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Agenda

Monday 10 november 2014

10:00-11:00 **Registration**

11:00-11:30 **Welcome and Introduction to the workshop**
Silvia Catinella
IMaSS President
Andrea Camporese, Fochesato Stefania
Istituto della Ricerca Pediatrica (IRP) Città della Speranza
Giorgio Perilongo, Eugenio Baraldi
Dipartimento di Salute della Donna e del Bambino (SDB)

Chairman: Eugenio Baraldi, Giuseppe Giordano

11:30-12:30 Anisha D, Wijeyesekera
"NMR AND MS-BASED METABOLIC PROFILING IN SYSTEMS MEDICINE"

12:30-12:45 M1-GUT MICROBIOTA METABOLOME IN PEDIATRIC DISEASES
Pamela Vernocchi

12:45-13:00 M2-METABOLOMIC APPROACH TO THE STUDY OF ALLERGIC DISEASE IN PEDIATRIC RESEARCH
Irene Costa

13:00-14:00 **Lunch**

14:00-15:00 **Assemblea soci IMaSS**

Chairman: Pietro Traldi, Enrico Davoli

15:00-15:45 Oscar Yanes
"METABOLOMICS: ONLY SUITABLE FOR MULTIDISCIPLINARY TEAMS"

15:45-16:00 M3-A SUCCESSFUL METABOLOMICS STUDY: FROM BENCH TO BEDSIDE IN POST-CARDIAC ARREST
Roberta Pastorelli

16:00-16:15 M4-METABOLOMICS OF GRAPE BERRY POSTHARVEST WITHERING
Flavia Guzzo

16:15-16:30 M5- ASSESSMENT OF SAMPLE PREPARATION STRATEGIES FOR FAST GAS CROMATOGRAPHY-MASS SPECTROMETRY BASED "METABOLOMICS STUDIES
Stefano Dugheri

16:30-16:45 M6-METABOLOMIC PROFILE OF NEURAL STEM/PRECURSOR CELLS (NPCS) UNDER INFLAMMATION: IMPLICATIONS FOR THE DISCOVERY OF NOVEL MECHANISMS OF THE NEURO-IMMUNE CROSS TALK
Denise Drago

16:45-17:00 M7-EFFECT OF K-RAS SPECIFIC MUTATIONS ON METABOLIC FINGERPRINTS IN LUNG CANCER CELLS: IMPLICATIONS FOR SUSCEPTIBILITY TO ANTICANCER TREATMENTS.
Laura Brunelli

17:00-17:15 **Coffee break**

Chairman: Roberta Pastorelli, Giuseppe Pieraccini

17:15-17:30 M8-A TARGETED LIPIDOMICS ANALYSIS TO STUDY NEURODEGENERATIVE DISORDERS
Ruggero Ferrazza

17:30-17:45 M9- NOVEL BIOINFORMATICS TOOLS FOR METABOLOMICS AND LIPIDOMICS USING ION MOBILITY-MS
Giuseppe Astarita

17:45-18:00 M10-ADDING COORDINATES TO THE METABOLOME: ION MOBILITY DERIVED COLLISION CROSS SECTIONS.
Giuseppe Paglia

18:15 **Departure to down town**

19:30 **Happy Hour (Caffè Pedrocchi)**

Tuesday 11 november 2014,

Chairman: Pietro Franceschi, Valentina Mileo

9:00-10:00 Wehrens Ron
"METABOLOMICS, BIOINFORMATICS AND STATISTICS: THREE SIDES OF THE SAME COIN"

10:00-10:15 T1-METABOLOMIC PROFILE IN INTRAUTERINE GROWTH RESTRICTION FETUSES
Marianna Tucci

10:15-10:30 T2-MARKERS OF ENDOTHELIAL DAMAGE AND METABOLOMIC PROFILE IN MONOCHORIONIC TWIN PREGNANCIES WITH SELECTIVE INTRAUTERINE GROWTH RESTRICTION
Marianna Tucci

10:30-10:45 T3-NMR SPECTROSCOPY FOR METABONOMICS: A POWERFUL TOOL FOR BIOMARKERS INVESTIGATIONS.
Debora Paris

10:45-11:15 **Coffee break**

Chairman: Silvia Catinella, Riccardo Zecchi

11:15-12:15 Stocchero Matteo
"CHEMOMETRICS IN NMR BASED METABOLOMICS: DATA PRE-TREATMENT AND PROJECTION METHODS"

12:15-12:30 T4-TARGETED LIPIDOMICS PROFILE AND NMR METABOLOMICS OF INJURED MOUSE BRAIN
Fabiana Piscitelli

12:30-12:45 T5-METABOLOMIC ANALYSIS FOR THE STUDY OF THE FREQUENT WHEEZING IN PRE-SCHOLAR CHILDREN
Paola Pirillo

12:45-13:00 T6- STANDARDIZED METABOLIC PHENOTYPING SOLUTIONS FOR BIOMARKER RESEARCH IN MULTIFACTORIAL DISEASES
Stefan Ledinger

13:00-14:00 **Lunch**

Chairman: Enrico Davoli, Marco Roverso

14:00-14:15 T7- METABOLOMIC PROFILING OF ANIONIC METABOLITES IN ORAL CANCER CELLS BY CAPILLARY ION CHROMATOGRAPHY HR/AM MASS SPECTROMETRY
Marc Günder

14:15-14:30 T8-ABSOLUTE REAL-TIME ETHYLENE AND FURANEOL DETECTION WITH PTR/SRI-MS
A. Algarra Alarcon

14:30-14:45 T9-A NEW METHODOLOGY FOR QTL ANALYSIS OF FRUIT VOLATILE SECONDARY METABOLITES BY PTR-TOF-MS COUPLED TO DATA MINING
Luca Cappellin

14:45-15:00 T10-A FAST LC-MS METHODOLOGY FOR MEMBRANE LIPID PROFILING THROUGH HILIC CHROMATOGRAPHY
Andrea Anesi

15:00-15:15 T11- MASS PROFILER PROFESSIONAL FOR BIOMARKERS DISCOVERY: LC-QTOF AND ION MOBILITY SYSTEM FOR CLINICAL AND FOOD APPLICATIONS
Nicola Cimino

15:15-15:30 T12-EXPLORING METABOLOMIC SIGNATURES OF ALTERED METABOLISM IN BREAST CANCER CELL
Maria José Caramujo

Chairman: Pietro Franceschi, Giuseppe Giordano, Enrico Davoli, Giuseppe Pieraccini

15:30-16:30 **Group discussion**

16:30-16:45 **Closing Remarks**

16:45 **Coffee break**

ABSTRACT

NMR and MS-based Metabolic Profiling in Systems Medicine

Anisha D, Wijeyesekera

Division of Computational and Systems Medicine Department of Surgery and Cancer Faculty of Medicine Imperial College London South Kensington Campus SW7 2AZ

Individual and population-level prognosis, diagnosis and responsiveness to therapy require deep exploration of gene, environment and lifestyle factors. Taken together, these influences shape human health and physiology, and impact on patient outcome. Systems medicine involves the conceptualisation of these influences, and is by definition a holistic approach to furthering understanding of health and disease based on knowledge of deep patient biology. Metabolic profiling using Nuclear Magnetic Resonance (NMR) Spectroscopy and Mass Spectrometry (MS), facilitates a systems medicine approach, by capturing the small molecular species representing non-genetic (i.e. environment, diet, lifestyle) exposures as well as downstream mediators and consequences of those exposures. As in other areas of health research, NMR spectroscopy is a primary platform for analysis of human biological specimens, benefitting from good reproducibility and robustness, acquisition of untargeted global metabolic phenotypes (enabling wide metabolome coverage), and the capacity to provide quantitative data. Quantification of biomarkers in metabolic profiling studies to augment characterisation of metabolic phenotype, is invaluable in better understanding alterations to biological processes in relation to disease. Among the available analytical techniques, MS offers a combination of sensitivity and selectivity of analysis and quantification of molecules at low levels of concentration. In this talk, some of the main applications of NMR and MS in Systems Medicine research related to human health and disease are highlighted, including metabolic profiling in interventional studies, metabolic phenotype characterisation for prognosis and diagnosis, and patient journey phenotyping to aid in clinical decision making.

M1

Gut Microbiota Metabolome in Pediatric Diseases

P. Vernocchi[1], F. Del Chierico[1], D. C. Accardi[1], G. Catesini[1], A. Russo[1], C. Rizzo[1], P. Paci[2] M.Muraca[1], V. Lucidi[1], V. Nobili[1], B. Dallapiccola[1], L. Putignani [1]

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The microbiota plays an important role in child health by acting as a barrier against pathogens and their invasion with a highly dynamic modality, exerting metabolic functions and stimulating the development of the host immune system, which influences all of the growth and aging processes. Indeed many pediatric diseases are associated with changes in gut microbiota. High-throughput meta-omics-based approaches advent may assist in unveiling the gut microbiota alteration.

The aim of this study was to investigate the gut microbiota of cystic fibrosis (CF), non-alcoholic steatohepatitis/non-alcoholic fatty liver disease (NASH/NAFLD), juvenile idiopathic arthritis (JIA) and inflammatory bowel disease and syndrome (IBD/IBS) patients by metagenomic and metabolomic combined analyses. The gut microbiota composition and modulation were investigated by metagenomic and metabolomic combined analyses in relation with healthy children (HC). The metabolomics was performed by GC-MS/SPME and ^{1}H-NMR, while metagenomics was carried out by 454 pyrosequencing platform.

About 250 VOCs, for each group, were detected by GC-MS/SPME and 20 molecules characterized with ^{1}H-NMR for CF patients. The inter-individual variability of VOCs levels resulted high. The level of esters, alcohols and aldehydes were higher in CF, NAFLD/NASH IBD/IBS and JIA patients. On the contrary in HC the SCFAs were more expressed than patients. ^{1}H-NMR analysis in CF patients showed lower levels of AA and uracil. The metagenomics on patients indicated specific OTUs profiles associated to disease characteristics, showing a high correlation between gut microbiota and disease. The integration of metagenomic, metabolomic and clinical data allowed to establish a typical phenotype patient-related. By this integrated approach it's possible to generate personalized "omics" charts to provide a translational medicine tool for personalized therapy.

M2

Metabolomic approach to the study of allergic disease in pedatric research

Irene Costa[1], Francesca Tirelli[2], Paola Pirillo[1], Mauro Naturale[1], Antonina Gucciardi[1], Silvia Carraro[2], Eugenio Baraldi[2] and Giuseppe Giordano[1].

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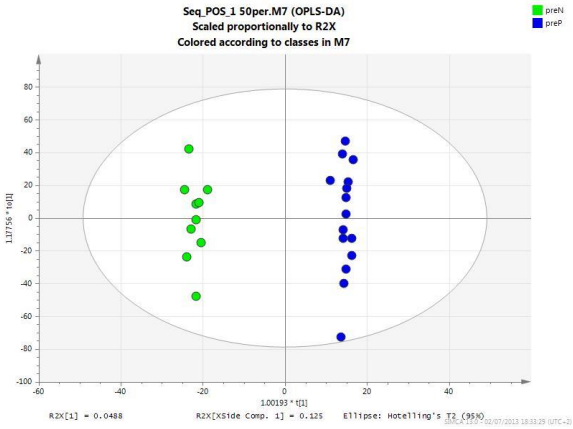
The metabolomics approach is based on the global identification, not driven by a priori hypothesis, of a high number of metabolites[1] in a biological fluid; this allows the characterization of the metabolic profile typical of a certain condition, enabling the identification of metabolites or pattern of metabolites that could be useful in discriminating different groups of subjects. The potential impact of this science in the field of food allergy is particularly interesting. Food oral challenge is considered the gold standard test for the diagnosis of food allergy; however, this test is time consuming, expensive, must be performed in a guarded setting, and exposes the child to the risk of developing severe allergic reactions. However no accurate biomarkers have been identified capable of predicting immediate allergic reactions caused by the ingestion of a certain food in children on an exclusion diet for that food. The main purpose of the study is to identify urinary and blood metabolic patterns predictive of response to milk challenge test in children with milk protein allergy, by using metabolomic analysis. Metabolomic[2] analysis was applied to characterize a urinary basal metabolic pattern that could discriminate between children who failed the challenge test from those who passed it. This is a prospective study including 25 children (19 males and 6 females, average age 4 years) with clinical indication for milk challenge test for previous generalized clinical reaction to milk; all of them had been following an elimination diet for this food for an average time of 3,7 years. An urine, plasma and dry blood spot sample was collected for each patient before and after the milk challenge test. Every child underwent a milk challenge test, carried out in standardized way and according to the response, the patients were classified in positives (15 children) and negatives (10 children). The metabolomic analysis of the urine was performed by using a high sensitivity mass spectrometry platform coupled with UPLC (Ultra Performance Liquid Chromatography). For UPLC, 2 chromatographic columns with different hydrophilicity and hydrophobicity were used, and the samples were analysed in positive and negative polarity. Metabolomic data were analysed with multivariate statistical methods (unsupervised and supervised). Clinical data were analysed using classical statistics (due to their non-normal distribution, non-parametric tests were used; tests were considered significant for $p<0,05$).



4 dataset were obtained (deriving from the combined use of two chromatographic columns, each with two polarities); for each of them, it was possible to build a robust model enabling the discrimination between children with positive response to challenge test and children with negative test. 4 variables emerged as particularly relevant in the differentiation of the two groups. Moreover, using the 2 most relevant variables it is possible to create a decisional tree which permits the correct prediction of food challenge response in every children but one. This study demonstrates for the first time that metabolomics analysis is able to predict food challenge test response, on the basis of basal urine metabolomics profile, in Children with cow's milk protein allergy.

Anyway, apart from the identification of the involved metabolites, the most relevant finding is the existence of a "metabolic fingerprint"[3] that individuates children with higher risk of positive response to challenge test. The development and validation of this method could allow avoiding the food challenge test in children who have high probability of failing it developing a generalized allergic reaction.

Reference

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Metabolomics: Only Suitable for Multidisciplinary Teams

Oscar Yanes

Centre for Omic Sciences, Spain

Metabolomics is defined as the comprehensive and quantitative analysis of metabolites in living organisms. Among the omic sciences, metabolomics is possibly the most multidisciplinary of all, involving knowledge about electronic engineering and signal processing, analytical and organic chemistry, biostatistics and statistical physics, and biochemistry and cell metabolism. Here an untargeted metabolomics workflow will be detailed that provides examples of this multidisciplinarity to understand disease pathogenesis.

M3

A Successful Metabolomics Study: from Bench to Bedside in post-Cardiac Arrest

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3. Department ofCardiovascular Research, IRCCS-Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy.

The majority of patients resuscitated from cardiac arrest die within 72 h, due to what is termed “post-cardiac arrest syndrome”. Such pathophysiological state is characterized by myocardial dysfunction with circulatory shock, systemic inflammation with activation of the clotting system, and evolving brain injury. Morbidity and mortality after successful cardiopulmonary resuscitation (CPR) largely depend on recovery of neurologic function. As many as 30 %of survivors of cardiac arrest, in fact, suffer permanent brain damage. Due to the complexity and interplay of events occurring during the post-cardiac arrest syndrome, predicting survival and functional outcome in patients resuscitated from cardiac arrest remains a difficult task, especially in the early postresuscitation phase. The comprehensive quantitative assessment of plasma metabolites might help fill this information gap as metabolite differences in plasma provide the closest link to cellular metabolism in the whole body and its response to resuscitation.

In our earlier investigation in a rat model of cardiac arrest and CPR, we adopted a mass spectrometry-based untargeted metabolomics approach (LC-MS/MS) to identify perturbations in post-resuscitation circulating metabolites. Following resuscitation, we identified alterations in a major route of the tryptophan (TRP) catabolism, namely kynurenine pathway (KP) [1]. KP is mainly activated upon inflammatory stimulation and is implicated in instances accounting for neurological injury (i.e. stroke and cerebral dysfunction) in intensive care unit (ICU) patients and in adverse prognosis in patients with coronary artery disease. We then used a targeted strategies (LC-MRMMS with isotope-dilution) to investigate KP activation after cardiac arrest and its relationship with the severity of post-cardiac arrest syndrome, by a fully translational approach [2]. More specifically, KP was assessed during the initial hours and days following resuscitation from cardiac arrest in rats, pigs, and few humans samples. We demonstrated that KP is activated following resuscitation from cardiac arrest in three different species. Accordingly, KP activation was initially observed in resuscitated rats, was then validated in pigs subjected to cardiac arrest and CPR, and was ultimately

confirmed in a small cohort of human patients as proof-of-concept. Indeed, increases in plasma levels of KP metabolites, kynurenine (KYN), kinurenic acid (KYNA) and 3-Hydroxyanthranilic acid (3-HAA), occurred during the initial hours following resuscitation and persisted up to 3–5 days following cardiac arrest. KP activation showed an equivalent time course in rats, pigs, and humans, and was significantly related to the severity of post-resuscitation myocardial dysfunction, cerebral injury, functional outcome and survival. Then the KP has been profiled in a large population of patients resuscitated from out-of-hospital CA (245 patients enrolled in a prospective multicenter observational study in 21 intensive care units in Finland). The KP is activated early after resuscitation from out-of-hospital CA. Patients who died before ICU discharge exhibited significantly higher levels of KP metabolites at ICU admission compared with those who survived. All KP metabolite levels predicted ICU death after resuscitation, whereas KYNA and 3-HAA seem to have the potential to further predict long-term neurological outcome after CA [3]. Further studies are in progress to elucidate whether the KP may be amenable to targeted therapeutic interventions. Mass spectrometry-based metabolomics strategies (from untargeted to targeted) once again offer promises for the discovery of potential biomarkers helping in clarifying unexplored biochemical pathways relevant to the human diseases.

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M4

Metabolomics of Grape Berry Postharvest Withering

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Grape postharvest dehydration is a technique used to enhance specific berry quality traits for the production of special wines. In Verona (Italy) province this technique is traditionally employed for producing premium wines like Amarone, obtained mainly from Corvina grape characterized by the capability to withstand a long period of postharvest dehydration.

Many of the modification affecting berries during postharvest withering are obviously related to water loss and consequent berry juice concentration. However, since the cells of the berries keep the ability to modulate their gene expression for a long time during the dehydration process, many other peculiar traits of dehydrated grapes are achieved in berries which are, at least in part, under strict genetic control. From the point of view of grape berry cells, the dehydration process is characterized by severe stresses probably partially overlapped, at least at the beginning of the process, with an extended ripening program.

In order to understand what happens during the postharvest withering process in different grape varieties that differ for the withering performances we analyzed and compared transcriptomics and metabolomics changes during the process in Corvina, Sangiovese, Oseleta, Merlot, Cabernet Sauvignon and Shiraz, placed in the same environmental conditions. The untargeted metabolomics analyses reveal that, besides the obvious metabolite concentration due to the water loss, only in some varieties the "constructive" processes (*de novo* accumulation of certain metabolites) prevail on the metabolite degradation. Furthermore, a general dramatic rearrangement of the berry transcriptome was observed during the postharvest period in all grapes. Corvina was characterized by a much higher induction of gene expression compared to the other cultivars. Moreover, in this cultivar the highest metabolite and gene expression modulation require a long lasting process, being abolished by artificially induced short withering.

M5

Assessment of Sample Preparation Strategies for Fast Gas Cromatography-Mass Spectrometry Based Metabolomics Studies

Stefano Dugheri [1], *Vincenzo Cupelli* [2], *Giulio Arcangeli* [2], *Ilenia Pompilio* [2] *Alessandro Bonari* [2]

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The role of urinary metabolic profiling in systems biology research is expanding. This is because of the use of this technology for clinical diagnostic and mechanistic studies and for the development of new personalized health care and molecular epidemiology (population) studies. The methodologies commonly used for metabolic profiling are NMR spectroscopy, liquid chromatography mass spectrometry and gas chromatography mass spectrometry (GC/MS). For the comprehensive metabolite profiling of human plasma, sample preparation is a crucial step. Here the newest trends and innovative miniaturization of extraction techniques, like single-drop microextraction (SDME), hollow fiber liquid-phase microextraction (HF-LPME), dispersive liquid-liquid microextraction (DLLME), solid phase microextraction (SPME) and micro extraction by packed sorbent (MEPS) are discussed, including the derivatization by *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA), *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA), trimethyloxonium tetrafluoroborate, 1-pyrenyldiazomethane (PDAM), pentafluorobenzylbromide, for analysis of urine metabolites and thoroughly developed and optimized from automatic preparation to detection with Fast GC. The availability of the GC/MS electron impact spectral library further facilitates the identification of diagnostic biomarkers and aids the subsequent mechanistic elucidation of the biological or pathological variations. Particular attention is given to ecological aspects therefore the use of new methodological solutions and the related instruments and devices for the efficient sample preparation and a friendly use of mass spectrometry apparatus. For an innovative powerful meaning in high-throughput routine, the generality of the structurally informative mass spectrometry fragmentation patterns together with the chromatographic separation and software automation are also investigated.

References (1 line spacing)

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M6

Metabolomic Profile of Neural Stem/Precursor Cells (NPCs) under Inflammation: Implications for the Discovery of Novel Mechanisms of the Neuro-Immune Cross Talk

Denise Drago[1,5], *Christian Frezza*[2], *Edoardo Gaude*[2], *Veronica Basso*[3], *Anna Mondino*[3], *Angela Bachi*[4], *and Stefano Pluchino*[5]

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Neural stem/precursor cells (NPCs) have recently shown great potentials in central nervous system (CNS) disorders treatment approaches. NPCs might be therapeutically efficacious through a number of bystander mechanisms, alternative to cell replacement, which include the secretion of immune modulatory or neurotrophic paracrine factors, resulting from highly sophisticated mechanisms of cell-to-cell communication. This paracrine hypothesis has inspired an alternative outlook on the use of stem cells in regenerative neurology. It is in fact becoming increasingly accepted that stem cells secrete a vast array of proteins – including growth factors, cytokines, chemokines, metabolites and bioactive lipids – that regulate their biology in an autocrine or paracrine manner, while orchestrating multiple interactions with the surrounding microenvironment (therapeutic plasticity) [1]. Our goal is to deeply focus on intercellular neuro-immune cross-talk by secretome-based screening and analysis of intracellular soluble small metabolites in adult mouse NPCs by metabolomics. This analysis provided us a "metabolic signature" associated with NPCs phenotype in different cytokine-enriched conditions, which we have shown to affect NPC biology, both *in vivo* and *in vitro* [2]. Exo- and endometabolome of NPCs in basal condition or after conditioning with either pro-inflammatory Th1 (e.g. IFN-γ, TNF-α and IL-1β) or anti-inflammatory Th2 (e.g. IL-4, IL-5 and IL-13) cytokines were analyzed by both

untargeted and targeted mass spectrometry (LC-MS/MS and GC-MS/MS). Consistently with our previous proteomics studies [3], we found that Th1 inflammatory environment can strongly affect the NPCs cellular metabolism leading to a significant and specific impairment of glycolysis, compared to either basal and Th2-condition. In addition, this study suggests an alteration in the arginine pathway in Th1 treated NPCs. This pathway is well known in myeloid tumor cell metabolic reprogramming which is essential to promote immune suppression and tumor evasion. Besides the arginine pathway alteration, the secretion of Arginase I in the NPCs secretome enhances the suppressive microenvironment depleting arginine and leading to a significant anti-proliferative effect on target T lymphocytes. Taking a look on well-established cancer cells metabolic reprogramming in the immune response and performing this metabolomic analysis on NPCs, we expect to improve our knowledge on NPC-mediated mechanisms of immune regulation. A more in-depth metabolomic characterization of the neurotrophic, regenerative and immunosuppressive factors including metabolites and bioactive lipids within stem cells is mandatory in the attempt to select and identify the molecules responsible for their therapeutic effects. Elucidation of these aspects may be a crucial and essential step in the control and improvement of NPCs to maximize therapeutic strategy in regenerative medicine of the CNS.

References

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M7

Effect of K-RAS Specific Mutations on Metabolic Fingerprints in Lung Cancer Cells: Implications for Susceptibility to Anticancer Treatments

Laura Brunelli[1], *Elisa Caiola*[2], *Mirko Marabese*[2], *Massimo Broggini*[2], *Roberta Pastorelli*[1]

1. *Department of Environmental Health Sciences; "Mario Negri", Milano, Italy*
2. Department of Oncology; IRCCS-Istituto di Ricerche Farmacologiche "Mario Negri", Milano, Italy.

Background. Lung cancer is the leading cause of cancer-related mortality worldwide. Non-smallcell- lung cancer (NSCLC) accounts for approximately 85% of lung cancer cases. Most patients with NSCLC have locally advanced and distant metastatic disease at the time of presentation, which is associated with a 5-year survival rate of less than 10% and 5%, respectively. To improve clinical endpoints for patients with lung cancer, targeted therapies are being used increasingly with encouraging results, particularly in patients with specific molecular features. KRAS mutations are present in about 20% of NSCLC patients, mostly at codon 12 and have been associated to tumor progression and resistance to chemotherapy in many types of cancer. Increasingly evidences seem to highlight that the definition of KRAS status is not sufficient to determine its predictive and/or prognostic role in NSCLC, and indicate that different KRAS mutations could differently impact on crucial cellular processes [1]. The occurrence of metabolic deregulation driven by KRAS mutations in the context of lung cancer and a systematic characterization of the metabolic pathways active in lung cancer cells harboring different KRAS mutations are lacking.

Methods: Two independent clones from the human NSCLC cell line NCI-H1299 overexpressing WT KRAS, and the three most common NSCLC KRAS mutations (G12C, G12V and G12D) were screened. All the clones had comparable KRAS expression levels and activity. We applied an explorative untargeted metabolomics approaches by liquid chromatography/tandem mass-spectrometry to characterize the largest possible number of metabolites from relevant or potentially affected metabolic pathways in NSCLC cells clones expressing mutated forms of KRAS at codon 12 (G12C, G12D, G12V).

Results: Each KRAS mutant clone has its own metabolomics signature, distinct from KRAS WT. KRAS G12C, G12D and G12V were significantly separated from KRAS WT by respectively 74, 58 and 48 singular metabolic species, identified by database searches (METLIN, HMBD). The

majority of identified metabolites were shared by all the KRAS mutant clones even if, mutant G12C had the largest number of specific unique metabolites. When the relative fold changes in the significantly altered metabolites in each KRAS mutant clone were compared to the WT, it was clear that KRAS mutations generally downregulated the amount of metabolites compared to the WT clone. To interpret the biological significance of the metabolite changes in the three KRAS mutant clones, we used MetaboAnalyst tools to link metabolites to metabolic pathways. Our strategy highlighted the involvement of both glutaminolysis and glutathione pathways as the main processes affected by KRAS mutations [2]. Based on our metaboolomic evidence, we examined the glutamine dependency of KRAS clones. Results of glutamine deprivation on cell growth in vitro showed that removal of glutamine strongly reduced the growth of both WT and mutant KRAS expressing clones.

Conclusion: The preliminary findings reported herein indicate that specific KRAS mutations at codon 12 are associated to different metabolomics profiles that might have an impact on the response to anticancer treatments. Our explorative strategy allowed us to indicate the presence of fragile points in the metabolic network of different KRAS mutants in lung cancer. Based on our untargeted metabolic evidences, we are now applying a targeted metabolic profiling (Biocrates platforms) to identify and quantify specific changes in metabolic pathways associated with the different susceptibility of KRAS mutant clones in response to different anticancer treatments relevant in clinical settings.

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M8

A Targeted Lipidomics Analysis to Study Neurodegenerative Disorders

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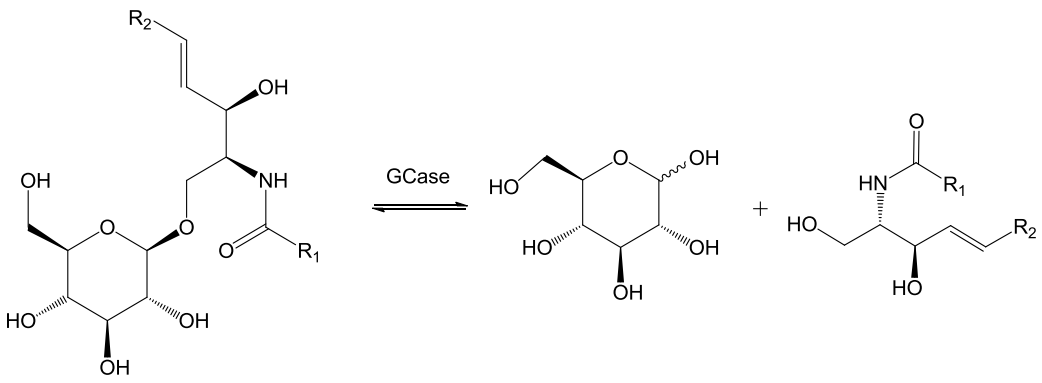
Parkinson's disease (PD) is a degenerative disorder of the central nervous system resulting from the death of dopaminergic neurons in the *substantia nigra*, and it is characterized by the accumulation of the protein α-synuclein in neurons, creating the inclusions called Lewy bodies. PD is an idiopathic syndrome, having no known cause, with sporadic PD being much more common than familial PD [1].

Among the recognized risk factors for PD are deficiencies in the lysosomal enzyme β-Glucocerebrosidase (GCase), encoded by the glucocerebrosidase (GBA) gene [2]. This enzyme is responsible for the breakdown of glucosyl-ceramides (Glc-Cer) to glucose and ceramides (Cer), according to the following scheme:



Insufficient activities of GCase trigger Gaucher's disease (GD), the most common lysosomal storage disorder, characterized by the accumulation of the substrate Glc-Cer in the lysosomes [3]. The association between GD and PD is not yet clear [2] [4], and several experiments have been carried out so far with the aim of better understanding the connection between GCase and PD [5] [6].

In this presentation we report a targeted lipidomics analysis with the aim of assessing whether deficiencies in the GCase enzyme in primary neuronal cultures from mice result in detectable changes in the relative amount of glucosyl-ceramides and ceramides. To this purpose, we analyzed two different preparations of lipid extracts coming from different treatments:

Two biological replicates of primary neuronal cultures from wild-type mice (control samples)
Two biological replicates of primary neuronal cultures from GBA knockout mice (treated samples)
The samples, dissolved in methanol, were subjected to HPLC-ESI-MS analysis by using a Triple Quadrupole Mass Spectrometer (API3000, AB SCIEX) as mass analyzer. The targeted sphingolipids were detected in Precursor Ion Scan mode, isolating the ions with m/z=264.3 in the third mass analyzer. The absolute amount of each species was determined using an external mixture of the commercially available standards Cer(d18:1/24:0) and Glc-Cer(d18:1/16:0). For each sample, at least two technical replicates were acquired.
The relative molar ratios for any sphingolipid couple [Glc-Cer]/[Cer] for the controls were found to be significantly lower than those for the treated samples, hinting that inhibition of GCase indeed results in an accumulation of its substrates in the lysosomes. We suggest that the abovementioned ratios may be used as a tool to pinpoint deficiencies in the GCase enzyme. Further research is underway in our Lab to validate these interesting results and to evaluate if they can be extended in such a way as to take also PD into the picture.

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M9

Novel BioInformatics Tools for Metabolomics and Lipidomics using Ion Mobility-MS

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Ion mobility (IM) is an analytical technique that separates ions in the gas phase according to their charge, shape and size. The ion mobility separation occurrs in the milliseconds timeframe making it compatible with liquid chromatography (LC) and mass spectrometry (MS). IM-MS can be integrated in current workflows for metabolomics and lipidomics, leading to high-throughput approaches with improved peak capacity and specificity of fragmentation over conventional MS analyses. Furthermore, ion mobility-derived collision cross sections (CCS) provide orthogonal physicochemical measurements on the shape of the metabolites that can be used in addition to retention time and accurate mass values to increase the confidence of metabolite identification. The large amount of ion mobility-derived data in MS-based metabolomics studies requires novel methods and computational tools. Here, we present the current approaches for IM-MS-based metabolomics and lipidomics research, illustrating workflows and bioinformatics solutions for metabolite screening and identification.

M10

Adding coordinates to the metabolome: ion mobility derived collision cross sections.

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Metabolomics and lipidomics are rapidly-evolving analytical approaches in life and health sciences. Here, we present the implementation of ion mobility techniques in typical metabolomics/lipidomics workflows.

Ion mobility allows for the measurement of the rotationally-averaged collision cross-section (CCS), which gives information about the ionic shape of a molecule in the gas phase. The CCS value is a unique physicochemical property of a molecule and can be used as an orthogonal molecular descriptor in addition to retention time and mass-to-charge ratio (m/z) to improve the identification confidence and the reproducibility of analysis.

Multiple travelling-wave ion mobility mass spectrometers (TW-IM-MS) located in independent laboratories were used to derive CCS information for a variety of small metabolites and lipids. Chromatographic separation was achieved using an ACQUITY UPLC system based on HILIC (polar metabolites) and reversed phase (lipids) UPLC. CCS values obtained in nitrogen were experimentally determined using polyalanine oligomers as the TW mobility calibrant. Data analysis and processing was performed using novel bioinformatics solutions.

We created a CCS database containing over 300 common metabolites and lipids using TW-IM-MS. CCS measurements were highly reproducible on instruments located in multiple independent laboratories (RSD <5% for 99%). We also determined the reproducibility of CCS measurements in various biological matrices including urine, plasma, platelets, and red blood cells, using UPLC coupled with TW-IM-MS. The mean RSD was <2% for 97% of the CCS values, compared to 80% of retention times. Finally, as proof of concept, we used UPLC/TW-IM-MS to compare the cellular metabolome of epithelial and mesenchymal cells, an in vitro model used

to study cancer development. CCS data were filtered and matched against the CCS database to confirm the identity of key metabolites potentially involved in cancer.

Experimentally-determined CCS values were used as orthogonal analytical parameters in combination with retention time and accurate mass information to improve identification confidence and reproducibility of analysis. Adding CCS data to searchable databases for metabolomic phenotyping improves the identification confidence and the reproducibility of analysis compared to traditional analytical approaches. In addition, we generated a publicly available database, containing CCS and accurate-mass values, to support metabolite/lipid identification.

Metabolomics, bioinformatics and statistics: three sides of the same coin

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Biometris, Wageningen UR

Metabolomics is one of the youngest examples of the transformation of biology from a qualitative to a quantitative discipline. Now, we have the tools to quickly and efficiently profile biological samples, thereby obtaining relative concentrations of all metabolites (small chemical molecules) of interest, which opens up whole fields of applications: this ranges from practical examples like health monitoring and disease diagnosis to fundamental research questions on basic biological processes. Unfortunately, this is the theory: practice is quite different. With such ambitious goals, we are operating at the cutting edge of what is analytically possible, and the result is that important and complicated data processing is necessary before the data can be interpreted. In this presentation I will focus on some of the issues that come up in mass-spectrometry based untargeted metabolomics, and will show what is currently possible. The good news is that many tools are being developed by the open-source community, enabling rapid progress and an excellent exchange of knowledge and experience.

T1

Metabolomic Profile in Intrauterine Growth Restriction Fetuses

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It is well know that adverse conditions during intrauterine life, such as intrauterine growth restriction (IUGR), can result in permanent changes in the physiology and metabolism of the newborn, which in turn leads to an increased risk of disease in adulthood. Metabolomics studies have revealed an altered metabolism in IUGR patients compared to controls. In this study a un-targeted metabolomic profiling was used to investigate fetal and/or placental metabolism alterations in IUGR fetuses by liquid chromatography highresolution mass spectrometry (LC-HRMS) analysis of cord blood collected from 22 IUGR and 21 appropriate for gestational age (AGA) fetuses. Birth weight differed significantly between IUGR and AGA fetuses ($p<0.001$). Serum samples were deproteinized by mixing with methanol; supernatants obtained after centrifugation were lyophilized and reconstituted in water for analysis. LC-HRMS analyses were performed on an Orbitrap mass spectrometer connected to a Surveyor Plus LC; fullscan mass spectra were acquired over the m/z range of 50–1,000 at a speed of 0.45 s/scan. To identify the most significant differences between IUGR and AGA samples, LC-HRMS data were analyzed by a principal component analysis model. Significant differences in relative abundances of the essential amino acids phenylalanine, tryptophan, and methionine between IUGR and AGA fetuses were highlighted. Logistic regression coupled to a receiver-operating characteristic curve identified a cut-off value for phenylalanine and tryptophan, which gave excellent discrimination between IUGR and AGA fetuses.

T2

Markers of Endothelial Damage and Metabolomic Profile in Monochorionic Twin Pregnancies with Selective Intrauterine Growth restriction

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Selective intrauterine growth restriction (sIUGR) is a major complication of monochorionic pregnancies, with potentially high risk of intrauterine fetal death or neurological dysfunction in both fetuses. The aim of this study was to assess the aortaintima thickness and serum metabolomic profile in selective intrauterine growthrestricted (sIUGR) monochorionic twin fetuses presenting Doppler velocimetry alterations. 24 twin fetuses were enrolled in the study: 4 were sIUGR with abnormal umbilical artery (UA) Doppler waveforms (Group 1), 4 were sIUGR with normal UA Doppler (group 2) and 16 were control fetuses appropriate for gestational age (AGA, Group 3). Serum samples were obtained by the fetal blood obtained from the umbilical vein immediately after fetal extraction. Fetal metabolism alterations were investigated by using a non-targeted metabolomic approach performed by liquid chromatography–high-resolution mass spectrometry (LC-HRMS) analysis. The study showed that aortic wall thickening is higher in sIUGR twins with UA Doppler velocimetry alterations than in sIUGR twins without UA Doppler anomalies or AGA co-twins. Moreover, an upregulation of serum phenylalanine, sphingosine, and glycerophosphocholine levels in sIUGR with UA Doppler waveforms was observed compared with AGA co-twins. Although for metabolomics data only a statistical tendency (and not a statistical significance) was reached due to the small sample size, our results can represent a valid starting point for further in-depth metabolomic and proteomic investigations of sIUGR in monochorionic fetuses.

T3

NMR Spectroscopy for Metabonomics: a Powerful Tool for Biomarkers Investigations

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Nuclear Magnetic Resonance (NMR) spectroscopy has emerged as a powerful tool for understanding metabolic process in living systems. Recently, metabonomics has been put forward as a new approach to elucidate metabolism and its mechanism. Such analysis is based on a minimum number of assumptions on the biochemical processes that occur in a living system, mainly investigated by advanced spectroscopic techniques including mass spectrometry and NMR spectroscopy. Metabonomics is formally defined as "the quantitative measurement of the multi-parametic metabolic response of living systems to pathophysiological stimuli or genetic modification" [1]. It has been coined to describe the combined application of spectroscopy and multivariate statistical approaches to investigate the multicomponent composition of biofluids, cells and tissues. In particular, NMR-based metabonomics has proven to be particularly suited for the rapid analysis of complex biological samples. Indeed, the so generated NMR spectra yield a unique metabolic fingerprint for each complex biological mixture. According to the possible changes occurring in a status of a given organism, such as in a disease state or following exposure to a drug, the unique metabolic fingerprint or signature reflects this change, thus supplying relevant biochemical indications.

Here we present a general overview of how NMR spectroscopy is apposite for a plenty of different metabolomic studies, showing a great versatility in being applied to a wide range of samples. We report some different applications which required specific kind of samples such as biofluids, tissue extracts and *in vivo* cells and organisms. In particular, as example of biofluids, we propose the case of exhaled breath condensates (EBC) whose NMR analysis can be used to study low airway diseases [2,3], and discuss the related methodological aspects to establish the feasibility of a non-invasive and reproducible method of investigation [4,5]. On the other hand, in the case of application to human hepatocellular carcinoma (HCC) study, a variety of samples of different tissue extracts were selected and acquired, in order to monitor progressive liver alterations during hepatic tumorigenesis, trying to discriminate metabolic profiles of primary HCC, chronic hepatitis-C virus related cirrhotic tissues, hepatic metastases from colorectal

carcinomas, and non-cirrhotic normal liver tissues adjacent to metastases as controls [6]. Finally, as example of *in vivo* application, we report the case study of the extremophile protozoo *Fabrea salina*, known to be among the strongest UV-resistant microorganisms, though the molecular mechanisms of such resistance are almost unknown. We investigated the induced changes in the metabolic profile of living *F. salina* cells exposed to visible light and to a polychromatic UV-B+UV-A+Vis radiation for several exposure times, thus detecting and highlighting sequential defense mechanisms against UV radiation [7,8].

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Chemometrics in NMR Based Metabolomics: Data pre-Treatment and Projection Methods

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NMR spectroscopy is largely applied in metabolomics. Specifically, 1d-HNMR can be used to obtain robust fingerprints where a large number of small molecules are quantified in complex biological matrices with a minimal sample preparation, fast data acquisition and high reproducibility. Also, different 2d-NMR techniques can be used in combination with 1d-HNMR for compound identification or structural elucidation. The transformation of the raw data into suitable fingerprints for data analysis requires *ad hoc* pre-treatment procedures. Depending on the biological matrix, data alignment, intelligent or regular bucketing, scaling or block scaling have to be applied to obtain high quality data sets. Normalization is usually applied to reduce the effects of systematic variations across samples.

The complex data sets obtained after pre-treatment must be investigated by using suitable multivariate techniques. PCA [1] is probably the most known and used projection technique to model metabolomics data. However, it is not able to include directly the design of the experiment in the data modeling and unsatisfactory data analysis are often produced. One of the current challenges in chemometrics is to provide new statistical approaches able to take into account the overall design of the experiment in data modeling. Specifically, new latent variable approaches based on projection were recently introduced to cover this shortcoming in the toolbox available for exploring metabolomics data. Simple designs used in cross-sectional studies can be exhaustively treated by OPLS-DA [2] while *ad hoc* techniques are required for more complex designs. Longitudinal studies for example can be investigated by ASCA model [3] while cross-over designs are still under investigation. In this communication new projection methods will be introduced focusing on the design of experiments. Two data sets [4,5] will be considered to discuss the effects of data pre-treatment on the obtained models and to present interesting improvements in data modeling.

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T4

Targeted Lipidomics Profile and NMR Metabolomics of Injured Mouse Brain

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Traumatic brain injury (TBI) is the leading cause of death in the young age group and the most commonly identified cause of epilepsy in adult populations older than 35 years. At present, there are no effective drugs to treat brain injury [1]. The role of the endocannabinoid system in neuroprotection is well established. Several groups reported enhanced levels of the endocannabinoid anandamide (AEA) after acute injury, and in response to TBI there is local and transient accumulation of the other endogenous agonist of cannabinoid receptors, 2-arachidonoylglycerol (2-AG) at the site of injury, peaking at 4 h and sustained up to at least 24 h [2]. Furthermore, very recently, Naqvi and co-workers [3] found that cigarette smokers presenting with TBI, with damage at the level of the insula, experience a cessation of smoking. Given the reinforcing role of endocannabinoids and CB_1 receptors in nicotine self-administration, it is possible that TBI is accompanied by reduced endocannabinoid levels in the insula. The aim of this study was, therefore, to investigate further the alterations of endocannabinoid levels in a model of mouse TBI and to discover new endocannabinoid molecules possibly involved in this process through the use of very sensitive and specific "targeted lipidomics" methods involving high resolution LC-ESI-IT-ToF (Liquid Chromatography-ElectroSpray Ionization-Ion Trap-Time of Flight) and NMR based metabolomics. Mice underwent TBI using the weight drop model and were divided into three experimental groups: naïve (untreated brains), control (only surgically incised brains) and TBI (injured brains). Animals were decapitated one day after injury and brains were divided into two halfs. Hippocampus and insular cortex were dissected from one half for lipidomics analysis and the other half was used for NMR analysis. Interestingly, this mild model of TBI led to a reduction of AEA levels and no changes in 2-AG. NMR based metabolic profiling was performed on both hydrophilic and lipophilic brain tissue extracts in order to investigate possible neurochemical alterations induced in mice after brain injury. NMR samples were discriminated using Projection to Latent Structure Discriminant Analysis (PLS-DA). Control and TBI groups showed low levels of L-

Glutamine and L-Glutamic acid with respect to the naïve group, thus suggesting impairment in the glutamine-glutamate cycle. Individually, the control group exhibited high levels of neurotransmitters Taurine and GABA, together with N-Acetyl-L Aspartic acid and Creatine/Phosphocreatine, which are involved in lipid synthesis and energy metabolism. On the other hand, the TBI group presented high concentrations of short chain lipids and myo-Inositol, which probably act as neuro-protecting compounds in response to brain injury.

In conclusion, we coupled targeted lipidomics with NMR metabolomics as a joint strategy to achieve a more complete characterization of brain damage and, possibly, identify putative markers associated with the alteration induced by traumatic brain injury. Apart from the LC-MS results, which suggest that the reduced levels of endocannabinoids following TBI might be one of the underlying causes of decreased nicotine self-administration, we found significant changes in the levels of metabolites involved in multiple neuronal activities, including neurotransmitter activity, neuronal osmoregulation and signaling.

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T5

Metabolomic Analysis for the Study of the Frequent Wheezing in pre-Scholar Children

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In the last years a new type of approach has developed, the so called untargeted approach, besides the classical target approach used to quantify already known compounds. In particular metabolomic approach sees the study of the entire set of metabolites present in a biological sample, (metabolic profile)1 by means of highly sensitive and precise analytic techniques, such as high resolution mass spectrometry (HMS). This approach allows the characterization of the general metabolic profile of a group of subjects with a specific pathology as compared to a group of control and extracts the discriminating variables, thanks to multivariate statistical analysis techniques.2

This study was meant to investigate upon the metabolic profiles which characterize children with frequent bronchospasm or with wheezing in preschool age.

The wheezing is a common symptom for several respiratory diseases which occur in childhood, like respiratory infections, but it is also related to the diagnosis of asthma3, since the frequent bronchospasm can either be solved as the child grows up or it can evolve onto bronchial asthma after the preschool age. Despite the fact that there are empirical indicators, such as the API4 to appreciate which children with a frequent wheezing in the preschool age will end up with bronchial asthma when growing up, what is missing are the prognostic biomarkers which would help the pediatrician make the correct diagnosis.

The final purpose of this perspective study is that of extracting, by analyzing urine samples with the mass spectrometry, the compounds which characterize children affected by wheezing, and, with a later evaluation, appreciate whether they can be considered useful biomarkers in predicting the next development of asthma.

The preliminary results of this part of the study have been encouraging: thanks to the analysis of the urine samples with the hybrid Q-TOF mass spectrometer associated to the UPLC, we have had a clear distinction of the subjects affected by wheezing as compared to healthy children, thus obtaining a robust OPLSA-DA model. (Ex. R2:0,82, Q2:0.60 for T3NEG). (Fig.1)

The investigation of the variables m/z, by means of the comparison of the exact mass with the metabolomic data bases, has indicated a possible role of the phospholipidic compounds (PGD2, phosphatidic acid, phosphatidylglycerol) in distinguishing children affected by wheezing from healthy subjects.

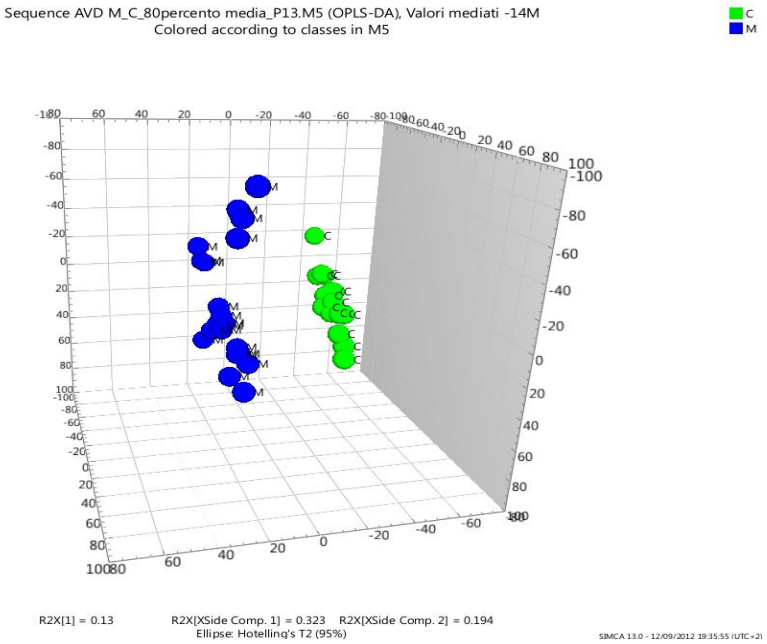

Fig. 1: Tridimensional representation of OPLS-DA

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T6

Standardized Metabolic Phenotyping solutions for Biomarker Research in Multifactorial Diseases

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In Metabolomics, there is an urgent need for standardization, and the lack of comparability between approaches currently limits impact. Biocrates is the only provider of standardized solutions in the field, which are used in dozens of laboratories around the world.
Further, researchers in both basic and clinical sciences are increasingly recognizing the need to collect comprehensive and quantitative phenotype data ("Deep Phenotyping"), with Metabolomics being one of the approaches that are employed to do so. Since Biocrates' solutions allow for the collection of comprehensive molecular phenotype data in a high throughput and high quality manner, they are referred to as Metabolic Phenotyping solutions.
The technology provides insights into many important pathophysiological processes, such as inflammation, cell cycle regulation and mitochondrial function. Applications include diverse indications such as Cardiology, Diabetes, Gynecology, and Neurodegenerative diseases, all of which being complex and multifactorial. The presentation, however, shall focus on applications in Oncology.

Metabolic Phenotyping in Oncology - Showcase

Many oncogenic and tumor suppressor genes have a profound effect on metabolism and several tumors are known to be dependent on specific metabolic processes. More recent findings that mutated forms of metabolic enzymes could promote cancer have also renewed interest to target cancer metabolism as approach for drug discovery.
Recent research has applied the Biocrates Metabolic Phenotyping platform to discover novel blood biomarkers that could potentially be used for non-invasive diagnosis and prognostic evaluation of cancers [1].
Metabolite profiles representing end-points of key cancer-related molecular pathways were analyzed and assembled to metabolite signatures serving as surrogate biomarkers for the tumor's biochemical activity. Those included markers signifying processes such as glutaminolysis, glycolysis, lipid biosynthesis, as well as nitric acid synthetase (NOS) activity.

As proof of principle study (prospective, NIH registered), 64 plasma samples of stage III breast cancer patients receiving a neoadjunvant taxane/anthracyclin-based protocol (NCT00820690), and 47 controls were utilized as training set for biomarker discovery and internal validation. The broad coverage of metabolite markers reflecting major metabolic dependencies of tumor biology enabled the delineation of biomarker signatures that displayed excellent characteristics in Breast Cancer diagnosis and prediction of treatment response, irrespective of mutation status.

The accurate and quantitative read-out of kit-based metabolomics analysis in blood allowed for a comprehensive reflection of a tumor's cellular activity. These advances hold promise to expand the armamentarium of blood tumor markers for diagnosis and prognosis. Furthermore, Metabolic Markers may serve as an invaluable tool for predicting and monitoring treatment response in Oncology and other fields.

A number of further recent works on tumor biology confirm the value of Biocrates' Metabolic Phenotyping solutions in cancer research.

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[1] Unpublished data

T7

Metabolomic Profiling of Anionic Metabolites in Oral Cancer Cells by Capillary Ion Chromatography HR/AM Mass Spectrometry

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Metabolomics approach has obtained increasing attention in oral cancer study. A highly analytically sensitive platform coupling capillary ion chromatography (Cap IC) with the Thermo Scientific™ Q ExactiveTM mass spectrometer has been developed, which is capable of metabolic biomarkers profiling of oral squamous cell carcinoma metastasis from cell lysates. The Cap IC has demonstrated outstanding separation for anionic polar metabolites and superior analytical sensitivities compared to RPLC and HILIC methods. Differential analysis finds significant changes in energy metabolism pathways, e.g., glycolysis cycle and tricarboxylic acid cycle. The experiments demonstrate Cap IC as a complementary separation technique by providing superior resolution and analytical sensitivity of polar metabolites combined with the high resolution and accurate mass measurement (HR/AM) capabilities to differentiate isobaric metabolites.

Anionic polar metabolites were used to optimize the MS conditions, Cap IC gradient and settings for minimal sample consumption and high analytical sensitivity.

Three OSCC cell lines, UMSCC1, UMSCC5, cancer stem-like cells (CSC), and according wild-type controls with biological replicates were harvested and counted. Cellular metabolites were extracted using liquid nitrogen snap-freezing method with methanol/water according the report [2]. IC was run at 25µL/min using an electrolytic suppressor to convert potassium hydroxide gradient to pure water. HILIC and RPLC methods were run at flow rates from micro flow to high flow.

Differential analysis was performed using Thermo Scientific™ SIEVE 2.1 and metaXCMS online. Components of interest (p-value<0.05, fold change>2) were identified using high resolution accurate mass, MS/MS and retention time.

Ionic analytes eluted from IC column and entered the MS in pure water. High temperature or organic solvent makeup flow could assist the desolvation and MS detection. Stable negative ESI (-) (TIC variation<10%) for micro flow (<50uL/min) on HESI source was obtained using -2800 V with 35 arbitrary unit sheath gas. However, temperature above 125 oC caused greater TIC variation. Makeup liquid at varying flow rates using acetonitrile and methanol were compared. Methanol containing 2 mM acetic acid at 10µL/min was found optimal for analytical sensitivity and stability.

Compared to RPLC and HILIC experiments, Cap IC had 100-fold increased analytical sensitivities. The detection limits for 40 standard metabolites ranged from 0.2 to 0.8 nmol/L (0.2 and 3.4 fmol) at S/N of 3. The inter day (n = 5) relative standard deviation (RSD) of retention time and intensity were below 8%.

Cap IC demonstrated outstanding separation and peak shape for anionic polar metabolites. Monophosphates and diphosphates with up to 10 isomeric species for a single m/z were well separated by Cap IC, as compared to a single peak or unsolved peaks in both high- and micro-flow rate RPLC and HILIC methods.

Meta-analysis is a powerful method to investigate the common changes in metabolomics study using multiple sample types [3]. Data from three OSCC cell lines UM1, UM5, CSC were analyzed using SIEVE 2.1 and metaXCMS online. Numbers of feature with significant change (p-value<0.05, fold change>2) are 4597 for UM5 KD vs. WT; 2856 for UM1 KD vs. WT and 3861for cancer stem cells (CSC) vs. control. The common feature number for all three is 218.

Cap IC was successfully coupled to HR/AM MS for untargeted metabolic profiling in oral cancer cells and polar metabolites characterization.

Keywords: Capillary Ion Chromatography, Q Exactive, Orbitrap, Mass Spectrometry, Metabolomics, Cancer stem cells (CSCs), Hydrophilic interaction chromatography (HILIC), SIEVE 2.1, metaXCMS, Human oral squamous cell carcinoma (HOSCC), Tricarboxylic acid cycle, glycolysis metabolism.

T8

Absolute Real-Time Ethylene and Furaneol Detection with PTR/SRI-MS

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Ethylene and furaneol are volatile organic compounds playing a crucial role in many systems and their detection and quantification is strategic for many research fields. We investigated the possibility to use Proton Transfer Reaction – Mass Spectrometry (PTR-MS) instruments modified by a Selective Reagent Ionization (SRI) upgrade [1] to detect such VOCs in real time and with a very high sensitivity. The detection limit of SRI-MS is analogous to that of commercial laser-based instruments, while SRI-MS provides superior performance in terms of dynamic range and response time. In force of our previous studies [2], we prove that using SRI coupled to a time-of-flight (ToF) detector it is possible to achieve absolute ethylene and furaneol detection without the need of time consuming instrument calibrations. We measured the reaction rate coefficients for reactions between the primary ions H_3O^+, O_2^+ and NO^+ and ethylene in the SRI-MS drift tube. The experimental reaction rate coefficients matches the theoretical collision rate in the case of O_2^+, while in the case of H_3O^+ and NO^+ the reaction is less efficient. Reaction product yields and their potential dependence on the buffer gas were investigated. New reaction product ions that were not previously reported were found. We concluded that among the explored instrumental settings, the preferable one for ethylene detection with SRI-ToF-MS is O_2^+ mode, considering the signal of the $C_2H_4^+$ ion at m/z 28.0307. We finally showed that SRI-MS is a powerful tool for ethylene and furaneol investigations in biological samples such as bacteria, fruits and plants [3].

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T9

A New Methodology for QTL Analysis of Fruit Volatile Secondary Metabolites by PTR-ToF-MS Coupled to Data Mining

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Volatile secondary metabolites represent major and complex components of fruit flavour. In many fruits, very few genes regulating their biosynthesis have been identified. Quantitative trait loci (QTLs) affecting fruit volatile emission are usually identified by crossing genetic and metabolomics data on germplasm collections or breeding materials. The gold standard for volatile organic compound (VOC) analysis in fruits is constituted by chromatographic methods, which are usually time consuming and therefore their use often becomes unrealistic in breeding programs. Proton Transfer Reaction – Mass Spectrometry (PTR-MS) is a very fast and high sensitivity analytical method for VOC detection. Early instrumental releases coupling PTR-MS with a quadrupole mass analyzer showed limitations in QTL studies due to the very low analytical power [1]. In the present work we show that the new high resolution generation of instruments equipped with time-of-fight mass analyzers (PTR-ToF-MS) [2], if coupled to proper data mining, is very effective for fast QTL investigations of fruit VOCs. The proposed methodology allows reducing the experiment time by about 100 times compared to standard chromatographic methods while maintaining an analytical power sufficient for VOC or VOC class identification in many cases. In the present study, it is employed to carry out a QTL investigations of apple VOCs on a full-sib 'Golden Delicious x Scarlet' population, resulting in the identification of several new QTLs, manly related to ethylene, estragole, carbonyl, alcohol and sesquiterpene emission. The proposed methodology has been successfully applied in recent studies [3] and it could be generally employed for analogous investigations, e.g. employing other fruits.

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T10

A Fast LC-MS Methodology for Membrane Lipid Profiling through HILIC Chromatography

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Lipidomic is an emerging field in the "omics" sciences. It has been defined as "the system level analysis and characterization of lipids and factors that interact with them" [1, 2]. In recent years, lipid analysis has gained more and more importance as lipid are involved in many cellular processes and an aberrant lipid metabolism is a determinant of the onset of some important diseases, such as diabetes, atherosclerosis, obesity, Alzheimer's disease and some cancer type [1, 3]

Various analytical platform have been adopted and adapted for lipid analysis, mainly nuclear magnetic resonance (NMR), gas chromatography coupled to mass spectrometry(MS)/flame ionization detector (FID) and high performance liquid chromatography (HPLC) coupled to MS/Diode Array Detector(DAD)/Evaporative Light Scattering Detector (ELSD). The use of HPLC has been widely exploited as it is possible to achieve a fast and consistent separation of many components of a mixture, given the ease of automation and the hyphenation with a wide array of detectors.

Lipid separation is achieved either by normal phase (NP) or reverse phase(RP)-LC. NP method exploit silica, diol or amine-bonded stationary phases in combination with mixture of hexane/isopropanol/water as mobile phases; under NP conditions, lipids are separated according to the different polarity of heads. RP-based separation on octasilyl (C8) and octadecylsilyl (C18)-derivatized stationary phases is achieved in combination with solvent systems composed by methanol/acetonitrile/water; under RP conditions, lipid are separated according to chemical features of acyl chains [4,5,6]

NP-LC has some drawbacks, as the scarce reproducibility due to low miscibility of solvent systems and the fact that solvent are less ESI compatible than those used for RP.

In recent years, the development of Hydrophilic Interaction Liquid Chromatography (HILIC) opened new possibilities for lipid research: the principle of HILIC separation is the same of NP-LC but solvent system are those of RP-LC.

Hereby, we present the development of a HILIC-MS method for the fast profiling of membrane lipids on a triple quadrupole MS using full scan, precursor ion and neutral loss scanning. 14 different lipid classes, which constitute cellular membranes of various micro- and macrorganisms, were tested on 4 different columns with different mobile phase combinations. The protocol was then further optimized on the column that gave the best results. The method was then tested on complex lipid extracts such as algae and plants, showing good separation for most lipids in 35 min run.

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T11

Mass Profiler Professional for Biomarkers Discovery by LC-QTOF and Ion Mobility System for Clinical and Food Applications

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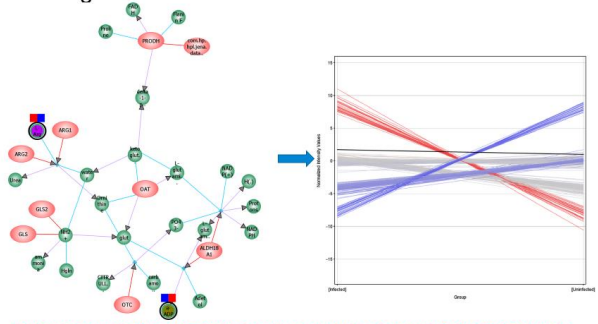
Chemometric data mining of high resolution LC/MS data sets is widely used in metabolomics and other "omics" applications in order to i.e. classify phenotypes or disease states. Since recently, multivariate statistical methods have found their way also into more "traditional" applied markets, such as environmental or food analysis.

This presentation shows examples on data mining workflow of feature extraction followed by multivariate statistical methods applied to Clinical, Environmental and Food analysis, such as Malaria infected Red Blood Cell, Lipidomics study of biological samples, food profiling, and investigation of penicillium expansum infected grapes and profiling of flavonoids in different bamboo leave qualities.

Samples analyzed by UHPLC/QTOF systems are processed by proprietary algorithm for data mining, feature extraction by Mass Hunter software, alignment and filtering by Profinder and multivariate analysis by Mass Profiler Professional (Agilent Technologies)

The utility of a combined un-targeted discovery-based LC/MS and targeted confirmation LC/MS/MS approach for mining metabolomics data was demonstrated using malaria-infected red blood cells as a model system. Several compounds, including arginine, citrulline, and ornithine, were identified as metabolites whose biosynthesis was altered in erythrocytes infected by the malaria parasite.

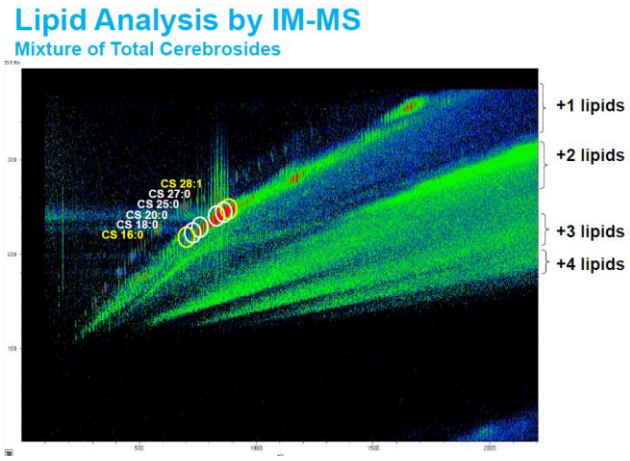
Translating Statistical Analysis Into Biological Meaning: Malaria Infected Versus Uninfected Cells



Differential analysis is submitted to pathway analysis to see metabolite relationships

The Agilent IM-QTOF 6560 LC/MS system is a major advance in the commercial development of analytical ion mobility‑mass spectrometry. Optimized development of a uniform drift field mobility cell and interface to a high resolution Q-TOF instrument gives a significant gain in ion mobility performance. The use of ion funnel technology pioneered by Agilent for both triple quadrupole and Q-TOF instruments over the past three years has been incorporated into the new IM-QTOF system. This has resulted in combined ion mobility separation and mass resolution with high sensitivity. The instrument delivers greater separation of lipids and glycopeptides (Lipidomics and Proteomics), more accurate collision cross section measurements enabling more confident characterization of structural conformations and isomeric compounds, greater numbers of trace level peptides in complex matrices, preservation of structural fidelity of metallo-proteins in liquid phase solutions.

Cerebrosides are a common class of glycosphingolipids called monoglycosylceramides which are important components in animal muscle and nerve cell membranes. They consist of a ceramide with a single sugar residue at the 1-hydroxyl moiety. Galactocerebrosides are typically found in neural tissue, while glucocerebrosides are found in other tissues and the IMS 6560 System delivers solution for a complex problem by the ability to separate these different cerebs based on the size of the head group.



Non-target metabolomics approach based on high resolution QTOF data followed by multivariate statistical analysis is a powerful tool in “multi-omics” applications. We could demonstrate the use of a non-targeted and targeted approach in characterizing complex samples with an improved confidence in identification and correlation of biological pathways.

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T12

Exploring Metabolomic Signatures of Altered Metabolism in Breast Cancer Cells

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Altered glucose and phosphatydilcholine (PtdCho) metabolism are metabolic hallmarks of various cancer phenotypes [Galluzzi et al. Nat Rev Drug Discov 2013; Glunde et al. Nat Rev 2011]. High level of intracellular lactate results from the up-regulation of glycolitic pathway. Both biosynthetic and catabolic pathways of PtdCho contribute to the increased phosphocholine (PCho) levels in cancer cells involving phosphorylation of choline (Cho) in *de novo* PtdCho biosynthesis, as well as by activation of PtdCho-specific phospholipase C (plc) [Podo et al. NMR Biomed 2011].

We assessed the metabolic signatures in breast cancer (BC) cells and their non-tumoural counterpart, and investigated the effect of plc inhibition on intracellular metabolism and cell growth to explore plc as a possible target for antitumor treatments.

High-resolution NMR experiments were performed (9.4 T, Bruker Avance) on aqueous cell extracts of BC cell lines MCF-7 and MDA-MB-231 and their non-tumoural MCF-10A cell line [Iorio et al., Cancer Res 2010]. Activity and expression of plc and choline kinase (ChoK) were assessed by biochemical assay and western blot. D609 (inhibitor of plc) was added (150 μM) after 24 hours of culture of plated cells (20000 cells/cm^2). Evaluation of necrotic, apoptotic and living cells were performed by flow cytometry after stained with Propidium Iodide (Lugini et al., J Immunol 2012). Principal component analysis was applied to explored the data.

Tumoural cell lines presented significantly higher content of PCho, ATP, ADP, succinate, acetate and lactate than MCF-10A. Both Chok and plc were up-regulated in the tumoural cells lines. The exposure to D609 decreased the difference in those metabolites (except lactate) and decreased the activity of plc in BC cells relative to MCF-10A. Multivariate analysis separated cell lines based mostly on their Pcho content (PC1) and their lactate content (PC2).

Tumoural cells lines show signs of both higher PtdCho metabolism and glycolysis than MCF-10A. The exposure to D609 elicits decrease in the PCho content in tumoural cells but not its suppression, thus pointing to biosynthetic pathway contribution to high PCho content of cells; the higher glycolitic metabolism of tumoural cells is mantained. Evidence of abnormal PtdCho metabolisms appears to have implications for BC biology and is a potential avenue to the development of non-invasive clinical tools for BC diagnosis and treatment follow-up.

Notes