomics platform, hundreds of lipids, including a vast amount of sphingolipids, could be accurately identified. It is demonstrated that the lung lipidome is significantly affected in patients suffering from chronic obstructive pulmonary disease (COPD).

**UNDERSTANDING THE ROLE OF METABOLITES IN HUMAN DISEASES – CLINICAL METABOLOMICS FROM A UNIVERSITY OF BIRMINGHAM PERSPECTIVE**

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Metabolomics is increasingly being applied as an integrative systems biology tool to investigate molecular processes related to human ageing and disease. The study of metabolites in mammalian samples (biofluids, cells, tissues) provides molecular and systems-level data on metabolism as well as other molecular processes (e.g. signalling and regulation). Clinical research groups at The University of Birmingham are applying metabolomics in laboratory and translational research to study a diverse range of diseases involving immunology and inflammation, endocrinology and cardiovascular systems as well as cancer and complications of pregnancy. Here three projects are described to highlight the diversity of clinical metabolomics research at The University of Birmingham.

1. Glucocorticoids (GCs) are a group of steroid hormones involved in many important biological functions in the human body including immune system suppression and the regulation of carbohydrate, lipid and protein metabolism. GCs have been shown to play important tissue-specific roles in insulin sensitivity/resistance, obesity and type 2 diabetes. Here, results from a UHPLC-MS untargeted serum metabolomics study of GC perturbations (e.g. diurnal variation) on endogenous metabolism will be discussed.

2. Twin-to-twin transfusion syndrome (TTTS) is a disease of the placenta that affects identical twin pregnancies who unequally share a common monochorionic placenta. Here, results from a study of amniotic fluid applying UHPLC-MS will be discussed which show metabolic differences related to treatment (pre- and post-laser treatment) and classification of symptoms and severity.

3. Fibroblast cells synthesize extracellular matrix and collagen and are the most common cell observed in connective tissues. We have studied the intracellular lipidome (applying UHPLC-MS) of fibroblast cells sampled from three different parts of the body (skin, synovial fluid, bone marrow). Clear differences in the lipidome are observed based on the site of sampling with glycerophosphocholines, glycerophosphoethanolamines, sphingolipids and acyl glycerides showing the greatest number of metabolic differences.



P11-23

**UNTARGETTED METABOLOMICS OF THE STZ MODEL OF DIABETES IN MULTIPLE TISSUE TYPES: 1 GAS CHROMATOGRAPHY-MASS SPECTROMETRY**

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A commonly used animal model (rat) of diabetes uses Streptozocin to cause selective destruction of pancreatic beta-cells, resulting in insulin deficiency and hyperglycaemia. This model is known to mimic many of the acute and some of the chronic complications of human diabetes. In this work we present an untargeted metabolomic study of multiple tissue types, comparing control and STZ treated animals (n=7 per group). Using a Folch-style extraction protocol, tissue (including brain, heart, liver and kidney) from was extracted in 50:50 MeOH:CHCl<sub>3</sub> using a Tissuelyser, and the extracts separated into polar and non-polar phases by the addition of water. Polar fractions were dried, subjected to MOX/TMS derivatisation following a previously described protocol and analysed by GC-MS using a 30m DB-17 column, a 35 min analysis time, and a LECO Pegasus HT GC-MS-TOF. (Non-polar fractions were analysed by UPLC-MS, as reported separately at this conference.) A reference table comprising 170 metabolite features was constructed, and used to allow reporting of a common list of features for all tissue types studied.

In most tissue types, simple PCA visualisations yielded clear separations between the STZ and control classes. Interestingly, the magnitude of the measured “fold-changes” for individual metabolites varied markedly between tissue types. This may reflect variation in the mechanisms of metabolic regulation available to the various tissues.

**<sup>13</sup>C<sub>16</sub>-PALMITIC ACID METABOLIZATION INTO CERAMIDE FOR THE DETERMINATION OF CERAMIDE SYNTHESIS IN TYPE 2 DIABETES PATIENTS**

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Lean tissues' fatty acid overexposure is involved in the development of insulin-resistance and type 2 diabetes. Unfortunately, fatty acid distribution and metabolism throughout the body is not well understood and merits further investigation. Therefore, a research protocol was developed to study the metabolism of palmitic acid into ceramide (N-hexadecanoyl-sphingosine or C16:0-ceramide).

A heavy palmitic acid tracer (<sup>13</sup>C<sub>16</sub>) was administered i.v. (0.01 μmol/kg/min) to three groups of patients: the first cohort was offspring of two parents with type 2 diabetes (n=11), the second cohort consisted of type 2 diabetes patients (n=12) and the third group consisted of healthy control subjects (n=13). Blood specimens were collected at different time points from -30 min to 240 min after the tracer administration, as part of a longitudinal metabolic evaluation study. Plasma samples were used for the mass spectrometry analysis.

An ultra-performance liquid chromatography coupled to a tandem mass spectrometry (UPLC-MS/MS) method was devised to quantify plasma C16:0-ceramide and (<sup>13</sup>C<sub>16</sub>)-C16:0-ceramide levels. (D<sub>4</sub>)-C16:0-ceramide was used as the internal standard (IS) for the sample preparation and analysis. Three ceramide standards were synthesized in-house from sphingosine and palmitic acid (C16:0, (<sup>13</sup>C<sub>16</sub>)-C16:0, and (D<sub>4</sub>)-C16:0) with a purity higher than 95%. Two hundred microliters of each plasma specimen was mixed with 20 uL of IS (5 uM) and purified by liquid-liquid extraction with CHCl<sub>3</sub> followed by a mixed-mode cation exchange (MCX, Oasis, Waters Corp.) cartridge. All samples were analyzed on an Acquity UPLC® I-Class system (Waters Corp.) coupled to a Xevo® TQ-S (Waters) tandem mass spectrometer. Reverse phase chromatography was performed on a BEH C8 column (Waters) using MeOH/5mM ammonium formate/0.1% FA and H<sub>2</sub>O/5mM ammonium formate/0.1% FA as mobile phases. The analysis was performed by multiple reaction monitoring (MRM) in positive electrospray mode. The study revealed that (<sup>13</sup>C<sub>16</sub>)-(C16:0)-ceramide was detected after tracer perfusion in plasma samples from patients of the three cohorts and its concentration gradually increased until the end of the study (240 min). Further investigations are underway with larger cohorts using a 24h-longitudinal timeframe.

P11-25

## **UNTARGETTED METABOLOMICS OF THE STZ MODEL OF DIABETES IN MULTIPLE TISSUE TYPES: 2 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY**

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The STZ rat model is one of the most widely used models of Diabetes Mellitus (DM). In our study we have used non-targeted metabolomic profiling utilising UPLC MS/MS to investigate the metabolic differences between control (n=7) and STZ-induced diabetic rats (n=6). We have chosen to investigate metabolic changes at the tissue level and as such have analysed the metabolic profile of 7 different tissues.

All animal experiments were conducted in accordance with the UK Home Office regulations for the care and use of laboratory animals. Treatment of the rats with intraperitoneal STZ resulted in elevated plasma glucose levels consistent with diabetes (> 39 mmol/L) in comparison to control rat plasma glucose levels (< 10 mmol/L). All tissue was harvested at 11 weeks after STZ administration, whereby tissue was dissected, washed in PBS, snap frozen and stored at -80°C until analysis.

An in-house Folch-based tissue extraction method has been developed which is applicable to a range of tissue types. Extracted metabolites were analysed by Ultra Performance Liquid Chromatography coupled to a LTQ-Orbitrap mass spectrometry in both positive and negative ion modes.

The number of metabolite features detected from each tissue type was deemed highly successful with on average > 5000 features in positive ion mode and > 2500 features in negative ion mode. The variability in the data and associated quality control (QC) samples was assessed by applying principal components analysis (PCA). In all tissue types the variation associated with the QC samples was less than the variation associated with the samples. Univariate statistical analyses were performed to identify metabolite features whose relative concentrations were statistically different ( $p < 0.05$ ) between control and STZ.

We have successfully identified a number of metabolite features which discriminate between control and STZ rat tissue and also a number of metabolite features which indicate differential responses between tissue types.

**P11-26**

**THE INTERACTION OF DIET AND AGE TO INFLUENCE THE METABOLIC PHENOTYPE OF ADIPOSE TISSUE**

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There is increasing evidence implicating obesity as the main underlying cause of the metabolic syndrome, leading to major interest in the biology of adipose tissue and the effects of dysfunction. Epigenetics has recently gained popularity as an alternative perspective on the etiology of metabolic disorders as there is an underlying, inherited susceptibility to obesity. Epigenetics refers to heritable changes in gene expression caused by modifications other than changes in nucleotide sequence. The epigenetic state of an organism is highly responsive to the environment (including nutrition), providing a link between genetic and environmental factors, which together influence the phenotype of an organism. The influence of high-fat feeding on adipose tissue function at metabolic and epigenetic levels was assessed in white adipose tissue (WAT) samples from ob/ob and control C57B1/6J mice fed on a high-fat diet over a ten-month ageing study. A metabolomics-based approach was used to define and understand the metabolic changes that occur in diet-induced obesity through characterisation of lipid metabolism in WAT. As methylation of DNA is the most commonly occurring epigenetic modification, the epigenetic state of WAT was assessed by analysis of DNA methylation processes, and measurement of concentrations of metabolites from the one-carbon cycle. Lipid metabolism was significantly altered by high-fat feeding, and differences were primarily due to diet rather than genetic background. Investigation into the metabolic link between nutrition and epigenetics in relation to adipose tissue function is on-going, and may help further understanding of the relationship between changes in metabolism, age, obesity and insulin resistance in the future.

**METABOLIC AND MOLECULAR TRAJECTORIES REVEAL THE LOSS OF METABOLIC FLEXIBILITY IN THE EARLY PROCESSES OF FRUCTOSE-INDUCED INSULIN RESISTANCE**

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Insulin resistance (IR) is one of the main components of metabolic syndrome. However, the early phases of its development such as the first metabolic alterations preceding its installation remain unclear. In order to analyse the early processes over time from the gene to the metabolite levels, we have pair-fed two groups of rats with either a control (65% starch) or a high-fructose (65% fructose) diets for 45 days. The final phenotype of the fructose-fed group consisted in fasting hyperglycaemia, hyperinsulinemia, and IR at the muscle level. Several major changes were also noticed over time before the IR installation: at the molecular level, the liver expression profile (90 genes related to lipid and glucose metabolism and insulin signalling) was strongly modified at D5. This drift was quickly compensated and the differences were no longer visible between D12 and D30. Nevertheless, this phenotypic flexibility was limited over time, and after 45 days of fructose feeding, the observed profile was identical as at D5, suggesting a final drift of the molecular phenotype probably linked to IR installation. This hypothesis was further supported by the metabolic trajectory observed in urine metabolomics analysis of the fructose-fed rats, which was completely different from that of the control group. This trajectory, together with the identified metabolites between D30 and D45 (when the IR was installed) allowed us to better characterize the metabolic flexibility of the fructose-fed rats. Finally, at the metabolic level, the fructose-fed phenotype was characterized by a rapid (D5) increase in the lipogenic and gluconeogenic potentials (enzyme activities and gene expression). All together, the events preceding the fructose-induced IR were characterized by an early (D5) shift of the metabolic phenotype (at the molecular and metabolite levels) mainly reflected in a highly-induced hepatic lipogenesis. While the global phenotype partially recovered the initial profile in the middle of the trial (D12-30, concomitantly with hyperglycaemia), a final shift was observed at D45 when IR was fully installed. This final phenotype was characterized (despite fasting hyperinsulinemia) by uncontrolled beta-oxidation and gluconeogenesis and impaired insulin signalling.

P11-28

**HIGH THROUGHPUT IMPLEMENTATION OF REVERSED-PHASE UHPLC–CSH-QTOF MS/MS FOR COMPREHENSIVE ANALYSIS OF COMPLEX LIPIDS IN BLOOD PLASMA**

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Lipid analysis can be classified into two major groups: shotgun lipidomics performed by direct infusion or pre-separation chromatography, the latter allows for more robust identification and quantification of lipid species. Limited instrument dynamic range and sample quantities are also a challenges associated with lipid quantification. There is a need for development of new instrumental methods capable of carrying out quantitative, high-throughput lipidomic analyses in clinical studies. Here we demonstrate a truly high-throughput method for extraction, data collection, compound annotation, and quantitation of >400 lipid species. Lipids were extracted using biphasic solvent extraction by cold methanol, methyl tert-butyl ether and water. Lipids were separated using a Waters CSH C18 column with acetonitrile/isopropanol gradients with 15 min run-to-run cycle times. Mass spectra were acquired using QTOFMS/MS operated in ESI(+) and ESI(-). Lipid identification was completed using in-house software, LipidBlast with accurate mass search windows of 5 mDa or better. Lipids were quantified using complex lipid class-specific internal standards (n=15) in combination with Agilent MassHunter software. Experiments were performed and optimized to produce information-rich chromatograms with narrow chromatographic peaks (8-17s). This approach was used to successfully identify >400 lipid species including mono-, di-, and triacylglycerols, lyso- and diacylglycerophospholipids, sphingolipids, cholesterol esters, ceramides, and fatty acids. Individual lipid species were quantified based on a ratio response to their complex lipid class-specific odd chain or isotopically labelled surrogates. A linear range for quantification of 3.5 orders of magnitude was observed using both an Agilent 6550 and 6530 QTOF. Sample volumes as low as 5uL of plasma were shown to be sufficient to maintain high analytical precision and repeatability for species identification. This method is currently used on the type 1 diabetes TEDDY study with 12,000 subjects.

**LC-MS/MS WORKFLOW FOR NON-TARGETED LIPIDOMICS: VALIDATION AND APPLICATION TO VERY LONG-CHAIN ACYL-COA DEHYDROGENASE DEFICIENT (VLCADD) MICE**

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The goal of this study was to optimize and implement a workflow for non-targeted lipidomics of any biological samples using high-resolution HPLC-quadrupole-time-of-flight (QTOF). The non-targeted approach, which aims at dissecting metabolite species that differentiate two conditions (for e.g. disease or therapy), was selected because it is generally assumed to provide some advantages in biomarker or pathway discovery compared to a targeted approach. First, our entire workflow has been tested for its reproducibility and robustness using chemical standards and plasma samples (n=5 on 3 days). By collecting MS data in the *m/z* range of 150-1700 Da. in both the positive and negative modes, to ensure maximal coverage of all lipid classes, ~1500 features were detected in all 15 plasma samples with a median intensity CV <20%. Then, this workflow was used to test whether compared to controls, VLCADD mice exhibit plasma lipid alterations beyond LC-acylcarnitine accumulation, which is commonly assessed as a marker of this  $\beta$ -oxidation defect. This analysis was prompted by our recent finding of a significantly lower level of the omega-3 polyunsaturated fatty acid docosahexaenoic acid in cardiac phospholipids of VLCADD mice (Gélinas *et al. AJP* 301: H813, 2011). Plasma samples from VLCADD mice (12) and their littermate counterparts (13) fed a standard or high fat diet were analysed along with QC samples. After extraction and alignment of MS signals, statistical analysis of the dataset was achieved using Agilent Mass Profiler Pro (MPP) software and the following criteria:  $p < 0.05$  using an unpaired *t*-test with Benjamini-Hochberg correction and a fold change >2. The number of unique features that met these criteria was greater in the high fat (92) than the standard (8) diet. LC-acylcarnitines (for e.g. C18:2 & C18:3), which are amongst the lipid species that have been identified to date by tandem MS, have *p* values < 0.0001 and fold-changes >3 in VLCADD vs. control mice fed the high fat diet. These results demonstrate the capacity of our workflow to identify lipid species between two conditions. However, more importantly, our findings of additional unique and previously unreported lipid species (identification in progress) offers great promise of extending our understanding of the biological impact of VLCAD deficiency, which is associated with a high risk of sudden cardiac death in infants.

## EVALUATION OF T2 DIABETES DRUG TREATMENT RESPONSE BASED ON METABOLOME

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Background: Diabetes mellitus type 2 (T2D) is an enormous and increasing societal and economic burden. Insulin resistance in muscle and liver and beta-cell failure represent the core pathophysiological defects in T2D. Treatment focuses on lowering blood glucose levels by reducing glucose synthesis or increasing insulin secretion. Globally the first line pharmaceutical treatment is metformin (MET). If needed, mostly a sulphonylurea (SU) is added to metformin or a combination of metformin and/or SU and other drugs is applied at later stages. If physicians could predict the patient's responsiveness to treatment, a more efficient stepped approach could be established.

Aim: To explore the variability in response to 5-year treatment of 352 screen detected type 2 diabetes patients within the ADDITION – NL study with the goal of predicting the individual response to the medication.

Methods: A metabolomics approach was used to identify predictive markers in the metabolome of 352 T2D patients. GC-MS analysis of baseline plasma samples resulted in a dataset containing relative concentrations of 173 metabolites for each patient. Different univariate and multivariate statistical methods were used with the 5-year change in HbA1c as outcome.

Results: Patients with a larger dysregulation of glucose metabolism (high glucose and 1,5 anhydro-glucitol) were more prone to decrease in HbA1c status after 5 years, regardless of medication. Stratification of patients based on their medication revealed that in subjects treated with MET only several liver markers (2- and 3-hydroxybutanoic acid) at baseline were associated with HbA1c decrease at 5 years. None of these liver markers were statistically significant in the SU group; their associations with HbA1c decrease at 5 years were statistically significant in the MET and SU combination group. Free fatty acids, mainly derived from adipose tissue, (C14:0, C17:0, C18:0, C20:1) and oxidative stress related markers (mannose, xanthine and myo-inositol) were only correlated to 5-years HbA1c decrease in subjects treated with the MET and SU combination. This study shows that metabolomics can be used to gain valuable insight into mechanisms underlying the differential responsiveness to different medications in screen detected type 2 diabetes.



P12-1

**GLUTAMINE, LACTATE, AND GLUCOSE AS METABOLIC MARKERS FOR METABOLOMIC STUDY USING <sup>1</sup>H NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY**

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NMR is a versatile method to conduct metabolomic study. It has been used for decades for metabolic profiling of cells, tissues, organs, and human bodies utilizing various nuclei in metabolites. NMR-based metabolomic study will lead to discovery of new metabolic markers, therapeutic targets, and ultimately a more thorough understanding of diseases. A study was conducted to test new metabolites such as glutamine, lactate, and glucose for metabolic markers using proton NMR spectroscopy for metabolomic study.

P12-2

## **FOOD METABOLOMICS: CHARACTERIZATION AND AUTHENTICITY ASSESSMENT OF COFFEE BY HIGH RESOLUTION LC-MS**

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After water and black tea, coffee represents the third most important beverage with a worldwide consumption of 4.5 million tons per year. Analytically, coffee constitutes a very complex mixture of small molecules which differ in composition and quantities based on the different coffee cultivars, cultivation regions, and processing procedures.

Metabolomics studies have gained major importance in food analysis. We analyzed 13 different types of coffee capsule extracts, assigned by their manufacturer to different intensity categories, using a new benchtop QTOF System. The first task was to correlate high resolution LC-MS data to the coffee manufacturer's description via a non-targeted metabolomics approach.

Samples were differentiated by PCA and PLS based on their assigned coffee flavour intensity. Compounds responsible for sample grouping and differentiation were tentatively identified by generating unique molecular formulas, based on accurate mass and isotopic pattern information in MS and MS/MS spectra. Subsequent in-silico fragmentation generated single candidate structures. The reliable identification of these compounds saved future analysis time and the cost of purchasing multiple reference materials to confirm the identity of the target compounds.

Some coffee types can be highly prized which increases the importance of quality control and authenticity assessment. The statistical models were used to classify coffee samples from the same vendor extracted on a different coffee machine. In a blind experiment, seven out of eight coffee samples were assigned to the correct coffee type based on the PCA model. A corresponding PLS model predicted the correct coffee intensity.

In summary, untargeted metabolomics enabled differentiation of coffee types based on their assigned flavour intensity and to readily identify target compounds responsible for the differentiation. The established model was successfully applied to classify coffee samples in a blind experiment.

**P12-3**

**A CE-MS-BASED METABOLOME ANALYSIS OF THE ANTI-DIABETIC EFFECT OF *PANAX NOTOGINSENG* EXTRACT IN KKAy MICE**

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We already revealed that *Panax notoginseng* extract including panaxatriol had an anti-diabetic effect on the Type2 model mice and improved fasting and postprandial blood glucose in a human study. Our several studies, to date, suggested that the underlying mechanism of the effect depends on improving insulin resistance, so we used a metabolomics approach by capillary electrophoresis mass spectrometry (CE-MS) to understand the anti-diabetic effect on the KKAy mice.

P12-4

**HIGH RESOLUTION MASS SPECTROMETRIC PROFILING IN A STUDY OF THE METABOLISM OF FERULIC ACID IN RAT HEPATOCYTES**

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Ferulic acid (FA) is a widely distributed dietary antioxidant compound. Its metabolism has been the subject of a number of previous in vivo and in vitro studies. However, the full extent of its metabolism has not been previously elucidated. FA was incubated with rat hepatocytes between 0 and 120 min and the metabolites formed were analysed by using both reversed phase and hydrophilic interaction chromatography in combination with an LTQ Orbitrap-FTMS in negative ion mode. Sieve software was used to identify metabolites formed in comparison with hepatocytes where no drug had been added. Using this approach 18 metabolites of FA were observed. The most abundant metabolite was the sulphate of FA and this was followed by glucuronide and glycine conjugates. Some of these metabolites could also be detected in urine at low levels. A wide range of low level metabolites were produced in the hepatocyte incubations some of which have not been described before. The structures of the metabolites were elucidated by using MS<sup>2</sup>. Novel metabolites resulted from side chain oxidation and reduction of the carboxylic acid group to an alcohol. In addition a glutathione (GSH) adduct was formed by within the hepatocyte incubation and was also metabolised to its glucuronide. Incubation of a solution of ferulic acid with GSH also resulted in formation of this adduct indicating that it could be formed purely by chemical reaction via nucleophilic addition. The adduct was characterised by MS<sup>2</sup> and was also prepared in sufficient quantities for NMR. The impact of FA on GSH levels within hepatocytes was also assessed.

## **METABOLOME ANALYSIS AFTER HYPER- AND HYPOGLYCEMIC FOOD INTAKE IN HEALTHY YOUNG MEN**

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Background: Postprandial hyperglycemia contributes to the onset and progression of metabolic syndrome such as diabetes mellitus and cardiovascular disease. Effect of hyper- or hypoglycemic food intake on serum metabolites was not well known.

Objective: This study aimed to investigate the effects of ingesting foods with different glycemic and insulin responses on postprandial endogenous metabolites by performing metabolomic analysis using capillary electrophoresis-time-of-flight mass spectrometry (CE-TOFMS).

Design: A randomized crossover study was conducted on 5 young healthy men. Blood glucose (BG), serum insulin and non-esterified fatty acid (NFFA) concentrations were measured following ingestion of a test meal of water (WAT), glucose (GLU), white rice (WR), or barley (BAR). Glu, WR and BAR are containing 75g available carbohydrate. We defined Glu and WR as hyperglycemic foods, BAR as hypoglycemic food, and WAT as control food. Hunger and satiety levels were also surveyed using a visual analog scale (VAS) immediately before blood sampling. Metabolome analysis of serum samples was then performed using CE-TOFMS.

Results: Compared to fasting, leucine and isoleucine significantly decreased in GLU and/or WR, but no change was observed in WAT or BAR. Interestingly, these metabolites recovered until baseline level at 360 min. Pyruvate and lactate levels in GLU or WR resulted in high compared to those in WAT or BAR. Ornithine levels in GLU were significantly lower than baseline level, and citrulline level was significantly diminished in GL or WR compared to baseline. Low citrulline readings were also observed for GL compared to WAT and BAR, and for WR compared to BAR. Citrulline increased from 120 min after GLU and WR intake, and no difference could be seen between test meals at 360 min.

Conclusions: These results suggest that serum metabolites are affected by insulin secretions as a result of ingesting foods with different postprandial blood glucose and insulin responses.

**“WHAT’S WRONG WITH MY DECAF?” – A METABOLOMICS-BASED COMPARISON OF CAFFEINATED AND DECAFFEINATED COFFEE AND ITS IMPLICATIONS FOR ALZHEIMER’S DISEASE**

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Epidemiologic studies have increasingly demonstrated therapeutic effects of caffeine and coffee against Alzheimer’s disease (AD). However, it was only recently that researchers started to compare caffeinated and decaffeinated coffee for their therapeutic roles in AD, and one study reported synergistic interaction between caffeine and unknown components in coffee. We postulated that decaffeination process removed caffeine and the synergistic components and hence rendered decaffeinated coffee less effective against AD. In this study, we employed GC-TOFMS-based metabolomics approach to characterize small-molecule metabolites present in both caffeinated and decaffeinated coffee. Commercially available freeze-dried coffee and its decaffeinated counterpart were used. Caffeine had been naturally removed with water as claimed by the manufacturer. Multivariate data analysis was used to compare the two coffee types to elucidate the differences in their metabolic profiles. Extensive overlapping between total ion chromatograms generated for both groups of samples indicates that both coffee types were acquired from similar source. However, one-component PCA ( $Q^2(\text{cum})=0.798$ ) and OPLS-DA ( $Q^2(\text{cum})=0.997$ ) demonstrated robust separation between the two coffee types. 73 discriminant metabolites ( $\text{VIP} \geq 1.00$ ) were identified based on OPLS-DA and all achieved significance (Bonferroni-adjusted) when their means were compared using independent t-tests. Besides caffeine, 39 other metabolites were diminished in decaffeinated coffee. Many of them have important implications for AD, such as malonate, 4-hydroxybutanoate, 2-deoxygalactose, L-rhamnose, picolinate, 2-furoate, and several phenolic compounds, just to name a few. On the other hand, 34 metabolites were augmented in decaffeinated coffee. They include a panel of amino acids (glycine, alanine, aspartate, valine and proline), as well as D-galactose, which was associated with accelerated aging. This study is the first to globally compare small-molecule metabolites present in both caffeinated and decaffeinated coffee. Our findings provide important revelations about different coffee types and their individual components as potential therapeutic agents against AD.

P12-7

## **ANALYSIS OF 1071 SERUM SAMPLES WITH LC-ESI/MS REVEALS AN AGE DEPENDENT LINEAR DECREASE OF DEHYDROEPIANDROSTERONE SULFATE**

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Background: The Centre for Translational Genomics is a research venture of the University of Tartu whose aim is to create a database that represents the metabolical, genealogical and genomical data of 5% of the Estonian population. Analysis of 1071 volunteers' serum samples with LC-ESI/MS might give insight into the biological age and overall health state of 1071 volunteers.

Aim: The aim of this study was to find metabolites from blood serum that correlate with age and that give insight into the relative biological age and current health state of the subject.

Methods: 1071 serum samples were analysed with LC-ESI/MS QTRAP 3200 in positive and negative ionization modes. Data was analysed with RStudio version 0.97.248. Many m/z values had a good correlation coefficient with age but the m/z value of 368 had the highest negative correlation ( $r = -0.54$ ,  $p = <0.0001^{***}$ ) and was selected for fragmentation. The acquired spectra were compared against spectra in metabolomics databases to determine the exact structure of the corresponding metabolite.

Results: m/z value of 368 showed an age dependent linear decrease in intensity in 1071 serum samples of voluntary men and women. Fragmentation spectra comparison in the METLIN database revealed the m/z value of 368 to be dehydroepiandrosterone sulfate (DHEAS) – a known natural steroid prohormone that is produced from cholesterol by the gonads, brain, adrenal glands and adipose tissue. In addition to its hormonal functions, DHEAS has been shown to reduce reactive oxygen species, possess an anti-inflammatory function and modulate a variety of synaptic transmissions.

Discussion: The age related decrease in intensity of dehydroepiandrosterone sulfate is expected as the levels of the hormones testosterone and estrone/estradiol decrease with age in men and women respectively. Although the decrease is age dependent, it is interesting to observe that for some younger subjects the intensity value is comparable to subjects of higher age and for some elderly subjects the intensity is unusually high. Analysing the intensity of DHEAS in people might reveal an insight into the subject's biological age and current health state.

**URINARY METABOLOME ANALYSIS: WORKFLOW OF A POWERFUL TOOL FOR THE SCREENING OF ANABOLIC PRACTICES IN CATTLE**

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The use of anabolic agents in meat producing animals is forbidden in Europe since 1988 (Council Directive 88/146/EEC). Nevertheless, the possibility of widespread abuse of hormonal substances still exists, mainly due to economical benefits. The detection of illegal practices classically relies on residue monitoring in a targeted approach. However, this strategy fails when faced to new xenobiotic growth-promoting agents or new ways of application, such as administration of low-dose cocktails, therefore new analytical tools to detect such abuse are today necessary. The use of "omics" approach is a promising approach to highlight anabolic hormone abuse by indirectly detecting their physiological action. In this context, the aim of this work was to set up a liquid chromatography - mass spectrometry (LC-MS) based metabolomics workflow for the screening of a combined trenbolone acetate/estradiol implant abuse in cattle urine. Therefore, an untargeted metabolomics approach combining the information provided by reverse-phase liquid chromatography (RPLC) and hydrophilic interaction chromatography (HILIC) both coupled to high resolution mass spectrometry was developed and applied to characterize and compare cattle urinary metabolic profiles from untreated and treated animals. Herein, normalization to specific gravity was assessed as an alternative to the time consuming freeze-dry step frequently used for normalization purposes in cattle urine. Data from RPLC and HILIC were found to be complementary. The combination of both separation modes improved the metabolite information richness. The use of pooled samples (quality control) not only allowed the assessment of the performance of the analytical methodology but also the instrumental drift correction between batches. Discrimination between treated and untreated animals was observed by application of multivariate statistical analysis. Moreover, OPLS models permitted to highlight the candidate biomarkers appearing as the ions which contribute the most in the observed discrimination. From the results obtained, metabolomics approaches can be considered as a powerful strategy for the detection of fraudulent anabolic treatments in cattle since global urinary metabolic response provides helpful discrimination.



## FOOD METABOLOMICS – VALIDATION STRATEGIES FOR MARKERS OF COMMON FOODS

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Objective markers of food intake are necessary to reveal food-related health effects. However, very few food-specific markers are currently known. Metabolic profiling is a useful explorative technique in the search for new food-specific markers. The identification of useful markers is complicated by the common observation that many putative markers are not robust.

We have explored a number of smaller and larger dietary intervention studies for urine markers of foods and food components, including crucifers, rye, oat, strawberry, root beet, coffee, pomes, citrus, trans fats, meat and cheese. Some of these markers have been further validated in an observational study or in a model study where volunteers selected foods in a controlled supermarket setting. Only a 20-30% of the markers were sufficiently robust to be validated by this approach.

The validated markers include sulforaphane mercapturic acid for broccoli and other specific mercapturates for other crucifers, a fragrance compound for strawberry, a colour metabolite for root beet, cafestol and kahweol glucuronides for unfiltered coffee, 2-aminophenol sulphate for rye, 2,6-dihydroxybenzoic acid for wholegrain, and some terpenoids as well as proline betaine for citrus. For several of these we have also evidence of a dose-response.

Marker validation is a multi-step process and must be verified not only by internal cross-validation or use of a separate test set but also by external validation in independent studies. Further sensitivity, specificity, dose-response and individual variability studies are also needed for final validation. We have observed several promising markers but additional studies are needed to ascertain dose-response, interfering factors and inter-individual variability.

Validation strategies will be discussed for biomarkers detected by metabolomics.

P12-10

## TRACING THE STAGES OF CHRONIC KIDNEY DISEASE (CKD) USING <sup>1</sup>H NMR-BASED SERUM METABOLITE PROFILING

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<sup>1</sup>H nuclear magnetic resonance (NMR)-based metabolomics is a powerful tool to investigate the perturbed metabolic patterns in a complete set of metabolites in a body fluid or serum to clarify the pathogenesis of many diseases. We applied <sup>1</sup>H NMR-based metabolic profiling to investigate the altered metabolic pattern in serum from patients with chronic kidney disease (CKD). Each type of CKD patients was divided into 3 stages (low stage; > 60 (ml/min/1.73m<sup>2</sup>), mid stage; >15 (ml/min/1.73m<sup>2</sup>), high stage; ≤15 (ml/min/1.73m<sup>2</sup>)) by estimated glomerular filtration rate (eGFR) and was profiled using <sup>1</sup>H-NMR spectroscopy. Principal Components Analysis (PCA) and partial least square-discriminant analysis (PLS-DA) score plots derived from NMR data showed that the metabolic patterns were gradually distributed by CKD stage. PLS-DA model showed the 70% accuracy rate. Creatinine, pyruvate, leucine, urea, and TMAO were important variables for determining stage of CKD (VIP > 1). The levels of creatinine, trimethylamine oxide (TMAO) and urea were consistently increased in the serum from CKD patients, depending on severity of disease. Also, the levels of leucine and pyruvate were consistently decreased in the serum from CKD patients, depending on severity of disease. Creatinine, pyruvate, TMAO, and leucine were correlated with eGFR (| r | > 0.4). This study demonstrates that <sup>1</sup>H-NMR-based metabolomics approach may be useful for detecting CKD stage and understanding the alternation of metabolites by disease.

P12-11

**PhytoHUB: A NEW DATABASE DEDICATED TO DIETARY PHYTOCHEMICALS AND THEIR HUMAN METABOLITES FOR NUTRITIONAL METABOLOMICS**

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The “food metabolome” comprises all metabolites present in biological fluids that are directly derived from the digestion of food. A large proportion of the food metabolome consists of phytochemical metabolites, which are products of intestinal and hepatic or microbial metabolism of molecules such as polyphenols, terpenoids and alkaloids. Identification of unknowns in metabolome profiles is a laborious step-by-step process and often a bottleneck in biomarker discovery. One major limitation for the interpretation of the food metabolome profiles is the incompleteness of existing databases with regard to phytochemical metabolites.

As part of the ANR PhenoMeNep project, we have designed an online database called PhytoHUB, dedicated to the study of the phytochemical part of the “food metabolome”. The database will contain all phytochemicals present in edible plants and their known metabolites manually extracted from the literature. Since the metabolism of many phytochemicals has not been studied in humans, a list of predicted metabolites will be generated from expert knowledge of the metabolism of each phytochemical class and analysis of precursor functional groups on precursor phytochemicals. Mass spectral data will be included from various sources: literature, other databases on plant phytochemicals and experimental data from our collaborative platforms.

Built with MySQL and Perl processing chains, an efficient relational design will underpin a powerful and intuitive web interface. For a queried monoisotopic mass or molecular formula, the database will return a list of possible metabolites, along with their physic-chemical properties, spectral data and possible dietary precursors linked to food sources. For a queried food, it will return a list of metabolites likely to be present in biofluids after consumption.

PhytoHUB will be the first publicly accessible database to collate information on phytochemical metabolites from a metabolomics standpoint, and should facilitate identification of unknowns in non-targeted profiling. A first version of the database is planned for 2013.

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P12-12

**IMPLEMENTATION OF METABOLOMICS TO THE HORSE BIOLOGICAL PASSPORT: MONITORING OF GROWTH FACTORS ADMINISTRATIONS IN URINE BY UHPLC-HRMS**

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In horseracing, use of growth hormone (GH) and others growth factors (IGF-1, GHRPs) is strictly banned. Routine analyses based on analytical methods have already been developed to detect these molecules due to an exogenous origin [1][2]. However, it is not always possible to detect the molecules because of technical aspects (i.e. short half-life, low doses, endogenous compound-like structures). Thus, the development of new methods, such as metabolomic approaches which focus on the effects of growth factors, could bring new insights to improve doping controls [3][4].

To explore the potential of metabolomics in horse doping control, administrations of reGH (recombinant equine GH), rpGH (recombinant porcine GH) and IGF-1 (Insulin-like Growth Factor 1) were achieved in horses under controlled conditions. Urine samples were collected from both treated and non-treated animals along kinetics and analyzed by LC-HRMS (UHPLC-ESI-QTOF). Following data processing using XCMS, multivariate analyses were carried out (PLS-DA and OPLS-DA, SIMCA, Umetrics) to identify the most important variables allowing discrimination between treated and non-treated animals for each treatment. A single statistical model was then developed from the common variables (about 500) to enable reGH, rpGH and IGF-1 use suspicion. This model was improved by adding new non-treated samples, reducing the number of variables up to 90 and maintaining a good predictive probability ( $Q^2Y=0.8$ ).

At L.C.H., each year 50 horses selected in terms of Prize money by SECF are followed up during a whole year to establish the "equine biological passport". In this context, normalization strategies of LC-HRMS data based on the use of quality control (QC) and pool samples allowed us to apply the growth factors model every month on the 50 horses making it possible its use for a longitudinal study. As far as we know, in the doping control field, it is the first and unique approach that allows a regular monitoring of the use of GH or IGF-1 as a complement of direct methods.

To go further, the model will be extended to others growth factors such as GH Releasing Peptides (GHRPs) and the same approach is being developed to a wider range of doping molecules.

**P12-13**

**THE fRAILL PROJECT - USING METABOLOMICS AS A TOOL FOR INVESTIGATING FRAILITY, RESILIENCE AND INEQUALITY IN LATER LIFE**

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The concept of ageing well in later life is becoming a key strategy of public health policy in many developed countries. Wellbeing and frailty are hypothesised to be interlinked to socioeconomic inequalities and addressing this area is an important step towards meeting the challenges (social, financial & medical) posed by the UK's ageing populations.

To this end, the fRAILL Project has been designed as a multilayer analysis of socioeconomic and biological determinants to develop an integrated understanding of processes leading to positive and negative outcomes in later life in the context of social inequalities.

Empirical data from the English Longitudinal Study of Ageing (ELSA) alongside genomic and metabolomic analysis will be used to examine genetic influences and their relationship with markers of metabolic processes, test gene-environment interactions, and identify biological characteristics related to resilience and vulnerability in the face of adverse life events.

A cohort of over 6000 patients have provided annual biological samples over a period of 6 years and metabolomic analysis using UHPLC-FTMS alongside GC-MS will investigate any potential biological descriptors contained within blood sera. This dataset and subsequent statistical testing will provide the basis of an initial cross-sectional investigation to determine a potential biological link to the wellbeing-frailty axis. This initial experiment will set the foundations for a further longitudinal study that will examine and how these biological changes are related to positive/negative patient outcomes over time.

**POSTPRANDIAL FATTY ACID-SPECIFIC CHANGES IN CIRCULATING OXYLIPINS IN LEAN AND OBESE MEN AFTER HIGH FAT CHALLENGE TESTS**

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Oxylipins, such as eicosanoids, are oxidative products of poly-unsaturated fatty acids (PUFA) and act as lipid mediators, which are known to be involved in inflammatory processes. They circulate in plasma, but also occur esterified in triglyceride-rich lipoproteins which are increased after a high fat meal depending on BMI and fatty acid type. Circulating oxylipins could have an effect on peripheral tissues and are assumed to play an important role in endothelial function. Yet, it is unclear which oxylipins appear in circulation after high fat meals differing in fatty acid composition. In a double-blind randomized cross-over challenge study we characterized the postprandial oxylipin response after different high fat challenges in lean and obese men. Subjects received three high-fat milkshakes, which were either high in saturated (SFA), mono-unsaturated (MUFA) or n-3 PUFA. Plasma oxylipin profiles were determined at baseline, 2 and 4 h after shake consumption. High fat shake consumption acutely altered circulating oxylipin profiles. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) derived oxylipins clearly increased after n-3 PUFA shake consumption. MUFA shake consumption increased levels of CYP450-mediated oxylipins. SFA shake consumption led to strong increases of LA-derived HODEs. No differences were observed between lean and obese individuals.

This study is the first demonstrating that circulating oxylipins profiles are acutely affected after high fat meal challenges and that these changes are strongly influenced by different dietary fatty acids.

P12-15

## **IMPACT OF EXERCISE ON THE PLASMA METABOLOME OF PATIENTS SUFFERING FROM MULTIPLE SCLEROSIS**

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It has been proposed that a programme of physical exercise could have beneficial effects on the health of multiple sclerosis (MSc) patients. We have conducted a metabolomic analysis on the blood plasma from MSc patients to evaluate if physical activity has an impact on the plasma metabolome and to identify any associated biomarkers. The volunteer MSc patients were allocated to either an exercise group (n=60) or no-exercise control group (n=60) and blood was collected pre and post intervention giving a total of 240 plasma samples. The intervention period lasted for 12 weeks and consisted of a three 1-hour sessions per week, including both supervised gym and home exercise sessions. Nuclear Magnetic Resonance (NMR)-based metabolomics and mass spectrometry analyses were performed on the plasma samples to see which biological pathways are influenced by the intervention.

Significant changes in several intermediates and end-products of beta-oxidation and glycolysis were observed after intervention: some changes were found in both non-exercise and exercise groups but more changes were found in the exercise group only, these indicative of physical activity (lipids and carbohydrates are used as fuels). It is possible that some patients may have increased their physical activity even if they were assigned to the non-exercise regime.

Xanthine levels were lowered by exercise. This outcome opens an encouraging path of research and leads the way to a follow-up study focusing on the effect of exercise on the purine metabolism in MSc patients.

**APPLICATION OF QUANTITATIVE METABOLOMICS IN HUMAN MATERNAL, PRENATAL AND NEONATAL DISEASE BIOMARKER STUDIES**

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Metabolomics has shown significant promise for the discovery of new biomarkers for the detection of a number of complex clinical disorders. However, the use of metabolomics in obstetrics is a relatively new phenomenon. TMIC (The Metabolomics Innovation Centre), Canada's national metabolomics platform, specializes in performing quantitative metabolomics assays on human biofluids using a wide range of technologies.

Recently, TMIC has participated in several maternal, prenatal and neonatal disease biomarker studies. These include two studies on first trimester prediction of early- and late-onset PE, where NMR based metabolomic analysis was performed on first trimester maternal serum between 11-13 6/7 weeks of gestation in a case-control study. There were 30 cases each of early and late onset PE and 60 unaffected controls. The concentrations of 40 metabolites were compared between the two groups. In both studies significant differences in the first-trimester metabolites were noted in women who had subsequently developed early- and late-onset PE.

Another two studies are performed to determine whether the metabolomic profile is altered in Trisomy 18 and 21 pregnancies and whether these maternal serum biomarkers can predict T18 and T21. In both studies, NMR based metabolomic analyses of maternal serum showed an extensive group difference in metabolomic profile and metabolomics appears to be a novel tool for aneuploidy prediction.

TMIC has also participated in metabolomic profiling of umbilical cord blood in neonatal Hypoxic Ischaemic Encephalopathy (HIE). The study population was divided into those with confirmed HIE (n=31), asphyxiated infants without encephalopathy (n=40) and matched controls (n=71). A combined DI and LC-MS/MS assay was used for the metabolomic analyses and results showed a significant alteration between study groups in 29 metabolites from 3 distinct classes (Amino Acids, Acylcarnitines and Glycerophospholipids). This study highlights the potential for metabolomic technology to develop a diagnostic test for HIE.

These applications will be presented in detail. Here we intend to show the potential that metabolomics holds in contributing to the understanding of maternal and neonatal health.



**GLOBAL PROFILING OF THE SKELETAL MUSCLE METABOLOME: METHOD DEVELOPMENT AND VALIDATION FOR THE ASSESSMENT OF METABOLIC CHANGES IN INTERVENTION STUDIES**

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Metabolomics, supported by modern analytical methods, has become a key element to understand pathophysiological processes but also more subtle metabolic alterations within physiologic states like exercise, nutrition challenges and aging. Skeletal muscle tissue is the largest insulin-sensitive tissue in the body and a major user of glucose, needed to meet the high-energy demands for locomotion and organ support. Understanding energy metabolism in muscle tissue provides insight into the mechanisms governing muscle in health and/or disease. Recent studies position muscle tissue as an endocrine organ, suggesting that analysing its metabolome might provide insights into mechanisms that go beyond the local tissue environment.

In order to study the metabolome of muscle tissue we first evaluated different extraction methods reported in literature to provide good results with tissue samples. In our hands, methanol/chloroform/water (MCW) proved to be the best method in terms of (non)selectivity, reproducibility and metabolite recovery. MCW extraction was also the most eclectic method enabling the extraction of a wide range of metabolites: organic acids (GC-MS), amines (UPLC-MS/MS), nucleotides/co-enzymes (IP-LC-MS/MS), acyl-carnitines (UPLC-MS/MS) and oxylipins (HPLC-MS/MS). We developed and validated this extraction method further being able to detect and quantify >100 metabolites from 10 mg of muscle tissue distributed between the classes previously mentioned. Biological validation was assessed by determining the intra-tissue (multiple muscle tissue biopsies within an individual) and inter-tissue (biopsies across multiple individuals) variations. These parameters were found to be <20% and <50% for most metabolites.

In this work we validated the extraction of a broad range of metabolites from muscle tissue. Our approach can now be applied to muscle biopsies from intervention studies (e.g. exercise, nutrition) enabling us to investigate the metabolic changes operated by these challenges in muscle.

**P12-18**

**TARGETED AND NON-TARGETED QUANTITATIVE METABOLOMICS USING MULTI-PLATFORM APPROACH**

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TMIC (The Metabolomics Innovation Centre) is Canada's national metabolomics platform. It was officially launched in the summer of 2011. As a national platform, TMIC has several mandates including the provision of low-cost metabolomics services to academic and industrial researchers, the maintenance of freely available metabolomics databases and web servers (HMDB, DrugBank, T3DB, MetaboAnalyst) and the development of improved or more comprehensive metabolomics assays. TMIC specializes in performing quantitative metabolomics assays on human, animal, plant and microbial samples using a wide range of technologies including NMR, GC-MS, LC-MS/MS, LC-FT/MS, HPLC-UV/FD, ICP-MS and HPLC-ELSD-FAMES-MS. In order to keep pace with the rapid technology developments in metabolomics, TMIC is constantly working towards developing, acquiring, testing and implementing new metabolomic technologies. Most recently, TMIC developed and adapted several quantitative assays to expand its list of detectable metabolites. These metabolites include vitamins (B, C, D, E, A), lipoproteins (LDL, HDL, VLDL), volatiles, oxylipins and steroids. TMIC is also working towards the developments of improved lipid profiling, the creation of custom metabolomics "kits" for specific combinations of 20-30 metabolites and the expansion of bile acids, polyphenols and plant secondary metabolites.

TMIC is also involved in the systematic characterization of human biofluids. This effort, which is an extension of the Human Metabolome Project, is aimed at providing reference values for all identifiable or quantifiable metabolites in blood, urine and cerebrospinal fluid. Using multi-platform metabolomic techniques along with extensive literature surveys, TMIC reported the measurement of 4229 metabolites in the serum metabolome and a total of 419 metabolites in CSF metabolome. Recently, for urine metabolome, 2882 metabolites have been identified, quantified and catalogued. Together, these efforts represent the most comprehensive metabolomic characterization of any human biofluid achieved to date. The biofluid data are now publicly available through the TMIC website ([metabolomicscentre.ca](http://metabolomicscentre.ca)). Description of TMIC's new assays and technologies along with a brief description of their applications in human health, agriculture, nutrition and other fields will be presented.

**COMPARISON OF THE URINE METABOLOMES OF STRATIFIED SU.VI.MAX2 SUBJECTS TO IDENTIFY BIOMARKERS OF FRUIT AND VEGETABLE INTAKE**

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The "Food metabolome" is the subset of all in vivo metabolites originating from the digestion of food components. Global analyses of these metabolites by high-resolution mass spectrometry, coupled with multivariate statistical methods, allow individuals with different dietary patterns to be distinguished. Further, the comparison of groups of low and high consumers of a given food can then be used as a basis for the identification of biomarkers of consumption. In a proof-of-concept study on citrus, we showed that urine profiling of cohort subjects stratified by consumption could be a more effective strategy for discovery of sensitive biomarkers of intake than intervention studies. As part of the ANR PhenoMeNEp project, we further tested this approach for the intake of 20 selected plant foods. Using dietary questionnaire data (1994-2009), 144 high and 66 low consumers of fruit and vegetables (F&V) were selected from the SU.VI.MAX2 cohort. Morning spot urine samples were analyzed by UPLC-QTOF with positive and negative electrospray ionisation. Subgroups of low and high consumers were selected for each of the 20 foods from reported consumption, excluding from each selection any subject with a high intake of other foods. Data were treated with both univariate (Anova with BH correction) and multivariate analysis (PLS-DA) performed after an Orthogonal Signal Correction (OSC). Good discriminations were observed for most foods, but particularly for 10 foods that are frequently consumed and rich in phytochemicals. The number of significant ions ranged from 133 for coffee to 428 for apple. Some of these discriminants, although highly correlated with consumption of the target food, were not specific enough to make good candidate biomarkers. The long-term low and high consumption of F&V were also clearly reflected in the urine metabolomes, mainly through variations in endogenous metabolites. The most discriminating and specific ions are being currently identified and several new biomarkers have already been identified in coffee. The study provided a useful insight into the conditions for success and the limitations of the approach of applying metabolomics to cohort samples for rapid discovery of a wide range of nutritional biomarkers.

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**P12-20**

## **METABOLOMICS BASED IDENTIFICATION OF PATTERNS OF DIETARY INTAKE**

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Accurate assessment of dietary intake is necessary for understanding the relationship between nutrition and disease. Traditional methods of dietary assessment are prone to errors such as under-reporting. In recent years dietary biomarkers have been proposed as a means for unbiased and objective measure of dietary intake and metabolomics offers a means to identify novel biomarkers. Classification of subjects into dietary patterns has emerged as a useful tool in nutrition epidemiology. These patterns have been used to uncover complex disease processes that are more likely related to a combination of dietary factors rather than a single dietary factor. The objective of the research was to investigate the link between dietary intakes patterns and metabolic profiles. Urine samples and dietary intake data from the National Adult Nutrition survey (NANS) were used in the analysis (n=600). Dietary data was collected using 4-day weighed food diaries. Samples were analysed using <sup>1</sup>H nuclear magnetic resonance (NMR) spectroscopy. The resultant metabolic profiles were clustered into four groups using k-means cluster analysis. Examination of the food group intake across the 4 clusters revealed that: cluster 1 had high intakes of wholemeal, brown breads, eggs and fruit, cluster 2 had high intakes of yoghurts potatoes, fruit, breakfast cereals, porridge, fish and fish dishes, cluster 3 had high intakes of savouries cheese, chips and processed potatoes, meat products and confectionary and cluster 4 had high intakes of chips and processed potatoes, meat products and high energy beverages. The use of metabolomics has identified certain food groups that can be linked to metabolic profiles. The use and reliability of metabolic profiles in dietary pattern intake will further be explored and is set to have a large impact in nutrition research.

P12-21

**THE ACCUMULATION OF CEREAL BRAN DERIVED METABOLITES IN PLASMA AND TISSUES AND IMPACT ON ENDOGENOUS METABOLISM IN TWO HUMAN DIETARY STUDIES AND AN ANIMAL FEEDING TRIAL**

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Growing epidemiological evidence shows that diets rich in whole grains can protect against chronic diseases including type 2 diabetes, cardiovascular diseases, and certain cancers. The bran and germ compartments are rich in micronutrients and bioactive phytochemicals, which most likely contribute to the beneficial health effects, although the exact biological processes involved are not known completely. This is partly due to the fact that the rich phytochemical composition of grain brans is not yet comprehensively characterized, and the effects of such diverse mixture on various biochemical processes are difficult to interpret with any presently used clinical biomarker analyses.

We have used the non-targeted LC-MS based metabolite profiling approach to characterize the composition of human plasma after consumption of bran to study the circulating phytochemical metabolites modulated by intestinal microbiota and phase II metabolism. Additionally, this approach has been used to investigate how the endogenous metabolism is affected by the bran-rich diet. The observed circulating metabolites after bran-rich meal include derivatives of alkylresorcinols, phenolic acids and benzoxazinoids, and the endogenous pathways that are most affected are certain lipid, amino acid and carnitine metabolism. The response is varied during several hours postprandially and at the fasting status. To complement the human studies, we have also done mouse trials using feeds enriched with rye and wheat bran to detect the appearance of the bran derived phytochemicals in various organs and whether they cause any changes on the endogenous metabolism of the organs. We have conducted the LC-MS metabolite profiling on liver, pancreas, heart, muscle, different adipose deposits, as well as several parts of the intestinal tissue. The data reveals interesting accumulation patterns in various organs, accompanied with endogenous metabolite changes, which will aid in interpreting the metabolic events taking place after bran consumption.

## EFFECT OF NUTRITIONAL INTERVENTION AND ETHERLIPID DEPLETION ON LIPID METABOLISM IN MOUSE MODEL

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The objective of the study was to investigate how tissue specific lipid pathways and lipidomic profiles are affected by etherlipid depletion in mouse model. Global lipidomic profiles were studied in plasma and tissues of gnpat knock out (KO) mice which lack the first enzyme involved in etherlipid biosynthesis. Additionally, plasma and HDL lipoprotein fractions of gnpat KO mice and their wild type littermates were investigated under different nutritional interventions, high fat diet (HFD) and HFD enriched with  $\omega$ -3 fatty acids.

Ether phospholipids are abundant lipid species characterized by the presence of alkyl chain bonded via ether linkage in position sn-1 and a usual acyl chain in the sn-2 position. They contribute to membrane structural integrity and are involved in multiple cellular functions. They have also shown to play an important role in human health as a factor involved in aging, obesity, diabetes, and diseases of the central nervous system.

Global profiling of molecular lipids was performed in plasma and tissues (liver, heart, skeletal muscle) samples as well as plasma lipoprotein fractions from wild type and gnpat KO mice. Additionally, plasma and HDL fractions were investigated in WT and KO mice fed with HFD and HFD enriched in  $\omega$ -3 fatty acids. For lipidomics, ultra high performance liquid chromatography-quadrupole-time-of-flight mass spectrometry (UPLC-QTOFMS) was applied. The data were processed using MZmine 2 software and the identification of lipids was based on an internal lipid library. Additionally, MS<sup>n</sup> spectra obtained by LTQ-Orbitrap mass spectrometer was used for the identification of unknown lipids and for structural characterization of ether phospholipids. The data were analyzed using two-way ANOVA and tissue profiles of WT and KO mice were compared separately using t-test.

A clear depletion of ether lipids was observed in all studied tissues of gnpat deficient mice compared to WT mice. Significant changes in other lipids were not observed at lipid class level. However, certain neutral lipids, such as polyunsaturated fatty acids (PUFA) containing triacylglycerols were upregulated especially in gnpat deficient mice liver. As expected, lipid profiles were strongly affected by HFD. Dietary lipid composition affected also the levels of many etherlipids, especially those containing  $\omega$ -3 fatty acid as well as lysolipids. In gnpat KO mice fed with HFD enriched in  $\omega$ -3 fatty acids the levels of several etherlipids in HDL were recovered to the level observed in HDL fractions of WT mice on chow diet. However, the processing of data is still ongoing and the final results will be presented in the conference.

In conclusion, lipidomic profiling showed that the depletion of ether phospholipids in plasma and tissues of gnpat KO mice can be modulated by diet.

P12-23

**STUDY OF THE METABOLOMIC CHANGES IN RED WINES DURING AGING UNDER DOMESTIC AND CELLAR CONDITIONS THROUGH AN UNTARGETED-TARGETED METABOLOMIC WORKFLOW**

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Storage conditions and duration have a great influence on the quality and nutritional value of food. Wine may improve its value during aging only if occurring under optimum condition. Temperature, relative humidity and time are important factors affecting wine quality. In order to evaluate the global effects of storage on wine composition, 20 red wines (10 bottles each) were stored under two conditions for a period of 24 months and sampled every 6 months. The wines were stored either under optimum temperature and humidity in a wine cellar or under typical domestic conditions with slightly higher and fluctuating temperature and humidity. The samples were analyzed first by untargeted UPLC-ESI-QTOF/MS analysis both in positive and negative mode, in order to find the putative biomarkers of wine aging.

The marker selection highlighted the influence of wine storage on a number of phenolic compounds (i.e. pigments, tannins, flavonols, etc) and others non-phenolic metabolites. Targeted UPLC-MS/MS analysis for the metabolites of the phenylpropanoid biosynthetic pathway, confirmed the results of the untargeted experiment concerning the pigments and other phenolic compounds, while providing a more detailed picture about the relative reaction rates occurring during the storage and revealing additional markers.

The results of the multivariate analysis (PCA plots) showed that the wines stored under optimum condition had small variation even after 24 months of storage, while the wines stored under typical house conservation developed approximately four times faster, reaching after 6 months a composition similar to the wines stored in cellar for 24 months.

In conclusion this project evaluated in an unbiased manner the chemical implications of the appropriate storage of red wines, highlighting a number of known and novel metabolites as markers. The data obtained following the kinetic of the biomarkers over 24 months at two conditions of storage, allowed us to build hypothesis about the metabolic changes in wine during storage, which can be useful for improving both the production and storage of red wines.

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## BIOMARKERS OF DIETARY INTAKE – A LIPIDOMIC APPROACH

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Reliable dietary assessment methods are essential when attempting to understand the complex links between diet and health. Metabolomics is an emerging technology that offers great promise in identifying novel biomarkers of dietary intake. Its application has identified a number of putative biomarkers of certain foods. Lipidomics is a discipline that falls under the umbrella of metabolomics. To date little attention has been given to link lipidomic profiles with dietary data to identify biomarkers of dietary intake. Therefore the aim of this study was to apply a statistical approach in an attempt to link lipidomic data with dietary data to identify novel biomarkers of dietary intake. We assessed the relationship between lipidomic profiles and dietary data in volunteers ( $n=34$ ) from the Metabolic Challenge Study (MECHE). A food frequency questionnaire (FFQ) was used to assess dietary habits over a period of 1 year. Serum samples collected at baseline were analysed by Biocrates Life Sciences AG using a targeted approach, with a total of 180 lipids identified and quantified by ESI/MS/MS. Principal component analysis (PCA), linear regression and heat map analysis were used to reduce the lipidomic data and investigate relationships between lipidomic patterns and dietary data. Six lipidomic patterns were identified by applying PCA, explaining 81% of the total variance. Linear regression and heat map analysis found that monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), saturated fatty acids (SFA) and fruit all have a strong relationship with LP1 (lipidomic pattern 1). Meat intake had a strong association with LP2, fish had a strong association with LP5, whilst meat, fish and vegetables all had a strong relationship with LP6.

Further investigation found that MUFA intake had a significant relationship with a glycerophospholipid (phosphoethanolamine acyl-alkyl C40:4,  $p=1.72 \times 10^{-4}$ ) and a sphingolipid (sphingomyelin C21:0,  $p=7.73 \times 10^{-4}$ ). PUFA intake had a significant relationship with a phosphoethanolamine diacyl (C36:5,  $p=4.0 \times 10^{-5}$ ), whilst SFA intake had a significant relationship with two sphingomyelins (C21:0,  $p=1.0 \times 10^{-3}$  and C14:0,  $p=1.0 \times 10^{-5}$ ). The identified lipids could be used in the future as biomarkers of dietary fat intake. Further work will be directed towards translation of the present findings into nutritional epidemiology.



**A STUDY OF METABOLOMIC CHANGES IN MATERNAL URINE BETWEEN 13-18 WEEKS OF GESTATION USING ZWITTER-IONIC HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY COUPLE TO ION TRAP TIME OF FLIGHT MASS SPECTROMETRY**

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Dynamic changes in maternal metabolism during pregnancy could be monitored via maternal bio-fluids. These changes not only reflect adaptation of mother to her foetus but also may reflect health of the growing foetus.

Zwitter-ionic hydrophilic interaction chromatography (ZIC®-HILIC) coupled to ion trap and time of flight (IT-ToF) mass spectrometry has been used to effectively detect the ionisation of metabolites present in 60 pregnancy urine samples. 2.1 mm x 150 mm, 5 µm ZIC® HILIC column was used. The flow rate was maintained at 0.2 mL min<sup>-1</sup> for gradient elution in 31 minutes separation time. 50 mM aqueous ammonium acetate was mixed with acetonitrile (95:5, v/v) and was used for mobile phase A. Eluent B was composed of mixture of 50 mM aqueous ammonium acetate: water and acetonitrile (50:45:5, v/v). The column was equilibrated for 5 min at 0.4 mL min<sup>-1</sup> flow rate with 5% aqueous solvent. In this unbiased, blinded analysis of urine, a comprehensive metabolic fingerprint has been generated. Noise reduction was carried out using Profiling Solutions software by limiting ion intensity threshold (20,000), retention shift (0.2 mins), *m/z* tolerance (+/- 25kD). Multivariate analysis (PCA-X, PLS-DA), model validation, variable score plots and classification tests was carried out using SIMCA P+. Minitab was used on raw data for multiple of median calculations.

On comparison of these normal pregnancy urine samples from 13-18th week of gestation it was found that five key features with *m/z* of 123.08, 230.14, 299.348, 514.78 and 653.71 expressed strong changes in pregnancy from start of 2<sup>nd</sup> trimester compared to mid 2<sup>nd</sup> trimester. Anatomical changes like exclusion of extra coelomic cavity may be attributed to the metabolomic changes observed in this study. Metabolomics of urine together with structural elucidation of potential small molecule biomarkers may help to understand the biochemistry and endocrinology pregnancy disorders and any potential risks associated during particular gestational age. No other published work is known to have used metabolomics as a tool for biomarker discovery in pregnancy urine.

**PATTERNS OF TIME SINCE LAST MEAL REVEALED BY SPARSE PCA IN AN OBSERVATIONAL LC-MS BASED METABOLOMICS STUDY**

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Overview : This study demonstrates the application potential of sparse PCA (SPCA) as a variable selection tool in a metabolomics study. The dataset was compromised of LC-MS plasma profiles of 270 subjects and the focus was extracting patterns related the time passed since the subjects had their last meal (TSLM). SPCA provided clear variable selection advantages over PCA. Also, SPCA extracted inter-correlated variables from different biochemical classes in different sparse components, i.e. amino acids and lyso-lipids in this case.

In metabolomics studies, liquid chromatography mass spectrometry (LC-MS) provides comprehensive information on biological samples. However, extraction of few relevant metabolites from this large and complex data is cumbersome.

Principal component analysis (PCA) has been widely used for both dimension reduction and for exploratory analysis of complex datasets. However, PCA represents each principal component as the linear combination of all original variables, such that all has non-zero contribution. Particularly in cases where the number of variables exceeds the number of samples as in metabolomics, it becomes unclear to extract only a few relevant features from the many irrelevant ones. In order to deal with this issue, SPCA was introduced to estimate a PCA-like model where sparsity is induced on the model parameters [1].

In this study, we have employed sparse principal component analysis (SPCA) to capture the underlying patterns and select relevant metabolites from LC-MS plasma profiles. The study involved a small pilot cohort with 270 subjects where each subject's TSLM has been recorded prior to plasma sampling. Our results have demonstrated that both PCA and SPCA can capture the TSLM patterns. Nevertheless, SPCA provides more easily interpretable loadings in terms of selection of relevant metabolites. Furthermore the metabolites associated with TSLM were identified as a group amino acids and a variety of lyso-lipids.

[1] Zou H, Hastie T, Tibshirani R (2006) Sparse principal component analysis. J Comput Graph Stat 15: 265-286.

**NMR METABOLOMICS TO STUDY THE REGULATION OF ENERGY METABOLISM ON CHICKEN LINES DIVERGENT FOR LOW OR HIGH ABDOMINAL FAT DEPOSITION**

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The body fat content and partition of lipid stored in the different tissues of the carcasses are important phenotypic traits to be considered for the sustainability of farm animal production. Both genetics and non-genetic factors, including the distribution of diets formulated under the optimal requirements, are able to modify body fatness of growing animals, through their actions on lipid metabolism.

This project aimed to characterize the response of lipid metabolism and body fat based on two types of diets more or less rich in fiber (high and low fat) and in two chicken lines divergent for carcass fatness (4 experimental groups). The goal was to detect overall metabolic perturbations of 48 chicken plasma samples. A metabolomic approach using the 1D <sup>1</sup>H NMR experiments was recorded on a Bruker Avance 500 spectrometer with a cryoprobe. NOESY 1D NMR sequence including water presaturation was used to obtain plasma metabolic profile. After identifying metabolites, a statistical approach was applied on data for metabolites discrimination. The statistic tests included univariate (ANOVA, BoxPlot) and multivariate analysis (PCA, PLS-DA) using the free R software and SIMCA P+ (Umetric®), respectively. Plasma metabolic profiles were different in chickens fed with high and low fat diets. Lipid concentrations were significantly increased whereas LDL (Low Density Lipoprotein) levels were decreased in plasma of chickens fed high fat diet (p<0.001). A significant difference was also observed in plasma metabolites (glutamine, histidine and betaine) of chickens issued from fat or lean lines. These results confirmed previous data obtained on these lines and suggested the implication of methyl donors on the regulation of lipid metabolism. This study provided new information on circulating metabolites, which may help in elucidating key regulators associated to variations in body fat content.

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**RESVERATROL METABOLISM IN A NON-HUMAN PRIMATE, THE GREY MOUSE LEMUR *MICROCEBUS MURINUS* BY ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-QUADRUPOLE TIME OF FLIGHT**

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The Grey Mouse Lemur (*Microcebus murinus*) is a non-human primate used to study the ageing process. Resveratrol is a polyphenol that might increase lifespan by delaying age-associated pathologies. The Restrikal study aims to investigate the long-term effect of resveratrol intake in *Microcebus* on the ageing process. However, no information about resveratrol absorption and metabolism is available in this lemur.

Resveratrol and its metabolites were qualitatively and quantitatively analysed in *Microcebus* plasma (after 200mg/kg resveratrol oral intake) by Ultra High Performance Liquid Chromatography (UHPLC) on line with a Quadrupole-Time Of Flight (Q-TOF) mass spectrometer used in full scan mode.

The data analysis showed an ion common to resveratrol and all its metabolites,  $m/z$  227.072. A semi-targeted study enabled to identify six hydrophilic metabolites of resveratrol, two monoglucurono conjugated derivatives, one sulfo conjugated and two mixed derivatives both sulfo and glucurono conjugated. The presence of such derivatives had already been described in the mouse, the pig and the human species. The compound concentrations have been determined with a standard curve specific to each compound when the corresponding standard molecule was available.

Conclusion: Free resveratrol is measurable for several hours in *Microcebus* plasma and its two main metabolites are trans-resveratrol-3-O-glucuronide and trans-resveratrol-3-sulfate.

**THE PHENOL-EXPLORER DATABASE ON THE POLYPHENOL METABOLOME AS AN AID TO IDENTIFY NOVEL BIOMARKERS OF DIETARY EXPOSURE FOR NUTRITIONAL EPIDEMIOLOGY**

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Thousands of metabolites found in blood or urine are directly derived from foods after digestion. They constitute the so-called food metabolome. Metabolomics has been used to discover in the food metabolome, metabolites to be used as novel biomarkers of dietary exposure. These biomarkers are needed in nutritional epidemiology to identify diet-related risk factors for diseases. Various open-access web databases aid in the annotation of metabolites in non-targeted metabolomic profiles. However, most contain little information on the food metabolome. We developed Phenol-Explorer, a specialist database on dietary polyphenols, a highly diverse group of antioxidants abundant in a large diversity of plant foods. We will report here the exploitation of the database to characterize the human polyphenol metabolome in both quantitative and qualitative terms. Data on 383 polyphenol metabolites were collected from 221 original intervention studies published in peer-reviewed journals. It includes information on the chemical nature of the metabolites, pharmacokinetic parameters and precursor-metabolite specificity. This information will be crucial to identify the most useful biomarkers of exposure to polyphenols in complex urine or plasma metabolic profiles. It will contribute to the development of metabolome-wide association studies in nutritional epidemiology. Hopefully, similar information will become also available for other classes of food compounds to provide a more comprehensive and highly detailed view of human dietary exposure.

## QUALITATIVE AND QUANTITATIVE DIFFERENTIATION OF SPECIES FROM VACCINIUM BY NMR SPECTROSCOPY IN AUTOMATION

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The genus *Vaccinium* contains approximately 450 species including bilberry, blueberry, cranberry, lingonberry, and huckleberry. The fruit of *Vaccinium* are widely eaten and touted for their health benefits, attributed to antioxidants and micronutrients. Blueberry leaf extract is a traditional Cree medicine, believed to have anti-diabetic properties. Extracts from *Vaccinium* plants are commonly added to foods, dietary supplements, and cosmetics. Given their widespread use in humans, it is important to have methods to verify the identity and purity of *Vaccinium* extracts.

Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool for characterizing mixtures such as plant extracts in full automation. Discrimination of different species, absolute identification & quantification of key compounds and verification if the determined parameters are in the allowed range is the basis for the quality control of a sample. The automation of this process minimizes failures in the analysis. A comprehensive report makes the results easy to interpret.

An NMR spectrum, such as a 1D <sup>1</sup>H spectrum, shows the superposition of the characteristic signals of all of the compounds in the mixture. When enough samples of a specific material are available, chemometric models can be build. Then new samples can be classified against the model to determine whether they represent the same material. The result is represented in different ways, e.g. as quantile plot.

Identifying and quantitating of key compounds from a mixture spectrum can be based on spectral analysis or using PLS-1. Both results can be used to verify if the sample is in the allowed range.

We will demonstrate the power of these techniques using examples from our ongoing study of *Vaccinium*.

**IS THE SLEEP APNEA DIFFERENT FROM COPD? THE METABOLOMICS STUDIES OF SERUM AND URINE BY <sup>1</sup>H NMR METHOD**

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Chronic Obstructive Pulmonary Disease (COPD) is characterized by persistent and progressive airflow limitation and by an enhanced chronic inflammatory response in the airways and in the lung to noxious particles or gases. Chronic inflammation causes structural changes and narrowing of the small airways. COPD is the fourth leading cause of mortality worldwide and results in an increasing economic and social burden. From the other hand obstructive sleep apnea is caused by obstruction of upper airflow and is characterized by repetitive pauses in breathing during sleep. This type of abnormal behaviour is reflected on reduction of blood oxygen saturation.

In these studies we used NMR spectroscopy to analyze the serum and urine from OSA and COPD group of 100 patients. The obtained data were evaluated by advance statistical analysis supported by unsupervised chemometric PCA and supervised chemometric OPLS-DA tools. The obtained preliminary data by OPLS-DA method have shown that OSA and COPD groups are differ slightly by both bio-fluids metabolites.

**A NMR BASED METABOLOMIC STUDY ON THE EFFECTS OF FATTY ACIDS INFLUENCE ON LIVER METABOLISM DURING OVERFEEDING**

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Gaining weight and especially fat accumulation in liver has many adverse metabolic consequences, which makes it an excellent target for metabolomic studies. Here we applied a comparative metabolomic analysis to investigate the consequences of increased intake of saturated (SFA) or polyunsaturated fatty acids (PUFA). The aims of the study was to investigate if there were any profile changes between the two study groups and also, if possible, correlate specific change in metabolites with liver fat assessed by magnetic resonance imaging (MRI).

In an overfeeding study, muffins containing high amounts of SFA or PUFA were added to the habitual diet of 39 healthy individuals for 7 weeks. Plasma samples were collected at baseline and post treatment. Precipitation with cold ACN were applied in order to ensure a high quality of data as reconstitution enables control over sample pH, ion strength etc. Samples were then analyzed with <sup>1</sup>H-NMR spectroscopy. Baseline-, post-treatment- and subtraction data (week 7-week 0) were evaluated with PCA, OPLS-DA and students t-test.

MRI assessments showed that the SFA group increased their levels of liver fat and total body fat while the PUFA group displayed instead increased levels of lean tissue. Differences in the serum profiles between the two groups were mainly due to amino acids, keton bodies and lactate. Correlations with MRI assessments (total body fat, liver fat and lean tissue) and several metabolites (for example valine, acetoacetate, isoleucine and 3-hydroxybutarate) were observed. These data indicated that the higher level of liver fat accumulation in the SFA group does not appear to be due to changed oxidation of fatty acids as SFA showed higher levels of keton bodies than the PUFA group.



P12-33

## **INTEGRATING FFQ AND FOOD EXPOSURE BIOMARKER TECHNOLOGY: ARE ALL FOODS APPROPRIATE TARGETS FOR BIOMARKERS?**

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Accurate measurement of habitual food intake, which is an essential component of much health-related research, is challenging. Dietary data recorded by self-assessment tools, such as Food Frequency Questionnaires (FFQs) and diet diaries, can be subject to participant bias. Recent reports have described biomarker discovery strategies using non-targeted metabolomics to analyse urines from cohort studies in which participants consumed a freely-chosen diet. However, the discovery and validation of new biomarkers has proved to be complex and to date putative biochemical markers are available for only a small number of food components. Here we describe a data-driven procedure combining diet information with metabolite fingerprinting by mass spectrometry and supervised multivariate data analysis to discover urine biomarkers indicative of habitual exposure to different foods. We explored diet diary data from the large, long term UK National Diet and Nutrition Survey (NDNS) to identify dietary components consumed frequently enough to be possible candidates for biomarker development, as well as segregating the main ingredients of commonly eaten complex foods/meals. Combined with FFQ data from existing smaller habitual studies, with associated urine samples, we identified that exposure to dietary components can be classified into general patterns differing in both range and consumption frequency, which has a major influence on the feasibility of biomarker discovery. Several metabolites proposed previously as acute exposure biomarkers were explanatory of habitual dietary exposure to the same food components. We conclude that our data-driven strategy provides a rapid and objective method to highlight dietary components recorded in FFQ data that may prove suitable for biomarker development.

**METABOLOMICS IN GENOME-WIDE ASSOCIATION STUDIES: REVEALING GENE-METABOLITE-DISEASE LINKS**

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While still in its infancy, the use of metabolic profiles in genome-wide association studies has improved the detection of, and provided biological context to, the sometimes poorly understood effects of genetic variants on clinical phenotypes. We ran a metabolome- and genome-wide association study on <sup>1</sup>H-NMR urine profiles from 835 individuals of the *Cohorte Lausannoise*. We used an *untargeted* approach, with the NMR signal intensities as the study's phenotypes, thereby carrying all available metabolomic information into the study. We then identified the metabolites underlying significant association using, in addition to manual identification, a newly developed automated identification method. The two novel gene-metabolite associations resulting from this study are of particular interest because both involve metabolites which we also found to associate with clinical phenotypes. These associations thus provide specific, statistically-determined, evidence of the propagation of genetic effect from gene to disease via the metabolome.

## PREDICTION OF METABOLIC PROFILE OF POLYPHENOLS AFTER WINE AND WINE PRODUCTS INTAKE USING THE PHENOL-EXPLORER DATABASE

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Wine intake has been related to a lower total mortality rate and reduced incidence of cardiovascular diseases in several epidemiological studies. Considering the important role of polyphenols abundant in wine, knowledge of the metabolic profile of polyphenols derived from the consumption of red wine could be key to understand its health benefits and associated mechanisms.

The goal of this study is to predict the wine polyphenol metabolome in human biofluids as accurately as possible by using Phenol-Explorer 2.0. The qualitative prediction is done by using those metabolites described in intervention studies with wine products, and those metabolites derived from the consumption of compounds present in wine.

A total number of 97 metabolites were predicted after wine products consumption in plasma and urine. Metabolites described after wine consumption were 37, while 90 different metabolites were identified in intervention studies with pure compounds known to be present in wine. Thirty metabolites were common to both groups. With all the metabolites retrieved from this analysis, a global pathway has been proposed. This pathway has allowed identifying and dismissing possible biomarkers for wine or wine polyphenol intake. It is essential to identify the range of metabolites formed from food consumption considering that some of these metabolites may show different biological activity when compared to their parent compounds.

In conclusion, the Phenol-Explorer database has allowed to build up a comprehensive pathway for wine polyphenol metabolites. Such an in-silico metabolite prediction will greatly facilitate the analysis of metabolic profile from samples collected from wine consumers in both intervention and observational studies. It should help identifying metabolites most promising as biomarkers of wine consumption. Analyzing the whole spectrum of metabolites derived from wine intake should also be key to understand the health effects of wine.

P13-1

**METABOLIC FINGERPRINTS OF SERUM, BRAIN AND LIVER ARE DISTINCT FOR MICE WITH CEREBRAL AND NON-CEREBRAL MALARIA: A <sup>1</sup>H NMR SPECTROSCOPY BASED METABONOMIC STUDY**

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Cerebral malaria (CM) is a life threatening disease in humans caused by *Plasmodium falciparum* leading to high mortality. *Plasmodium berghei* ANKA (PbA) infection in C57Bl/6 mice induces pathologic symptoms similar to that in human CM. However, experimental CM incidence in mice is variable and there are no known metabolic correlates/fingerprints for the animals that develop CM. Here we have used <sup>1</sup>H NMR based metabonomics to investigate the metabolic changes in the mice with CM with respect to the mice that have non-cerebral malaria (NCM) of same batchmates with identical genetic background and infected simultaneously. The metabolic profile of the infected mice (both CM and NCM) was separately compared with the metabolite profile of uninfected control mice of same genetic background. The objective of this study was to search for metabolic changes/fingerprints of CM and identify the pathways that might be differentially altered in mice that succumbed to CM. The results show that brain, liver and sera exhibit unique metabolic fingerprints for CM over NCM mice. Some of the major fingerprints are increased level of triglycerides, VLDL-cholesterol in sera of CM mice and decreased levels of glutamine in the sera concomitant with increased levels of glutamine in the brain of the mice with CM. Moreover, glycerophosphocholine (GPC) is decreased both in the brain as well as liver of animals with CM, *myo*-inositol and histamine are increased in the liver of CM mice. The metabolic fingerprints in brain, sera and liver of mice with CM point towards perturbation in ammonia detoxification pathway and perturbation in lipid and choline metabolism in CM specifically. The study helps us to understand the severity of CM over NCM and in unrevealing the specific metabolic pathways which are compromised in CM.

**P13-2**

**MOLLICUTES ADAPTATION TO LOW pH CONDITIONS**

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Since most microbes spend the vast majority of their life under environmental stress, further knowledge of stress responses is critical to the complete understanding of microbial physiology. Study of bacteria stress responses may contribute to the development of new vaccines, new disease therapies, new agricultural strategies, new food safety procedures, and new antimicrobial agents. Metabolome - a dynamic collection of all metabolites, cell or organism under these conditions. Thus, the metabolic profile of an organism at a given time is a living imprint of cellular activity. Determining the composition of metabolites coupled with data from transcriptomic analysis can provide valuable information about the processes of cell regulation and mechanisms of organism adaptation to various environmental stresses. The object of our research is a Mollicutes class bacteria (*Spiroplasma melliferum*, *Mycoplasma gallisepticum*, *Acholeplasma laidlawii*), a unique category of bacteria that have in common the absence of a cell wall, a reduced genome, and simplified metabolic pathways. We present a result of reliable measurement of a hundred Mollicutes metabolites, including components of sugar, amino acid, and nucleotide metabolism in normal and low pH conditions, in complex with data from transcriptomics studies and pathways reconstruction. For metabolomic analysis we selected liquid chromatography coupled with time of flight mass spectrometry approach. Hydrophilic interaction chromatography with silica column effectively separates highly polar cellular metabolites prior to their detection on a high accuracy mass spectrometer in positive and negative acquisition mode for each column.

**P13-3**

### **THE METABOLIC INTERPLAY BETWEEN PLANTS AND PHYTOPATHOGENS**

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Plant diseases caused by pathogenic bacteria or fungi cause major economic damage every year and destroy crop yields that could feed millions of people. Only by a thorough understanding of the interaction between plants and phytopathogens can we hope to develop strategies to avoid or treat the outbreak of large-scale crop pests. Here, we studied the interaction of plant-pathogen pairs at the metabolic level. We selected five plant-pathogen pairs, for which both genomes were fully sequenced, and constructed the corresponding genome-scale metabolic networks. We present theoretical investigations of the metabolic interactions and quantify the positive and negative effects a network has on the other when combined into a single plant-pathogen pair network. Merged networks were examined for both the native plant-pathogen pairs as well as all other combinations. Our calculations indicate that the presence of the parasite metabolic networks reduce the ability of the plants to synthesize key biomass precursors. While the producibility of some precursors is reduced in all investigated pairs, others are only impaired in specific plant-pathogen pairs. Interestingly, we found that the specific effects on the host's metabolism are largely dictated by the pathogen and not by the host plant. We provide graphical network maps for the native plant-pathogen pairs to allow for an interactive interrogation. By exemplifying a systematic reconstruction of metabolic network pairs for five pathogen-host pairs and by outlining various theoretical approaches to study the interaction of plants and phytopathogens on a biochemical level, we demonstrate the potential of investigating pathogen-host interactions from the perspective of interacting metabolic networks that will contribute to furthering our understanding of mechanisms underlying a successful invasion and subsequent establishment of a parasite into a plant host.

P13-4

## SERUM METABOLOME CHANGES IN ADULT PATIENTS WITH PRIMARY DENGUE INFECTION

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Dengue virus is the most widespread arbovirus with an estimated 100 million infections occurring every year. To better understand the molecular mechanisms and identify putative therapeutic targets of dengue infection, we performed metabolic profiling of serum samples at early febrile, defervescence, and convalescent stages of dengue infection from 44 adult dengue fever patients using both LC/MS and GC/MS, with 50 asymptomatic healthy subjects as references. Dengue infection causes significant serum metabolome changes in the patients. Dozens of differential metabolites were identified and the main metabolite classes were free fatty acids, acylcarnitines, phospholipids, and amino acids. Major perturbed metabolic pathways included fatty acid biosynthesis and  $\beta$ -oxidation, phospholipid catabolism, steroid hormone pathway, purine metabolism, and heme degradation pathway, suggesting the multifactorial nature of human host responses. These metabolites were significantly perturbed during the early stages, and normalized to control levels at convalescent stage, suggesting their potential utility as prognostic markers. Many of the differential metabolites are involved in acute inflammatory responses and our global analyses revealed early anti-inflammatory responses working in concert to modulate early pro-inflammatory processes, thus preventing the host from development of pathologies by excessive or prolonged inflammation. This study is the first example of how metabolomics approach can divulge the extensive, concurrent, and dynamic host responses elicited by dengue virus and offers plausible physiological insights to why dengue fever is self limiting.

**TOWARDS IDENTIFYING THE FULL METABOLOME OF THE PROTOZOAN PARASITE *LEISHMANIA* BY USING HIGH RESOLUTION LIQUID CHROMATOGRAPHY MASS SPECTROMETRY**

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*Leishmania* is a protozoan parasite of humans and other mammals transmitted by the sandfly. Extensive knowledge is available on the genomes and transcriptomes of the parasite species, and these have facilitated predictions on the metabolic pathways operating. Experimental investigations over many decades have provided much information on the metabolites in the parasite. However, these have largely been focused studies rather than global profiling. We have now taken advantage of the advent on improved technologies for analysing small metabolites in biological samples to provide robust data on the whole spectrum of metabolites that comprise the metabolome in this parasite. Initially we have focussed on the promastigote stage of *Leishmania major* and applied methods modified from those that we used to investigate the mechanism of drug resistance in *Leishmania* and a reversed phase chromatography method to analyse the non-polar metabolites. We have confirmed identities of metabolites by running pure compounds as standards and MS<sup>2</sup> for compounds where standards were not available. We have also quantified less expected metabolites via spiking with standards for these compounds over a calibration range. The analysis so far has firmly identified >400 polar and non-polar metabolites. The analysis will continue by applying IC methodologies to establish the identity of metabolites less well separated using our current HILIC methods. We expect that the analysis will provide for the parasitology community robust information on a large percentage of the metabolome together with key information on how the individual metabolites can be confirmed. Further sample manipulations will be carried out in order to extend metabolite coverage within this significant group of parasites.



**MULTI-PLATFORM METABOLIC FINGERPRINTING OF CANINE VISCERAL LEISHMANIASIS**

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Visceral leishmaniasis (VL) is a severe infectious disease with worldwide distribution and is caused by protozoan parasites of the genus *Leishmania*. Dogs are the main peridomestic parasite reservoirs in Americas and the Mediterranean region. In these hosts, the disease progress from asymptomatic infection to symptomatic severe manifestations, culminating with death. Although adopted in European countries, treatment is not permitted by public health authorities in Brazil, since asymptomatic, seronegative or treated animals remain as a source of parasites for sand fly infection. Culling of seropositive animals is one of the main epidemiologic control measures in some countries, but control programs may fail due in part to the low sensitivity of the diagnostic tests. In this context, early diagnostic tools, applicable to field conditions, are becoming increasingly necessary. Changes in metabolic profiles are a potential source of molecules with a high probability of later being useful as diagnostic/prognostic markers. In addition, the identification of pathways altered by the disease can improve the characterization of pathophysiological alterations associated with VL. As no single analytic technique covers the entire spectrum of a biological system either to their different physical-chemical properties, a multiplatform fingerprinting were performed. Using a novel approach combining four complementary metabolomics platforms, the extent of biochemical differences between positive (symptomatic and asymptomatic) and negative dogs was investigated. Using an approach combining three complementary metabolomic platforms (LC/MS, GC/MS and CE/MS), urine and plasma samples were fingerprinted to investigate the extent of biochemical diversity between the groups. Multiple metabolomic data sets were analyzed using multivariate statistical methods to identify discriminatory compounds related to the disease state. To achieve optimum disease-related differentiation, partial least squares discriminant analysis (PLS-DA) was performed. Score plots of PLS-DA of all observations revealed significant differences between groups. In total, more than 500 statistically significant compounds ( $p < 0.05$ ) were selected and searched against METLIN, KEGG, LIPIDMAPS, and HMDB databases. Some tentative compounds were found in different techniques. The identity of these compounds will be confirmed by MS/MS analysis.

## DIAGNOSIS OF ACUTE TYPHOID FEVER BY METABOLOMICS

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Typhoid fever is an infectious disease caused by the bacteria *Salmonella* Typhi and the closely related *Salmonella* Paratyphi. Typhoid fever can only be spread among humans, which implies that regional elimination of the disease should be possible. However, large efforts will be needed in many areas to be able to reach such a high set goal, one of them being improved diagnostic methods since current methods suffer from major limitations. Thus, there is a need for a rapid, more sensitive and specific diagnostic method for typhoid fever. Here, serum samples from patients with typhoid fever have been analysed with a metabolomics approach to investigate the possibilities of finding biomarker patterns suitable for use as a diagnostic test for typhoid fever. 75 human serum samples including: controls (no detected infection), *S. Typhi* and *S. Paratyphi* was analysed with GCxGC/TOF-MS for metabolite detection and quantification. The acquired metabolic fingerprints were subject to multivariate data analysis to search for metabolic differences between typhoid fever and control, as well as between *S. Typhi* and *S. Paratyphi*. 900-1000 metabolites were detected in the GCxGC/TOF-MS analysis and around 200 of these could be assigned with a molecular identity. Multivariate OPLS-DA models revealed a significant separation between the typhoid and control samples (*S. Typhi* vs. control,  $p=1.3 \cdot 10^{-21}$ , and *S. Paratyphi* A vs. control,  $p=9.5 \cdot 10^{-18}$ ). Interestingly, a separation (although less significant,  $p=5.0 \cdot 10^{-2}$ ) could also be seen between *S. Typhi* and *S. Paratyphi* A, something that is clinically highly relevant when it comes to treatment of the disease. The obtained results are promising for the continued work of searching for metabolic biomarkers for typhoid fever diagnostics.

P13-8

**QUANTITATIVE HIGH RESOLUTION <sup>1</sup>H NMR URINALYSIS FOR ALGORITHMIC DIAGNOSIS OF URINARY TRACT INFECTION USING AN AUTOMATIC ANALYZER**

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For the diagnosis of urinary tract infection (UTI), urine dipstick analysis is simple but limited by its diagnostic accuracy while urine culture takes time. There is no quantitative biochemical method for the diagnosis of UTI. In this study, we described a quantitative method for diagnosing bacterial UTI and specifically for *Escherichia coli* (EC).

We compared the urine metabolomes from 88 patients with bacterial UTI and 61 controls using <sup>1</sup>H NMR spectroscopy, followed by orthogonal partial least squares-discriminant analysis. The diagnostic accuracy of the markers was evaluated using receiver operating characteristic (ROC) curve analysis using the urine culture results as standard. Urine concentration of acetic acid/creatinine is a marker for bacterial UTI with an area under ROC of 0.95. At the optimal cutoff of 0.03 mmol/mmol, it correctly classified bacterial UTI with sensitivity of 91% and specificity of 82%. In addition, urine concentration of trimethylamine/creatinine is a marker to predict EC-associated UTI. It has an area under ROC of 0.90. At the cutoff of 3.0 μmol/mmol, it correctly identified EC-associated UTI with sensitivity of 85% and specificity of 84%. A new diagnostic algorithm based on the two cutoffs could identify 97% bacterial UTI, 82% EC-associated UTI and 86% non-UTI control with a predicted 74% reduction in workload for urine culture.

In conclusion, urine acetic acid is a neglected urine metabolite for the diagnosis of UTI. Urine acetic acid is not usually measured in hospital laboratories. We envisaged NMR based method for urine acetic acid will simplify the diagnosis of UTI and EC infection.

P13-9

## **METABOLOMICS STUDY OF SEPTIC SHOCK: FROM NMR AND MS DATA TO MORTALITY PREDICTION**

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Septic shock is a major life-threatening condition in critically ill patients and it is well known that early recognition of septic shock and expedient initiation of appropriate treatment improves patient outcome. Therefore, the identification of new diagnostic tools remains a priority for increasing the survival rate in intensive care units (ICUs). In the present study we tested whether <sup>1</sup>H NMR, GC-MC and targeted LC-MS could be useful for diagnosis and prognosis of septic shock. Serum and plasma samples were collected from septic shock patients within 24 hours of meeting criteria for septic shock and from ICU patients with systemic inflammatory response syndrome (SIRS) but not suspected of having an infection (ICU controls). The mortality of septic shock patients was tracked during their stay at hospital. By using statistical analysis methods such as Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) we were able to distinguish patient groups and detect specific metabolic patterns. Additionally, we have evaluated whether our approach could be applied to define metabolic variation between septic shock survivors and non-survivors and predict patient outcome on the first day of their admission to the ICU. A receiver operating characteristic (ROC) analysis indicated an excellent predictive ability of the constructed OPLS-DA models when compared to the conventionally used ICU scoring systems. Our findings suggest that metabolomics could become a promising approach for diagnosis and prognosis of septic shock in the ICUs and is deserving of further evaluation in other clinical settings, such as the emergency department.

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**P13-10**

**COMPLETE PROFILING OF THE *TRICHOMONAS VAGINALIS* METABOLOME USING LC-MS, LC-MS/MS AND GC-MS AND ITS USE AS AN ANALYTICAL STANDARD**

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*Trichomonas vaginalis* is an anaerobic protozoan parasite with a fermentative energy metabolism that also metabolises oxygen. It is the cause of trichomoniasis, which has been identified as a co-factor in the transmission of HIV/AIDS. The parasite uses phosphoserine and H<sub>2</sub>S to produce cysteine, which is used as an anti-oxidant defence mechanism. *Trichomonas vaginalis* is less well studied compared to other parasites, but its metabolism produces some interesting compounds, such as thioethers, that are not found in many parasites, making it an interesting area of investigation for metabolomic analysis.

There is currently a need for complex analytical standard mixtures in metabolomics, which can be used to identify unknowns in metabolomics samples and assess instrument and column stability. Current methods for producing these mixtures are to prepare a large number of analytical standards and combine them into a number of mixtures, with isomers separated between them. This is time-consuming and requires long-term oversight and repeated preparations as stocks are depleted. *Trichomonas vaginalis* is relatively safe to handle, can be grown overnight on a simple medium to a suitable cell density and initial results show that the metabolome is reproducible from culture to culture, making it a suitable candidate for use as a complex analytical standard.

This work seeks to identify as many metabolites as possible in the metabolome using a mixture of LC-MS, LC-MS/MS and GC-MS techniques by fragmentation and comparison to analytical standards. Current progress on this work, detailing the different metabolites found using the range of analytical methods is shown along with future plans for other analyses.

P13-11

**DIFFERENCE IN THE METABOLOME BETWEEN *ESCHERICHIA COLI* ISOLATED FROM PATIENTS WITH PYELONEPHRITIS AND CHOLANGITIS**

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*Escherichia coli* can lead to invasive infection including pyelonephritis and cholangitis. We postulate that there are differences between *E. coli* strains causing these different clinical syndromes. Using mass spectrometry, we have analyzed the metabolome profile of 11 blood culture *E. coli* isolates from patients with pyelonephritis (Group P), 11 blood culture *E. coli* from patients with cholangitis (Group C), and 10 stool isolates from patients without pyelonephritis or cholangitis (Group S). We have successfully distinguished between different groups of *E. coli* using principal component analysis and hierarchical clustering. We have identified 378 biomarkers that were different between the three groups using reversed phase LC-MS positive mode, 475 biomarkers using reversed phase LC-MS negative mode, 145 biomarkers using normal phase LC-MS positive mode, and 68 biomarkers using normal phase LC-MS negative mode. Analysis using different modes is important because some markers may only be found in the analysis with a particular ionization mode. The criteria for a difference between the three different groups were a P value of <0.01 and a fold change of >16 between at least two groups. We have manually inspected all these biomarkers to exclude false positive biomarkers in statistical analysis. After preliminary analysis, we have identified 20 biomarkers which highly expressed in Group P. Further characterization of these potential biomarkers may shed light on the specific virulence factors, and also may be used in the differentiation of *E. coli* isolates arising from different sites, which will help the clinicians to manage the patients

P13-12

## **METABOLOME ANALYSIS OF THE ENTOMOPATHOGENIC FUNGI FROM THE ISARIA FUMOSOROSEA SPECIES COMPLEX**

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Entomopathogenic fungi from the *Isaria fumosorosea* species complex are natural parasites of many economically important arthropods in temperate and tropical regions, which makes them interesting agents for biological control methods. Unlike chemical pesticides, very complex mode of action of the fungi prevents development of resistance in pests. Indeed, the global market for the biopesticides is rapidly growing at about 15 % annually and is expected to reach \$3.2 billion by 2017. The impact of these interesting biological agents and their metabolites on environment, humans and their potential as a pharmaceutical resource has been of great general interest including regulatory institutions.

However, knowledge of this species complex is still limited; crucial issues how the fungi kill insects, their primary and secondary metabolism, mode of action and toxicity of the secondary metabolites massively produced during mycosis remain poorly understood.

We investigated primary and secondary metabolite profiles in the mycelium of two *Isaria fumosorosea* strains *I.f. PFR 97* (known also as *Apopka 97*) and a new isolate *I.f. CCM -8367* which were cultivated under identical conditions. Seven LC-MS & GC-MS analytical platforms were employed to cover nonchiral & chiral profiling of L,D amino acids & organic acids, amines & purine & pyrimidine bases, sugars, sterols & steroids, phospholipids & glycerides, sugars, secondary metabolites & pigments in the extracted fractions of variable polarity and in their hydrolysates. More than 150 metabolites was identified in both strains that were clearly separated principal component analysis of primary and secondary metabolites and confirmed by cluster analysis. The metabolite data complement the analysis of the genetic background of the studied strains and the differences observed in their efficacy against particular host species.

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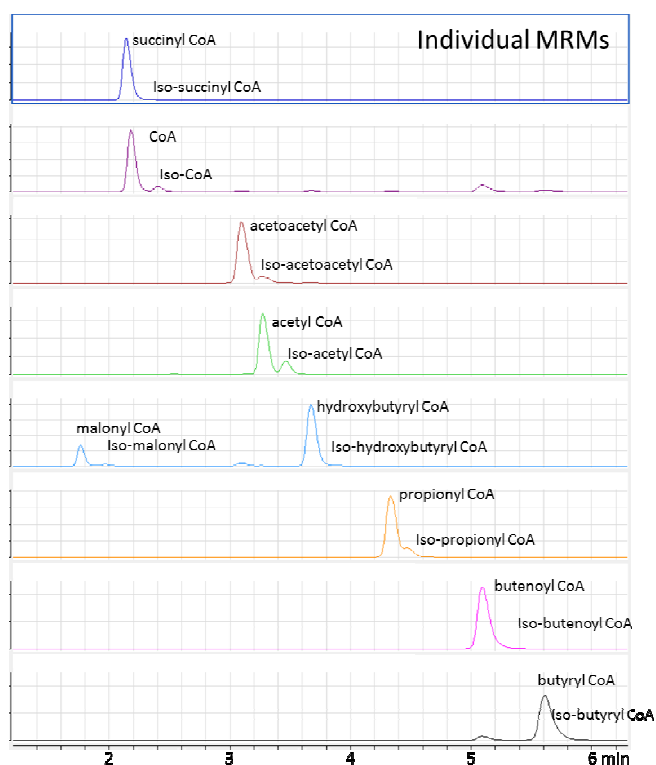
## IMPROVED METHODOLOGY FOR QUANTITATIVE SRM BASED LC-MS/MS FOR THE ANALYSIS OF AcylCoAs FOR RATIONAL DESIGN OF SYNTHETIC BIOLOGY PROCESSES

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The Acetyl-CoA node represents an important and highly regulated control point in central metabolism and thus represents a key parameter for monitoring the metabolic state of the cell and understanding the effects of metabolic engineering and synthetic biological manipulations. These various CoA Thioester derivatives play crucial roles as carriers of activated acyl groups in a number of pathways of lipid, amino acid and carbohydrate metabolism.

In previous work, a rapid, reproducible, sensitive method was developed for 10 Acyl CoAs based on LC-MS/MS with Ultra High Pressure Liquid Chromatography (UHPLC) and Selected Reaction Monitoring (SRM) on a Triple Quadrupole (QQQ) Mass Spectrometer. A novel TCA extraction methodology was developed for extracting the AcylCoA moieties from *E.coli* samples which is more efficient than conventional metabolomic extraction methods. The method allows quantitation of 10 AcylCoA derivatives in 5 minutes with a run-to-run cycle time of 8 minutes and a detection limit of approximately 100fg per component.



However, in the application of this method to characterizing the levels of AcylCoAs in *E.coli* cellular extracts with an engineered exogenous pathway transforming AcetylCoA to Butanol, it was found that each AcylCoA was detected as two isomeric forms; specifically the naturally occurring 3' phospho-acyl-CoA and the acid hydrolysis catalysed degradation product 2'-phospho-acyl-CoA (iso-acylCoA). In this work, the identification of these isomers and approaches to accurate quantitation are described.



## METABOLOMICS-BASED ASSESSMENT OF AQUATIC ANIMAL HEALTH

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Veterinary care and monitoring of aquatic animals faces many obvious limitations, from difficulties identifying symptoms to limits on knowledge of the molecular and biochemical indicators of disease. The most common diagnostic approaches include observable phenotypes and traditional clinical chemistry assays, with only the most basic measures in those assays well-characterized for any given species. Metabolomics is a promising, yet underexploited, method to advance our understanding of a variety of species-specific maladies, as well as our ability to monitor and diagnose aquatic animal health. The systems-scale and potentially untargeted nature of metabolomics makes it ideal to identify biomarkers in the understudied area of aquatic animal health, enabling earlier intervention whether in the field or in captivity.

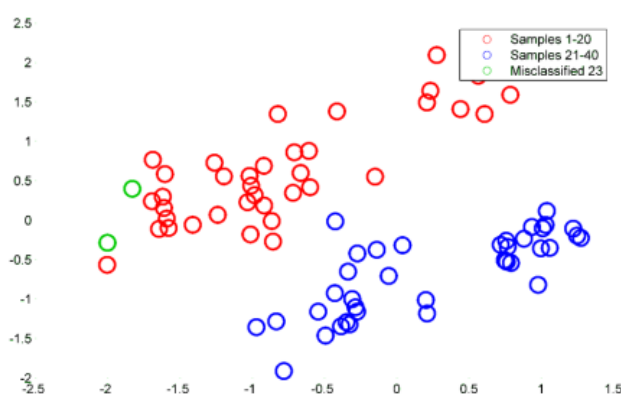


Figure 1: Separation of **bred** and **spawning** salmon based on metabolite profiles.

Here, we present our work in two different aquatic animal systems: the bottlenose dolphin (*Tursiops truncatus*) and the Atlantic salmon (*Salmo salar*). We have studied the impact of pathogenic infections on each species (lobomycosis and furunculosis, respectively), and of inappetance due to salmon spawning, on the plasma metabolite profiles of the animals using two-dimensional gas chromatography coupled to mass spectrometry. In each case, we were able to differentiate between treatment and control groups of animals. These studies have provided significant novel biological insights into each system. In lobomycosis-infected dolphins, the accumulation of fatty acids and depletion of specific vitamin metabolites has suggested the existence of a previously-unexpected catabolic state in such animals. Our investigations of the metabolic impacts of spawning-induced inappetance in salmon (Figure 1) – including changes in specific classes of fatty acids – suggest a distinct, but not dysfunctional, metabolic state in these animals, consistent with their natural life cycle. Taken together, our results suggest that metabolomics is a powerful tool to monitor, diagnose, and learn about aquatic animal health.

P13-15

## IMPACTS OF PARASITISM IN THE ENDANGERED FLORIDA SCRUB JAY

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The Florida Scrub-Jay (FSJ; *Aphelocoma coerulescens*) is an endangered species with less than 2500 breeding pairs left in the wild. Long-term population demography has been maintained for 43 years at Archbold Biological Station, Florida USA. New work in disease ecology has found two unidentified parasites; a *Trypanosoma* & *Filaridae* species. Greater than 60% of the FSJ population is infected with parasitic worms whose *filariids* are found in blood and 20% are infected by *trypanosome*. Initial data has demonstrated decreased breeding success for individuals infected with *filariid* parasites. This study has used a metabolomics approach in a wild species to examine the impacts of parasitism upon host metabolism with the aim of combining identified changes in metabolism with demographic and environmental data to determine affects upon breeding success and survival.

Baseline gender (M/F) and ontogenic (11 days, 85 days, 6 months and adult) metabolite profiles were generated by analysing samples of FSJ serum from 300 individuals using targeted LC-MS amino acid analysis and untargeted GC-MS and LC-MS profiling methods. Results indicate gross changes in metabolism related to age with more subtle effects observed for those individuals that are parasitized.

# LATE POSTER ABSTRACTS

## DROSOPHILA MODEL FOR HUMAN INBORN ERRORS OF METABOLISM DISEASES

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*Drosophila* or fruit flies offer a highly tractable genetic model organism for understanding the molecular mechanisms of human diseases. Previous studies have proven that *Drosophila* and mammals have many identical basic biological, physiological and neurological properties. The *Drosophila* genome has been completely sequenced and encodes for more than 14,000 genes on four chromosomes, one of which is a sex chromosome and three of which carry the bulk of the genome. Nearly 75% of human genes that cause disease are believed to have a functional homologue in *Drosophila*.

Inborn errors of metabolism are single gene (Mendelian) defects in metabolic enzymes. Most are exceedingly rare in the West, but can be a serious health burden in countries with traditions of consanguineous marriage. In this study, we selected maple syrup urine disease and phenylketonuria as two classes of important inborn errors of metabolism (IEM) diseases to establish whether *Drosophila* can be used as a model for human conditions. We have identified and obtained mutants for the *Drosophila* homologues of these diseases, and have performed whole-organism and tissue-specific global metabolomics on the mutants, using methanol:chloroform:water extraction, and zicHILIC chromatography coupled to an Orbitrap MS. We see both expected and unexpected changes in metabolite levels. Based on the results, we can critically evaluate whether *Drosophila* is a successful model of human metabolic disease. Then, the further research on *Drosophila* applications in human metabolic diseases by screening for possible therapies or by screening human samples using metabolomic techniques.

## IDENTIFICATION BY HIGH RESOLUTION MASS SPECTROMETRY OF A NEW METABOLITE IN THE STRICT AEROBIC BACTERIUM *ACINETOBACTER BAYLYI* ADP1

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We aim to complete the metabolic activities inventory in the soil bacterium *Acinetobacter baylyi* ADP1 (ADP1) by different methods (comparative genomics, high throughput growth phenotyping, RNAseq...), with a special focus on the discovery of new enzymatic activities through metabolomics.

We monitored metabolism adaptation by the use of an alternative carbon source by high-resolution mass spectrometry (LC-ESI-LTQ/Orbitrap). The comparison of a metabolome from cells grown on succinate (reference carbon source) or quinate (alternative carbon source) highlighted the different intermediaries of this well-known catabolic pathway.

Similar studies done on *Pseudomonas* strains showed that the major changes regarding the metabolite composition involve the degradation intermediaries of the carbon sources used [1-2]. However, in ADP1, we did not notice such a 'core metabolome': almost 40% of the detected metabolites in ADP1 present at least a 4 fold variation according to the carbon source used.

But the most important point is that many metabolites, not identified, are only present on quinate. We present here the structural elucidation of one of these metabolites. The database interrogation using accurate mass did not allow us to propose an identification consistent with the CID fragmentations. These fragmentations were analyzed by high resolution and led us to suggest that the compound is most likely a tyrosine substituted in benzylic position by an aminomethyl group. To our knowledge, this metabolite has never been described so far. The experimental approach to elucidate this structure is discussed.

Thus, high-resolution mass spectrometry can be a very powerful tool to elucidate new metabolites structure.

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Key words: microbial metabolomics, LC-MS, structural elucidation

## **ISOTOPIC RATIO OUTLIER ANALYSIS (IROA) TO INVESTIGATE CHANGES IN THE SECONDARY METABOLITE PROFILES OF *CYSTOBACTERINEAE***

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The IROA protocol has been applied to the analysis of *Cystobacterineae* secondary metabolomes. In this IROA analysis, bacterial strains were cultivated under high-Fe (95% <sup>13</sup>C) and low-Fe (5% <sup>13</sup>C) conditions. Biological compounds from samples associated with 95% <sup>13</sup>C and 5% <sup>13</sup>C media are differentiable and therefore high and low Fe samples can be pooled and prepared simultaneously, removing sample-to-sample variance and ion suppression. Artifactual information identified by their absence of isotopic signature is removed and the identification of each compound enabled by the use of ultra-high resolution mass measurement and the knowledge of the number of carbons in each molecule.

The ClusterFinder software identified 160 IROA peaks, at the most stringent level. The iron limitation in the media (5% <sup>13</sup>C culture) significantly changed the levels of many compounds. Complimentary to the IROA ClusterFinder, the analysis tool SmartFormula™ allowed for the verification of molecular formula suggestions by combining accurate mass and isotopic pattern information.

In this experiment all of the expected compounds were identified, and a number of novel molecules were identified by examination of their IROA patterns. The formulae of these peaks have been tentatively determined and the identity of these molecules is currently being examined.

## Comparative metabolomics of erythroid lineage: implications for malaria control

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*Plasmodium berghei*, a popular *in vivo* model for malaria preferentially invades reticulocytes. A discovery based comprehensive comparison of the metabolomes of uninfected reticulocytes (immature erythrocytes) and normocytes (mature erythrocytes) was performed to understand differences in intracellular host cell environments. Using LCMS and GCMS, 384 individual metabolites were identified in uninfected erythrocytes and more than half of them were found to be up regulated in reticulocytes. This implied that a reticulocyte is a more complex cell with huge metabolic reserves and possibly active metabolism which the malaria parasite may exploit. It was found that some of the already published apparent differences attributed to *P. berghei* and *P. falciparum* metabolism were due to the cell types they invade under laboratory conditions e.g, enzymes of glutathione biosynthesis are dispensable for *P. berghei* blood stage development *in vivo* but are essential for *P. falciparum in vitro* blood stage development which could be explained by the comparatively high levels of glutathione synthesis intermediates in reticulocytes.

Comparative metabolomics predicted that intermediary carbon metabolism and pyrimidine biosynthesis (targets for drug development) would be dispensable in *P. berghei* and we successfully knocked out key *P. berghei* genes involved in these pathways. The precise blood stage phenotype of these mutants is under analysis as is the extent of exo-erythrocytic development. This implies that many reticulocyte metabolites can indeed be scavenged by the parasite. The implications for intervention strategies which usually focus solely on parasite metabolism are:

1. Therapeutic treatment of blood stage malaria should differ according to the target host cell e.g. for *P. falciparum* (usually found in normocytes) and *P. vivax* (reticulocyte preferring).
2. Relapsing *P. falciparum* infections might result from parasite survival in immature normocytes.

## **Analysing durable anti-fungal resistance processes in cereals by metabolic fingerprinting**

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Introduction of durable resistance genes in crops is an important strategy to prevent yield loss and to maintain food security. The resistance gene *Lr34* of wheat, coding for an ATP-binding cassette transporter, confers durable resistance to four major fungal pathogens. The molecular mechanism leading to this resistance is not known. A metabolic fingerprinting approach based on ultra-performance liquid chromatography – high-resolution mass spectrometry on transgenic barley lines was chosen to identify the substrate transported by *Lr34*.

## THE SYSTEMATIC ANNOTATION OF THE METABOLIC COMPOSITION OF THE MODEL LEGUME *MEDICAGO TRUNCATULA* USING UHPLC-MS-SPE AND NMR

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The large-scale profiling of plant metabolites (i.e. metabolomics) is advancing our fundamental understanding of plant biochemistry, yielding discovery of novel metabolites and gene functions, and providing an advanced mechanistic understanding of plant responses to biotic, abiotic, and environmental stimuli. However, the current depth-of-coverage (~10-20%) is still a major limitation and there is a critical need to better define the metabolic composition of plants. The specific aim of this project is to address this need through the systematic identification of the metabolic composition of the leading model legume *Medicago truncatula* which is a close relative of alfalfa. This is being achieved through the use of an ensemble of technologies including GC-MS, UHPLC-MS, UHPLC-MS/MS, UHPLC-UV-MS-SPE, and NMR. As a result, the biological context of our metabolomics experiments are being increased and provide even greater opportunities and understanding of this model legume.

Our first approach to systematic identification is the co-characterization of authentic standards and the creation of custom spectral databases. Accurate mass measurements and isotopic ratios are being used to predict putative elemental formulas for all observed but unknown components within the metabolic profile. Predicted elemental formulas are being refined and validated through the co-analysis of <sup>13</sup>C isotopically labeled *M. truncatula* aerial tissues. Refined elemental formulas are used to search large web based chemical databases (PubChem, Chem Spider, MassBank, etc.) for putative metabolite identifications. Confirmation of the putative identifications are being performed through the purchase and co-characterization of authentic standards whenever possible/practical and added to our spectral libraries. UHPLC-QToFMS is also being coupled with automated solid-phase extraction (SPE) for the purification and concentration of unknown plant metabolites. Targeted recovery amount is typically 1-25 µg. Dried, targeted analytes isolated on SPE cartridges are eluted with deuterated solvents for further NMR analyses. Structural identifications are then made from combined UHPLC-MS/MS and NMR data.