Italian Mass Spectrometry Society

GIDRM
Gruppo Italiano Discussione Risonanze Magnetiche

Advances in NMR and MS Based Metabolomics

Lucca, San Micheletto Conference Center,
November 20th – 22nd, 2019

Program and Abstracts

Under the Auspices of

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Città di Lucca

ADVANCES IN
NMR AND MS BASED METABOLOMICS

Venue: Complesso San Micheletto, Lucca, Italy
Via San Micheletto, 3, 55100 Lucca

TUTORIALS

Day 1: Wednesday, November 20th, 2019

09:15 – 10:15

MS-based metabolomics: sample preparation, data acquisition, data pre-processing

Pietro Franceschi, Unit of Computational Biology, Research and Innovation Centre,
Fondazione Edmund Mach (FEM), San Michele all'Adige, Italy

Mass spectrometry, almost always coupled with chromatographic separation, is one of the techniques of election able to perform a comprehensive investigation of the "metabolome". MS is almost "universal" and characteristics like sensitivity and dynamic range make it an ideal tool for this task. For exactly the same reasons, however, the results of a MS-based metabolomic investigation are sensitive to any issue occurring during sample preparation, sample analysis and data preprocessing. Errors occurring in these phases will affect all the downstream statistical analysis following the well known "garbage in, garbage out" principle.
The aim of this tutorial is to highlight the most critical aspect which should be taken into consideration when designing a (successful!) MS-based metabolomics assay. The discussion will touch lab practice, quality assessment and data preprocessing. The objective is to describe the strategies which can be used to control the major sources of variability occurring in the early stages of an investigation, with the ultimate objective of providing a reliable data matrix to the subsequent statistical analysis.

10:15 – 11:15

NMR-based metabolomics: sample preparation, data acquisition, data pre-processing

Emanuela Locci, Department of Medical Sciences and Public Health,
Section of Legal Medicine, University of Cagliari, Cagliari, Italy

NMR spectroscopy is widely employed in metabolomic investigations. NMR shows some advantages in the analysis of biological samples, since it is non destructive and non selective. It allows to obtain a global profile of the low molecular weight metabolites present in the sample in one experiment without extensive sample manipulation. Moreover, it is extremely versatile, robust, and highly reproducible. However, compared to MS spectrometry, it has an intrinsically lower sensitivity, which requires high concentrations and precludes the analysis of small volumes. This tutorial addresses the principal steps and methodologies used in NMR-based metabolomics. In particular, sample preparation, data acquisition, and processing of spectra will be discussed.

11:15 – 12:15

Design of Experiments and Data Processing

Matteo Stocchero, Department of Women's and Children's Health,
University of Padua, Padua, Italy

The analytical platforms used for metabolomic investigations produce large data sets where variables are strongly correlated and redundancy is present in the data. Discovering the hidden information is usually a challenge, and suitable approaches for data analysis must be employed. Multivariate data analysis has been successfully applied to metabolomics. On the other hand, design of experiments is fundamental to obtain robust results in metabolomics. Confounding factors are often present in the design and it is not obvious how to take them into account in data analysis to avoid false discovery.

In this tutorial some relevant issues about design of experiments and data analysis are addressed. Principal Component Analysis and Projection to Latent Structures regression (PLS2) are introduced. Post-transformation of PLS2 is used to discover structured noise and to focus the model on the data variation useful to explain the response. Moreover, model validation is discussed. Constrained PCA and constrained PLS2 are introduced to explicitly include experimental design in model building.

ADVANCES IN
NMR AND MS BASED METABOLOMICS

Venue: Complesso San Micheletto, Lucca, Italy
Via San Micheletto, 3, 55100 Lucca

Conference Program

Day 1: Wednesday, November 20th, 2019

12:00 - 14:30 ***Participants Registration***

13:00 – 14:00 **Light Lunch**

14:30 – 15:00 **Welcome and Introduction to the Meeting**
Alessandro Tambellini, Lucca city Major
Pietro Franceschi e Marco Geppi, IMass and GIDRM Presidents

Chairpersons: Pietro Franceschi, Marco Geppi

15:00 – 16:00 **Hamed Pirimoghadam, University of Alberta, Canada**
(*Plenary Lecture 1)* Making quantitative metabolomics faster and cheaper

16:00 – 16:25 **Cristina Piras**, University of Cagliari, Italy
(Oral W1) Metabolomics analysis and modeling in fibromyalgia

16:25 – 16:50 **Alessia Vignoli**, CERM, University of Florence, Italy
(Oral W2) Serum NMR-based metabolomics as prognostic tool for acute myocardial infarction

16:50 – 17:15 **Claudia Napoli,** Bruker Italia S.r.l.
(Oral W3) New NMR tools for clinical research and integrated use of NMR and MS

17:15 – 18:15 **Augustin Scalbert, IARC, France**
(*Plenary Lecture 2)* The food exposome in cancer epidemiology: discovery, validation and application of dietary biomarkers

19:00 **Wine Tasting – Offered by Consorzio del Vino Montecarlo DOC**
"Una piccola DOC per grandi vini di territorio"

Day 2: Thursday, November 21st, 2019

Metabolomics in Clinical Medicine
Chairpersons: Luigi Atzori, Giuseppe Giordano

09:00 – 10:00 **Marta Cascante, University of Barcelona, Spain**
(*Plenary Lecture 3*) Unveiling the metabolic phenotypes and vulnerabilities underlying metastasis and drug resistance

10:00 – 10:25 **Daniel O. Cicero,** University of Rome Tor Vergata, Italy
(*Oral Th1*) Can a cardiac ischemic episode be anticipated by metabolic profiling? An untargeted NMR study

10:25 – 10:50 **Veronica Ghini,** CERM, University of Florence, Italy
(Oral Th2) NMR-fingerprint of blood: from methods to application in precision medicine

10:50 – 11:15 **Marco Roverso,** University of Padua, Italy
(Oral Th3) Metallome alterations in gestational diabetes: an investigation on maternal whole blood, placenta and cord whole blood samples

11:15 - 11:45 ***Coffee Break***

Chairpersons: Giuseppe Pieraccini, Emanuela Locci

11:45 – 12:10 **David Heywood,** Waters Corp.
(Oral Th4) Development of high throughput, multi-omic methods applied to a breast cancer study

12:10 – 12:35 **Giovanna Musco,** San Raffaele Scientific Institute, Milan, Italy
(Oral Th5) NMR metabolic studies of the renal cortices changes in a mouse model of Renal Cell Carcinoma (RCC)

12:35 – 13:00 **Elena Cannas**, University of Bologna and Italian Institute of Technology, Italy
(Oral Th6) LC-MS Lipidomics to characterize the altered lipid metabolism as a stress reaction to acid tumor microenvironment in osteosarcoma

13:00 – 13-25 **Greta Petrella**, University of Rome Tor Vergata, Italy
(Oral Th7) How NMR data could assist MS hit classification in an untargeted metabolomics analysis? Our case study: bladder cancer

13:25 – 14:25 ***Lunch and Poster Session I***

Food / Nutrition

Chairpersons: Augustin Scalbert, Vito Gallo

14:25 – 14:50 **Cinzia Ingallina**, University of Rome, Italy
(Oral Th8) Torpedino and San Marzano tomato fruit metabolite profiling through NMR and MS methodologies

14:50 – 15:15 **Elisabetta Schievano,** University of Padua, Italy
(Oral Th9) Innovative qNMR methodology for carbohydrates quantification in complex mixtures

15:15 – 15:40 **Anatoly P. Sobolev**, IMC-CNR Rome, Italy
(Oral Th10) A multi-methodological protocol to characterize the metabolite profile of "Bianco di Sperlonga" PGI white celery ecotype

15:40 – 16:05 **Nicola Cimino**, Agilent Technologies
(Oral Th11) A new LC/Q-TOF platform for metabolomics analysis: 6546 LC-QTOF workflows

16:05 – 16:35 ***Coffee Break***

Chairpersons: Andrea Armirotti, Cristina Airoldi

16:35 – 16:50 **Alberto Asteggiano**, University of Turin, Italy
(Oral Th12) HPLC-MS/MS untargeted metabolomic approach for disease-related molecular markers detection in quick decline syndrome

16:50– 17:15 **Laura Righetti**, University of Parma, Italy
(Oral Th13) Mass Spectrometry Imaging for Untargeted Plants Metabolomics: a Case Study in Mycotoxin Accumulation

17:15 – 18:15 **Fabien Jourdan, French National Institute for Agricultural Research, France (*Plenary Lecture 4)* Metabolic networks to interpret and predict metabolism**

20:00 ***Social Dinner***
Sala Refettorio del Campus San Francesco

Day 3: Friday, November 22nd, 2019

Metabolomics Data Analysis and Applications
Chairpersons: Matteo Stocchero, Andrea Raffaelli

9:00 – 10:00 **Jasper Engel, Biometris, Wageningen University & Research, The Netherlands (*Plenary Lecture 5)* One-class modeling in untargeted metabolomics: case studies in diagnosis and risk assessment**

10:00 – 10:25 **Vito Gallo**, Bari Technical University, Italy
(Oral F1) Harmonization of Data Processing procedures for non-targeted NMR analysis in metabolomics studies

10:25 – 10:50 **Antonio Pompeiano**, St. Anne's University Hospital, Brno, Czech Republic
(Oral F2) Multivariate data analysis of metabolomic data: data integration, feature selection and visualisation

10:50 – 11:20 ***Coffee Break***

Chairpersons: Paola Turano, Amalia Gastaldelli

11:20 – 11:45 **Marialuce Maldini,** SCIEX
(Oral F3) SWATH™: QUAL & QUAN metabolomics in the same run

11:45 – 12:10 **Alberto Chighine**, University of Cagliari, Italy
(*Oral F4*) Metabolomic profile of aqueous humour in a 24-hours period after death: an animal model for post-mortem interval estimation

12:10 – 12:35 **Valeria Righi**, University of Bologna, Italy
(Oral F5) From Actinic Keratosis to Squamous Cell Carcinoma: NMR analysis with clinical and histological aspects

12:35 – 13:00 **Gabriele Poloniato**, University of Padua, Italy
(Oral F6) An integrated software platform to improve the identification of metabolites, on the untargeted LC-MS metabolomics

13:00 – 14:30 ***Lunch and Poster Session II***

14:30 – 15:30 **Group discussion: the new Italian Metabolomics Network**

15:30 – 16:00 ***Closing Remarks***

POSTER LIST

P1. Gaia Meoni
From beans to brew: NMR based metabolomic approach to assess traceability of coffee producers within a restricted geographical area of Colombia

P2. Riccardo Frizzo
NMR-based metabolite profiles in *mytilus galloprovincialis*: experimental set-up and preliminary data

P3. Cristina Licari
The importance of bucketing procedure for NMR-based metabolomic fingerprinting

P4. Camilla Marasca
Blood microsampling for untargeted lipidomics

P5. Alana Pereira
MS metabolomic overview of chemical interactions from leaf cutting ants symbionts

P6. Erica Pitti
Development of a green method to extract lipids from human plasma

P7. Valeria Righi
Non canonical Cyclic Nucleotides Monophosphates in *Aphanizomenon flos-aquae*: nuclear magnetic resonance and mass spectrometry

P8. Silvia Sabatini
Lipidomic data analysis for non-alcoholic Fatty Liver Disease

P9. Mattia Spano
Metabolic profile of hemp flowers from Lazio: an NMR study

P10. Vito Gallo
Effects of sample preparation procedures on non-targeted NMR analysis of tomatoes

P11. Michela Buonocore
An NMR-MS metabolomic study of brain tissue from D-aspartate oxidase knock-in mouse model

P12. Emanuela Di Gregorio
Real time metabolomics analysis of breath volatile organic compounds (VOCs) by selected ion flow tube mass spectrometry (SIFT-MS) in cancer patients

P13. Anna Di Porzio
Metabolomic investigation of the effects of nutraceuticals and potential drugs in murine inflammatory models

P14. Nunzia Iaccarino
NMR and MS-based metabolomic study on the effects of structurally different mixed linkage β-glucan in hypercholesterolaemic rats

P15. Hocelayne Paulino Fernandes
Identification of Citrus metabolites associated with the defense against *Phyllosticta citricarpa* using NMR and GC-MS profiling techniques

P16. Eleonora Quartieri
Metabolomic profiling of human saliva

P17. Laura Righetti

Mass spectrometry imaging as a tool to visualize the plant metabolome changes in response to mycotoxin accumulation

P18. Fabio Spreafico

'Functional microbiomics' – assessing nutrition-microbiome-host interaction in blood and feces

P19. Elena Michelucci

A targeted LC-MS/MS analysis of circulatory lipid profile to highlight biomarkers for patient stratification according to coronary artery disease severity

P20. Andrea Armirotti

Quantification of twelve neurotransmitters in mouse cerebrospinal fluid

P21. Cristina Airoldi

NMR-driven identification of potential antitumoral and antiamyloidogenic activity of Cinnamon extracts

P22. Alessandro Palmioli

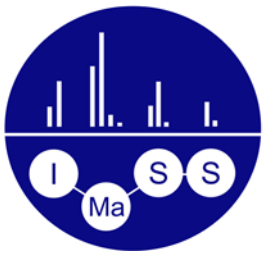
Metabolomic profiling of beers: combining ^{1}H-NMR spectroscopy and chemometrics approaches to discriminate craft and industrial products

P23. Giulia Ricciardi

Atrial fibrillation in the elderly: a metabolomic approach with GC-MS

P24. Lucie Vanickova

Mapping of elements in MeLiM tissues by LA-ICP-MS



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Plenary Lectures

Making quantitative metabolomics faster and cheaper

Hamed Pirimoghadam

University of Alberta, Dept. of Biological Sciences, University of Alberta, Edmonton, AB, T6G 2E9, pirimogh@ualberta.ca

Metabolomics can be targeted or untargeted, quantitative or qualitative, MS-based or NMR-based. Regardless of the choice of platform or the adopted approach to analysis, metabolomics continues to relatively slow, manually intensive and expensive. Indeed, compared to other 'omics technologies, metabolomics has not yet seen very profound "next-gen" changes in analysis speeds or costs. In this presentation I will look at some of the recent technological developments in metabolomics that are leading to faster and cheaper approaches to performing targeted, quantitative metabolomics. Unlike untargeted metabolomics, targeted, fully quantitative metabolomics can be almost fully automated – both for NMR and MS-based platforms. I will first describe a number of methods that are now available for fast, inexpensive and fully automated NMR-based quantitative metabolomics. These will include several commercial as well as several open-access NMR options that have been recently developed in my laboratory. I will then describe a number of approaches for fast, inexpensive approaches for semi-automated MS-based quantitative metabolomics. While generally not as cheap or automated as NMR methods, quantitative LC-MS methods (or kits) offer much greater metabolite coverage and are becoming increasingly cheaper. To conclude this presentation, I will provide some thoughts on where quantitative metabolomics needs to go if it is going to become more widely adopted in different clinical, environmental and industrial sectors.

The food exposome in cancer epidemiology: discovery, validation and application of dietary biomarkers

Augustin Scalbert

International Agency for Research on Cancer, Nutrition and Metabolism Section, Biomarkers Group, 150 cours Albert Thomas, F-69372 Lyon Cedex 08, France.

Estimating dietary exposures with high accuracy is essential in nutritional epidemiology to understand the effects of the diet on human health. The use of dietary biomarkers may limit biases and errors often associated with the more common use of dietary questionnaires. We recently developed the Exposome-Explorer database (http://exposome-explorer.iarc.fr/), in which information on dietary biomarkers extracted from the scientific literature is systematically curated. Over 450 dietary biomarkers have been used in various population studies. However, these biomarkers alone cannot describe the high complexity of the human diet. More than 80,000 compounds have been described in various foods, and many of them are absorbed through the gut barrier and found in blood, urine or tissues. These compounds can be specific of a particular food or food group and as such of potential interest as biomarkers. They constitute altogether the food exposome, a resource still poorly explored in nutritional epidemiology. Progress in mass spectrometry and in metabolomics makes today possible the identification of a large diversity of novel dietary biomarkers. Examples will be given on the discovery of biomarkers for various plant and animal foods in dietary intervention studies and in the European Prospective Investigation on Cancer and nutrition (EPIC) cohort. Implementation of these biomarkers in exposome-wide association studies conducted in large cohorts should allow to identify novel risk factors for diseases.

Unveiling the metabolic phenotypes and vulnerabilities underlying metastasis and drug resistance

Marta Cascante[1,2], Míriam Tarrado-Castellarnau[1,2],Cristina Balcells[1,3], Josep Tarrago[1,2] Igor marin de Mas[1,4+], Esther Aguilar[1+] , Pedro de Atauri[1,2] , Erika Zodda[1], Mònica Pons[5], Josep J. Centelles[1,2], Balázs Papp[5], Francesc Mas[3], Miquel Muñoz-Ordoño[1,2], Vitaly Selivanov[1,2], Jordi Perarnau[1+], Fionnuala Morrish[6], David Hockenbery[6] , Mariia Yuneva[7] , Silvia Marin[1,2] ,Timothy Thomson[2,5]

1. *Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, Universitat de Barcelona (UB) and Institute of Biomedicine of University of Barcelona (IBUB), Barcelona.*
2. *Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Instituto de Salud Carlos III (ISCIII), Madrid.*
3. *Department of Material Sciences and Physical Chemistry and Research Institute of Theoretical and Computational Chemistry (IQTCUB), Barcelona.*
4. *Synthetic and Systems Biology Unit, Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences, Szeged.*
5. *Department of Cell Biology, Barcelona Institute for Molecular Biology (IBMB-CSIC), Barcelona. [6]Fred Hutchinson Cancer Research Center, Seattle. [7]The Francis Crick Institute, London. [+]Former affiliations were the work has been done.*

Despite advances in therapy, progression to metastasis and acquisition of resistance to chemotherapy remain the greatest challenges in cancer. More specifically, new modalities of treatment are urgently needed for metastatic cancer and to cope with acquired drug resistance. Recent studies have highlighted metabolic reprogramming as a key player in the acquisition of metastatic potential and therapeutic resistance, which has led to propose metabolic adaptations as potential therapeutic targets. Here, we have applied a systems biology approach, including experimental data integration into genome-scale metabolic models, to unveil metabolic differences and potential vulnerabilities associated with metabolic heterogeneity of tumor cells subpopulations in metastatic prostate cancer and to cisplatin resistance. Using a dual model clonal cell model, consisting of a CSC-subpopulation with an epithelial phenotype and a non-CSC-subpopulation with hallmarks of stable EMT, we show that EMT and metastasis programmes can display distinct metabolic traits. Briefly, the major differences were observed in differential use of glucose and glutamine to fuel TCA cycle, mitochondrial respiration, one-carbon metabolism, beta-oxidation and eicosanoids metabolism. By applying similar approaches, we have identified metabolic adaptive responses associated with platinum resistance as actionable targets in combined therapeutic strategies. Finally, we have analyzed the metabolic reprogramming associated with the inhibition of the cyclin-dependent kinase CDK4/6 in colorectal cancer cells, having unveiled new mechanisms of cancer cell adaptation whose targeting averts the acquisition of pharmacological resistance to cell cycle inhibitors.

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Metabolic networks to interpret and predict metabolism.

Fabien Jourdan

INRA Toxalim (Research Centre in Food Toxicology) MetaboHub, Toulouse, France
E-mail: Fabien.Jourdan@inra.fr

Metabolic modulation is a cornerstone cellular response to genetic or environmental stresses. This plasticity is going beyond central metabolism and may involve complex processes spanning several metabolic pathways. Hence, it is a key challenge to be able to decipher metabolic modulations in a systemic and global perspective.

The aim of the computational methods and tools which will be presented is thus to consider the full complexity of metabolism. To do so, all metabolic reactions the cell is able to achieve are gathered in a single mathematical model call “genome scale metabolic network”. Based on this model it is then possible to identify metabolic specificities of different cell lines, predict metabolic behaviours, simulate metabolic responses to single or multiple knock-out and ultimately identify potential drug targets.

One-class modeling in untargeted metabolomics: case studies in diagnosis and risk assessment

Jasper Engel

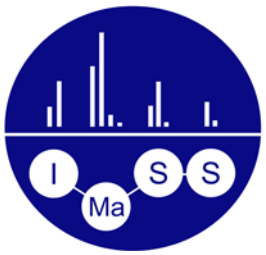
Biometris, Wageningen University & Research

You are not feeling well. Your physician finds clinical symptoms and refers you to hospital. How should your condition be diagnosed in a quick and efficient manner?

Do you mind being offered genetically modified (GM) food? If so, how can this be avoided?

These seemingly different problems will be discussed in the context of analysis of untargeted metabolomics data. Conventional case-control analyses will be contrasted to novel approaches. Metabolic profiles of suspecting subjects will be compared to a well-defined control group, e.g. healthy individuals or foods with a history of safe use. We will use a difference testing approach to show statistically significant deviations in a patient's metabolic profile compared to the healthy controls. This way, an untargeted signal to identify disease or other abnormalities in the patient is obtained. We will use an equivalence testing approach to show that there are no meaningful differences between GM and the safe foods, meaning that it is assumed that the GM is equally safe.

Key words: untargeted data analysis, one-class versus two-class modeling, difference versus equivalence tests



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Oral Communications

Metabolomics analysis and modeling in fibromyalgia

Cristina Piras[1], Pierluigi Caboni[2], Amit Kumar[3], Maria Laura Santoru[1], Enrico Pieroni[3], Enrico Cacace[4], Valeria Ruggero[4], Stella Conte[5], Luigi Atzori[1]

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2 Department of Life and Environmental Sciences, University of Cagliari, Italy.
3 Biomedicine Department, CRS4, Cagliari, Italy.
4 Department of Medical Sciences ''Mario Aresu'', University of Cagliari, Italy.
5 Department of Pedagogy, Psychology and Philosophy, University of Cagliari, Italy.

Abstract

Fibromyalgia (FM) is a highly disabling pathology with unknown and debated etiology, manifesting a variety of non-specific symptoms. In order to have a better understanding of this condition, a metabolomics approach was used. The plasma of FM patients was studied with LC-Q-TOF/MS and MVA. Some lysoPCs discriminated the plasma of patients with FM compared to controls. Using the most discriminant lysoPCs, protein docking and molecular dynamic (MD) studies were then performed. A binding to Platelet Activating Factor Receptor (PAFr) by the discriminating lysoPCs was observed. MD and docking indicate that the ligands investigated have potentialities to activate the PAFr. A further analysis was carried out on the plasma of patients suffering from FM with electromagnetism sensitivity (EFM). The aim of the present prospective analysis was to identify a possible hydrophilic metabolomics profile in subjects with EFM using NMR Spectroscopy. The results showed that EFM subjects were characterized by a distinct metabolic fingerprint (e.g. including glycine, pyroglutamate, choline, glutamine, isoleucine).

Altogether, our results indicate the capability of a metabolomics approach to identify distinct metabolic profiles in FMS patients, suggesting a possible role for lysoPCs as biomarkers and players to the disease phenotype by having a role in the pathogenesis of this condition. Moreover, discriminant hydrophilic metabolites are observed in fibromyalgia with electromagnetism sensitivity. So, these results might suggest new disease biomarkers and therapeutic targets in FM, helping the design of new approaches for the treatment.

Serum NMR-based metabolomics as prognostic tool for acute myocardial infarction

Alessia Vignoli[1,2], Leonardo Tenori[1,2], Betti Giusti[3], Rossella Marcucci[3], Anna Maria Gori[3], Claudio Luchinat[1,2]

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2. *Consorzio Interuniversitario Risonanze Magnetiche di Metallo Proteine (C.I.R.M.M.P.), Via Luigi Sacconi 6, 50019, Sesto Fiorentino (Florence), Italy*
3. *Department of Experimental and Clinical Medicine, University of Florence, Florence, Largo Brambilla 3, 50134, Italy*

Risk stratification and management of acute myocardial infarction (AMI) patients continue to be challenging despite considerable efforts made in the last decades. The aim of this study[1] was to investigate the metabolomic fingerprint of AMI patient using nuclear magnetic resonance spectroscopy (NMR) of serum samples and to evaluate metabolomics efficacy in the prognostic stratification of AMI patients. 978 AMI patients were enrolled in the frame of the Florence Acute Myocardial Infarction-2 (AMI-Florence 2) registry[2], among these 146 patients died, whereas 832 survived within 2 years from the AMI. Serum samples were analyzed via high resolution 1H-NMR and spectra were used to characterize the metabolic fingerprint of patients. In the training set, metabolomics showed significant differential clustering of the two outcomes cohorts (76.9% sensitivity, 79.5% specificity, and 78.2% accuracy). These results were reproduced in the internal validation set, obtaining 72.6% sensitivity, 72.64% specificity and 72.64% accuracy. The known prognostic factors were compared by Cox models with the metabolomic random forest (RF) risk score and the RF score shows the P-value by far more significant (P=2.16·10-16). In conclusion, this study demonstrates for the first time that patients with different outcomes after an AMI can be significantly discriminate using metabolomic technologies, and thus metabolomics can improve the standard stratification based on clinical and biohumoral parameters.

References

[1] A. Vignoli, L. Tenori, B. Giusti, P. G. Takis, S. Valente, N. Carrabba, D. Balzi, A. Barchielli, N. Marchionni, G. F. Gensini, et al., BMC Med. 17, 3 (2019).

[2] F. Cesari, R. Marcucci, A. M. Gori, R. Caporale, A. Fanelli, G. Casola, D. Balzi, A. Barchielli, S. Valente, C. Giglioli, et al., Thromb. Haemost. 109, 846–853 (2013).

New NMR tools for clinical research and integrated use of NMR and MS

Claudia Napoli

Bruker Italia, viale Lancetti 43, 20158 Milano (claudia.napoli@bruker.com)

The global profiling of metabolites is becoming increasingly important in human metabolomic and clinical research.

For this reason, Bruker has introduced a platform for the automated analysis of NMR spectra from clinically relevant biological fluids like urine, serum and CSF: the In-Vitro Diagnostics for research (IVDr) system.

In some cases, however, the evaluation of spectroscopic data obtained from bodyfluids, points to signals of compounds which cannot be identified readily by comparison to spectra in libraries or data bases. If the unknown signal belongs to a marker discriminating healthy individuals from patients suffering from a disease, it is of fundamental importance to solve the identity of this compound in order to reveal the biochemical reason for the onset of a disease.

If NMR and mass spectroscopic data are available for such a study, heterostatistical analysis can be employed to correlate molecular mass information to signals in the NMR spectrum of the sample of interest. In this way, valuable information can be obtained which can identify the marker.

When it is not possible to identify the compound of interest readily, the compound must be chromatographically isolated and the structure elucidated after acquiring MS/NMR required data.

Some examples will be shown.

Can a cardiac ischemic episode be anticipated by metabolic profiling? An untargeted NMR study

Daniel O. Cicero[1], Erica Pitti[1], Angelo Lembo[2], Gaetano Tanzilli[3], Nicola Viceconte[3]

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2. *IRBM S.p.A., via Pontina km 30.600, 00071 Pomezia (RM) Italy*
3. *Dipartimento di Scienze Cardiologiche, Respiratorie, Nefrologiche e Geriatriche, "Sapienza" University of Rome, Policlinico Umberto I, Rome, Italy*

Cardiac ischemia is a severe pathological condition characterized by a reduced oxygen flow to the heart that can lead to myocardial infarction [1, 2]. Classical diagnostic methods are affected by low sensitivity or by high dose of radiation [3]. Here we present a non-invasive metabolomics profiling of cardiac ischemia fingerprint in human plasma by 1H-NMR. Plasma samples of ischemic and healthy patients were collected before (basal dataset) and after a cardiac stress test (stress dataset). Multivariate data analysis was used to evaluate metabolic fingerprint discrimination between healthy and ischemic patients. Unexpectedly, we found a significant difference already in the basal samples between healthy and ischemic patients undergoing the cardiac stress test. This result suggests that metabolism is already anticipating the outcome of the stress test and can help in determining a risk factor useful for physicians to diagnose cardiac conditions

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NMR-fingerprint of blood: from methods to application in precision medicine

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The type and abundance of metabolites detected in a biological sample can be viewed as a global fingerprint that unambiguously describe the overall response of the individual to the different stimuli (i.e. pathology, treatment, etc.); this is particularly true for blood. In this framework, in our lab, IVDr platform (Bruker s.r.l.) is routinely used for the NMR-metabolomic fingerprinting of blood serum/plasma. To ensure intra- and inter-laboratory comparability, the IVDr platform uses standardized procedures for NMR sample preparation and spectral acquisition [1,2,3]. Here we will provide an overview of the critical aspects of both pre-analytical and analytical procedures. The former is designed to obtain high quality samples and includes all steps from collection to NMR sample preparation; the latter relies on instrumental optimization, NMR pulse sequence selection, and choice of acquisition parameters.

The strong potentiality of serum metabolomic-fingerprinting to approach precision medicine will be demonstrated making reference to a pilot study aimed at the accurate identification of responders to immunotherapy among patients with aggressive forms of lung cancer.

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Metallome alterations in gestational diabetes: an investigation on maternal whole blood, placenta and cord whole blood samples

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Gestational diabetes (GDM) is defined as a carbohydrate intolerance with onset or first recognition during pregnancy, resulting in hyperglycemia of variable severity. Although glycaemia normally returns to physiological levels after delivery, GDM might cause short-term and long-term complications for both the mother and the fetus. The molecular pathways and physiological processes involved in GDM development are still unknown. Omics sciences represent a promising strategy to study GDM at molecular level.

In this work, placenta samples, maternal whole blood and umbilical cord whole blood from GDM patients and controls were collected after delivery and analysed via ICP-MS to determine the metallome, i.e. the whole elemental content. Results were statistically evaluated to evidence the correlation between the elemental concentrations in all samples and the presence of the disease. The results obtained in whole cord blood showed that many elements were correlated with GDM: Ca, Cu, Na, and Zn were present in higher concentration in GDM cord blood than in control samples, whereas Fe, K, Mn, P, Rb, S and Si showed an opposite trend. It was also highlighted that the cord blood from GDM patients exhibited an elemental composition more similar to that of the mother blood compared with the cord blood from control subjects. These results, in part interpreted in the light of the literature, open the possibility to use cord blood as a GDM marker, thus helping to delineate more accurate nutritional guidelines for pregnant women and to explain the biochemical processes occurring in the fetus and placenta during GDM.

Development of high throughput, multi-omic methods applied to a breast cancer study

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Current discovery OMIC analytical methods provide broad sample coverage but often employ long sample cycle times to achieve this. Using these conventional methods for the analysis of large sample sets, imparts pressure on laboratory resources from personnel to instrumentation and subsequently require analysis in multiple batches leading to batch effects. Furthermore, the identification of disease biomarkers helps facilitate early detection, risk reduction and monitoring the efficacy of disease treatment. Genomics has led the way in disease biomarker detection and monitoring, however there is still a growing need for fast, robust and inexpensive blood tests to provide accurate biomarker candidates while having minimal impact on resource and data quality. Here, we demonstrate the application of rapid lipid and protein-based methodologies to plasma sourced from breast cancer patients, in order to provide greater insight into the pathogenesis of the disease.

Un-depleted plasma was prepared for LC-MS analysis using IPA for protein precipitation (lipid analysis) or tryptic digestion for proteomic analysis. Standard flow (1mm scale) chromatography was employed for both peptide and lipid analysis with various data independent (DIA) strategies [1,2] used as part of the MS acquisition for both analyte types. Data were subsequently processed and database searched. Further curation of the data was then conducted prior to pathway analysis. In summary, the results showed a number of lipid species to be differentially regulated (i.e. phosphatidylserines and phosphocholines). Complimentary proteomic data showed a variety of proteins to be expressed, including APOH, haptoglobin and serotransferrin as examples. Combined lipid/protein mapping to pathways highlighted several statistically significant pathways, including IL-6 signalling and associated immune responses.

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NMR metabolic studies of the renal cortices changes in a mouse model of Renal Cell Carcinoma (RCC)

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Renal Cell Carcinomas (RCCs) are commonly found in syndromic forms of cancer as Von Hippel Lindau (VHL) disease [1] and Tuberous Sclerosis Complex (TSC) [2]. In the Dr. A. Boletta's laboratory (San Raffaele Scientific Institute) is available a mouse model, in which TSC1 gene is inactivated specifically in kidneys, that recapitulates the progression of the RCC: from cysts formation to cystsadenomas to carcinomas. Metabolomic studies were performed at P50, time point corresponding to cystadenoma, and P80, time point corresponding to carcinoma. 1D 1H NMR noesy experiments were acquired on the polar extracts of murine renal cortices collected at P50 and P80. Partial least squares-Discriminant Analysis (PLS-DA) was performed comparing wild-type (WT) and knock-out (KO) renal cortices collected at P50 and at P80. Metabolic pathway analysis found 20 and 16 metabolic pathways deregulated at P50 and P80, respectively; 15 of them are in common and they are usually involved in cancer, such as TCA cycle. Moreover, targeted analysis was performed, and 46 metabolites, usually involved in kidney metabolism and cancer, were identified and quantified. Interestingly, KO samples at P50 and P80, at variance to WT, present an accumulation of fumarate, a known oncometabolite [3]. Further studies on the same mouse-model, performed in the lab of Dr. Boletta indicate that Tsc1 inactivation results in the mTORC-dependent downregulation of the TCA cycle enzyme fumarate hydratase (FH). Taken together this study reveals a role for mTORC1 in renal tumorigenesis, which depends on the oncometabolite fumarate [4].

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LC-MS Lipidomics to characterize the altered lipid metabolism as a stress reaction to acid tumor microenvironment in osteosarcoma

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Osteosarcoma (OS) is an aggressive bone malignancy with a high relapse rate despite combined treatment with surgery and multiagent chemotherapy [1,2]. Tumor-associated microenvironment may contribute to tumor initiation, growth, and metastasis. In this context, tumour-induced acidosis is increasingly recognized as a hallmark of cancer, and also in OS, it has been demonstrated to promote cancer progression. However, the mechanism has not been fully elucidated and might include a metabolic adaptation of the tumour cells to the hostile acid microenvironment [3-6]. Preliminary studies on OS spheroids have shown that acidosis causes lipid droplet accumulation, increased lipophagy and sphingomyelin synthesis and alterations of S1P/SphK signalling pathway. We are now investigating the alterations in lipidomic profiles of serum of patients diagnosed with OS, by performing untargeted high-resolution LC-MS lipidomics. The participants were sampled at two different time points: at diagnosis and after surgical and chemotherapeutic treatment. The aims of our activities are to: 1) confirm the association between tumor acidosis and lipid alterations in patients; 2) discover putative candidate biomarkers to be correlated to metastasis occurrence. The long-term objective of the project is to use lipidomics data to deeply dissect the OS machinery and to unravel novel therapeutic targets that are related to the altered lipid metabolism and, finally to identify entirely novel therapeutic targets and circulating lipid markers with prognostic value.

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How NMR data could assist MS hit classification in an untargeted metabolomics analysis? Our case study: bladder cancer

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Compound identification in mass spectrometry based metabolomics can be a problem if no authentic standard library is available. Many papers report the use of MS/MS or MS2 as a tool for the identification of known metabolites [1]. NMR and LC-MS are the analytical tool that are routinely, but separately, used to obtain metabolomics dataset due their versatility and accessibility. These two techniques are highly complementary and combining them is likely to improve the overall quality of a study and enhance the coverage of the metabolome [2]. Urine samples of patients affected by bladder cancer were used as an evaluation dataset to find new ways to combine NMR and MS information. In our work we propose an analytical tool where NMR information is used to help in the classification of hits from MS analysis. Our workflow is quite simple we used NMR dataset of quantified metabolites as a reference library for the classification of significant MS hits into biochemical pathways. To do that, we look for LC-HRMS hits which intensities show high correlation indexes with the NMR quantified metabolites. In this way we obtain a first picture of the pathways that are perturbed, also if they are limited to those covered by the available NMR metabolites. This approach is not intended to be a replacement for hit identification, but rather a preliminary analysis of the large LC-HRMS dataset. The correlation between NMR and MS datasets can enlarge also the metabolome coverage in order to reinforce our biochemical hypothesis related to cancer onset.

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Torpedino and San Marzano tomato fruits metabolite profiling through NMR and MS methodologies

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Torpedino di Fondi (TF) is a hybrid tomato variety recently introduced in Lazio area, developed along with the classical San Marzano (SM) variety, characterized by a peculiar sweetness and palatability.

The present study carried out in the frame of e-ALIERB OpenLab Project funded by "Regione Lazio" [1], aimed at the metabolite profiling and characterization of TM and SM tomato fruit varieties, at two developmental stages (ripe and unripe).

Blight-Dyer extraction protocol [2] was applied to tomato samples and the resulting organic and hydroalcolic fractions were analyzed by high field NMR (1D and 2D experiments 1H-1H TOCSY, 1H- 13C HSQC and 1H-13C HMBC) and ESI FT-ICR MS methodologies. Different metabolites were assigned in both extracts of tomatoes by mean of literature data [3,4], reference standards and 2D NMR spectra. From a qualitative point of view the 1H spectra of all tomato varieties appear to be conservative since the same metabolites are present in all the samples examined.

From a quantitative point of view, differences can be observed due to the different concentrations of the metabolites. ESI FT-ICR MS experiment confirmed most of metabolites identified by NMR analyses belonging to different classes of compounds (carbohydrates, organic acids, amino acids, fatty acids) and allowed the identification of several additional bioactive constituents, which might represent potential chemical markers for discrimination of SM and TF cultivar and their ripening stage.

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Innovative qNMR methodology for carbohydrates quantification in complex mixtures

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The knowledge on carbohydrates composition is of great importance to determine some properties of natural matrices, such as foodstuff or bio-fluids. Due to their structural similarity and to the high number of isomers they present, carbohydrate analysis is complex. While a number of analytical techniques and methods have been used to quantify them, most of these are expensive and time-consuming due to complex sample preparation procedures, which require the derivatization of the carbohydrates being analyzed. Here we present an innovative qNMR procedure based on highly selective chemical shift filters followed by TOCSY, which allows to acquire specific background-free signals for each sugar with no pre-treatment of the sample [1]. The method was validated on raw honey, the most complex natural matrices in terms of saccharides composition. Twenty-two main sugars present in honey were accurately quantified: four monosaccharides (glucose, fructose, mannose, rhamnose), eleven disaccharides (sucrose, trehalose, turanose, maltose, maltulose, palatinose, melibiose and melezitose, isomaltose, gentiobiose nigerose and kojibiose) and seven trisaccharides (raffinose, isomaltotriose, erlose, melezitose, maltotriose, panose and 1-kestose). This method was extended to other complex matrices: preliminary data on wine, milk and saliva will be also presented.

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A multi-methodological protocol to characterize the metabolite profile of "Bianco di Sperlonga" PGI white celery ecotype

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White celery is a widely used vegetable known for peculiar sensorial and nutritional properties. Here, the white celery (Apium graveolens L.) "sedano bianco di Sperlonga" PGI ecotype cultivated in Lazio region of Italy was investigated to obtain the metabolite profile of the edible part (blade leaves and petioles). A multi-methodological approach including high resolution NMR and ESI FT-ICR MS was applied to analyze celery extracts. The most abundant metabolites belonging to different classes of compounds (sugars, polyalcohols, amino acids, organic acids, phenols, sterols, fatty acids, phthalides, and flavonoids) were identified and quantified. The results of the study highlight differences between petioles and blade leaves chemical profile, thus suggesting specific nutritional and health properties of the different celery parts. Moreover, the detailed characterization of metabolite profile furnishes an additional value to this product with specific properties owing to the production area. The present study was supported by e-ALIERB OpenLab project funded by "Regione Lazio" [1].

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A New LC/Q-TOF Platform for Metabolomics Analysis: 6546 LC-QTOF workflows

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Agilent has introduced the 6546 Q-TOF this year. The biggest enhancement in the 6546 LC/Q-TOF is the implementation of 10 GHz ADC electronics. With the faster digitization, much greater resolution, especially at low m/z, is achieved while still maintaining broad dynamic range. This is clearly an advantage for metabolomics and lipidomics.
The 6546 offers accurate isotope ratio measurements with a specification of ±5%. This is valuable for confident identification of compounds, including empirical formula generation of unidentified compounds.
The capillary-gate valve allows capillary maintenance without venting the instrument thus easier maintenance and less downtime.
All of these improvements were made while still maintaining the same analytical sensitivity and robustness of the previous generation 6545 LC/Q-TOF, an instrument that has delivered consistently outstanding metabolomics results.
A major challenge in mass spectrometry-based metabolomics and lipidomics is the comprehensive characterization of metabolites or lipids, spanning a wide concentration range within a biological sample. Identification or annotation requires data acquisition at the MS/MS level to enable product-ion spectral matching against databases.
While LC separation helps elucidate isomeric species and reduce complexity, data-dependent high-resolution MS/MS data is limited by the number of precursors that can be selected for fragmentation during chromatographic elution. Therefore, it is still not possible to acquire all the MS/MS spectra of interest in a single analysis for complex samples. The Agilent iterative MS/MS mode achieves more comprehensive coverage by allowing repeat injections of a sample using an automatically-created rolling exclusion list to select new precursors in each injection. Precursors ions selected in the first injection are not selected again in injections 2 or 3. Instead, new less abundant precursors are chosen for MS/MS. In this manner, it is possible to more deeply identify and/or annotate metabolites and lipids.
In lipid profiling, lipid annotation is the biggest challenge. Agilent Lipid Annotator software is designed to rapidly and accurately annotate lipid MS/MS data and convert those results into an accurate mass, retention-time database. The AMRT database is created by acquiring comprehensive MS/MS for a representative sample or samples. This could be a pooled sample, a fractionated sample, a QC sample, or multiple samples but needs to be from the same sample source. This lipid AMRT database is then used to easily extract annotated lipids from MS1 data using the same chromatography as used for database creation. That lipid database is then used for either targeted extraction of the annotated lipids from MS1 data or to annotate features in a discovery workflow. This will be covered in more detail as will the Lipid Annotator software.

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HPLC-MS/MS untargeted metabolomic approach for disease-related molecular markers detection in "olive quick decline syndrome"

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Olives *(Olea europaea)* are being decimated in some regions since 2003 by a destructive disorder called "olive quick decline syndrome" (OQDS). [1]

Etiology of this disease is related to *Xylella fastidiosa,* a pathogenic gram-negative bacterium of American origin. The plant, once infected by this bacterium, starts to quickly dry up and could die. [2]
Nowadays the proliferation of *Xylella* lead Apulia's region economy to suffer for a sensible decrease in olive oil production. [3]

Given the complexity and multifactoriality of OQDS, the demand to understand the pathosystem and the need to identify and recognize infected plants, request a powerful tool not only to detect the bacterium itself but to find molecular markers for a healthy-infected discrimination.

HPLC-MS/MS techniques are nowadays capable of high-resolution profiling of an entire metabolome. They can provide a wide and in-depth knowledge of multiple samples comparison by to chromatograms and mass spectra interpretation software. Therefore, the primary purpose of this work is to develop an efficient HPLC-HRMS analytical method based on untargeted metabolomics to identify metabolites that are differentially expressed in olive trees and involved in disease development or defense responses to OQDS. The samples (olive leaves) are submitted to a preparation protocol developed by University of Salento to extract all the metabolites of interest from healthy (HP) and infected (OP) olive trees samples.

An HPLC coupled with an HRMS instrument (LTQ orbitrap) were used to set a method up by University of Torino for metabolites identification. All data coming from analysis were submitted to XCMS program for putative identification of the main metabolites involved in the pathogenic pathway.

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Mass Spectrometry Imaging for Untargeted Plants Metabolomics: a Case Study in Mycotoxin Accumulation

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Mass spectrometry imaging (MSI)-based metabolomics is a powerful tool for detection and visualization of metabolites, essential to assign their functional role. Nevertheless, MSI untargeted metabolomics is not as exploited as expected, mainly because MSI-data analysis still represents a remarkable computational challenge due to the size and complexity of the datasets.

To this end, some of us recently developed LipostarMSI (Molecular Horizon srl, Perugia, Italy): the first comprehensive, vendor-neutral software for MSI that covers all the steps required for MSI data analysis, including automated metabolite annotation, significantly streamlining biochemical data interpretation [1].

In this talk, a case study in food metabolomics will be presented to illustrate the bases of the LipostarMSI workflow.

In particular, to gain insight into plant resistance mechanism against mycotoxin accumulation, transversal cross-sections obtained from wheat roots, stems, leaves were treated with mycotoxin using an in-vitro plant model. To disclose the distribution of metabolites involved in the plant response, samples were analyzed using atmospheric-pressure (AP)-scanning microprobe matrix-assisted laser desorption/ionization MSI ion source (TransMIT GmbH, Giessen, Germany) coupled to a Q-Exactive HF orbital trapping mass spectrometer. By comparing the mycotoxin-treated and control samples in LipostarMSI, several differentially accumulated and distributed metabolites were found, demonstrating the analytical potential of combining innovative technique as MSI with comprehensive high-throughput software solution.

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Harmonization of data processing procedures for non-targeted NMR analysis in metabolomic studies

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Nuclear Magnetic Resonance (NMR) spectroscopy is a recognized analytical tool able to furnish a huge amount of information rapidly, in a non-destructive and reproducible way. Several algorithms have been introduced to facilitate the statistical elaboration of a large number of data. A pre-processing step, involving data bucketing and bucket scaling, is always required before submitting the data to the statistical elaboration.[1,2]
So far, the choice of the pre-processing approach has been based mainly on the observation of the best matches according to the initial goal, and no unique criterion has been introduced.
In this communication, we describe the results observed during a chemometric study conducted on a large amount of data deriving from an interlaboratory comparison involving 65 spectrometers, which were different in manufacturer and magnetic field strength. In particular, we discuss on suitable strategies to process data in such a way to stress the importance of different factors (nature of sample vs instrument features).

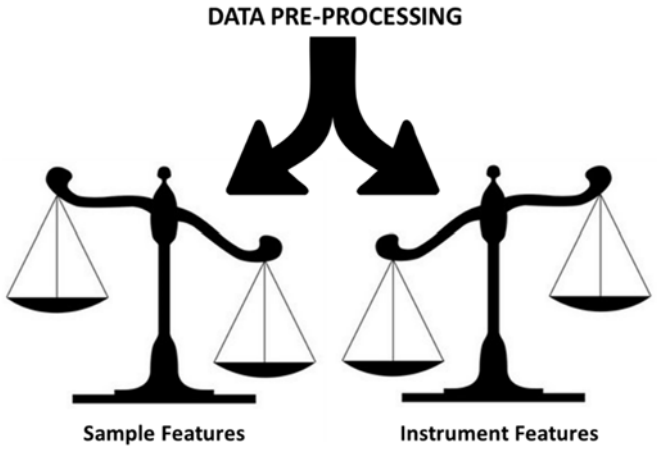

Fig. 1. Schematic representation of the influence of data pre-processing mode on the factors causing the discrimination of data distribution.

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Multivariate data analysis of metabolomic data: data integration, feature selection and visualisation

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Today a close interaction between statisticians, bioinformaticians and molecular biologists is essential to provide meaningful results. In particular, unlimited quantity of data from multiple and heterogeneous sources are publicly available, bringing computational issues to foresee, and biological interpretation issues for validation. In fact, molecular entities act together in biological pathways and need to be appropriately modelled using novel statistical techniques. Multivariate methods are well suited to large ‘omics data sets where the number of variables (e.g. genes, proteins, metabolites) is much larger than the number of samples (patients, cells, mice). Appealing properties of reducing the dimension of the data by using instrumental variables (components), which are defined as combination of all variables. In the present presentation, the modern tools dedicated to the multivariate analysis of biological data sets will explored with a specific focus on data exploration, dimension reduction and visualisation. A wide range of methods that statistically integrate several data sets at once to probe relationships between heterogeneous ‘omics data sets will be discussed.

SWATH™: QUAL & QUAN metabolomics in the same run

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Data independent acquisition (DIA) workflows are well adopted in quantitative discovery proteomics, but still not commonly used in discovery metabolomics. Data dependent acquisition (DDA) techniques are heavily employed in the field of metabolomics and workflows on mass spectrometers have been adapted so that as much data as possible can be captured. Researchers were limited by the speed of their QTOF mass spectrometers meaning a multiple injection workflow. Also, the stochastic nature of data dependent workflows often means MSMS of low abundant metabolites are often missed. Here, we describe how DIA enables the identification of a higher number of metabolites for untargeted metabolomics workflows compared to traditional DDA approaches thus enabling a broader profile of the metabolome.

DDA and DIA specific settings were chosen and evaluated. The data were acquired on a QqTOF mass analyzer. For the DDA acquisition, we selected the top 5, 10, 15, 20 and 25 precursor ions for MSMS. For DIA, we applied 15, 20 and 30 mass windows with either fixed window (fw) or variable window (vw) widths. Results were evaluated by the highest number of identifications and coverage of metabolites in plasma and urine extracts.

At the DDA level, the data demonstrate a significant improvement of metabolite coverage at the MSMS level when comparing the top5 to the top25 DDA method. We show over 100% increase of metabolite coverage in plasma extracts by increasing the number of selected precursor ions for DDA acquisition from top5 to top25. This result highlights the capability of the QqTOF mass analyzer for fast MSMS acquisition, which allows for the fragmentation of a large number of precursors in a single DDA cycle, leading to a larger number of metabolites identified.

In the second part of this study, we evaluated the DIA strategy with various fixed (fw) and variable window (vw) sizes with similar cycle time in a plasma extract. Increasing the number of fixed windows resulted in ~30% gain in metabolite coverage. Using the variable window method resulted in a ~70% gain in metabolite coverage.

We finally applied these experimental approaches to common matrices used in metabolomics studies, namely urine and extracted plasmaWe show that a DIA approach applying 20 variable windows can identify up to 55% more metabolites than a traditional top20 DDA acquisition (in a urine extract). More confident MSMS based identifications lead to higher quantifiable metabolites in a metabolite expression experiment, which at the end allows better understanding of the biology. When comparing the performance in extracted plasma it can be observed that applying a DIA approach with 20 variable windows allows significant gains in metabolite coverage (around 55%) versus the top20 DDA acquisition, similar gains as seen in the urine extract.

Metabolomic profile of aqueous humour in a 24-hours period after death: an animal model for post-mortem interval estimation

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The estimation of the time since death or post-mortem interval (PMI) remains a main issue in the forensic scenario being one of the most challenging points in question for forensic pathologists. Several forensic laboratories are involved in ongoing efforts to describe and to validate an objective analytical method for this purpose. Beside traditional methods, based on macroscopic corpse modification, different analytical procedure have been recently proposed for this aim, including the evaluation of mRNA or DNA degradation in post-mortem tissues, human tissues transcriptomes and gene expression patterns, muscle proteins degradation based on protein or peptide signatures, the estimation of the hypoxic inducible levels of vascular endothelial growth factor, and the study of post-mortem biochemical changes in body fluids, such as blood, synovial, pericardial, and cerebrospinal fluids. Ocular tissues and fluids (vitreous humour above all), have been frequently chosen as reference biological samples [1], due to their anatomical features. Recently, metabolomics has shown to be a potential tool to investigate the time-related post-mortem metabolite modifications in animal models [2]. While traditional techniques for PMI estimation are quite subjective in nature and other proposed methodologies are based on the estimation over-time of a single o few parameters (potentially prone to the influence of intrinsic/extrinsic factors), the analysis of metabolomic modifications, relying on multiple metabolites/biomarkers quali-quantitative changes, may be more informative.
Here we propose, for the first time, the use of a ^{1}H NMR metabolomic approach for the estimation of PMI from aqueous humour (AH) in an ovine model. A total of 59 AH samples collected at different PMI (spanning form 118 up to 1429 min, at 60 min pace). 38 out of them were used for the training set while the remaining 21 were employed as test set. ^{1}H NMR experiments were performed, and spectral data analysed by multivariate statistical tools. Exploratory data analysis was performed using Principal Component Analysis (PCA) to discover outliers and specific trends in the data. Thus, supervised data analysis based on Projection to Latent Structure regression (PLS2) was applied to evaluate the effects of PMI on the metabolomic profiles of the collected samples. In particular a multivariate oCPLS2 calibration model was built to estimate PMI on the basis of the metabolite content of the samples. The model was validated with the independent test set, obtaining a prediction error of 99 min (59 min for PMI <500 min, 104 min for PMI from 500 to 1000 min, and 118 min for PMI >1000 min). During the first 1000 min lactate is accumulated and strongly influences the sample distribution along PC1. Early PMI samples are characterized by high levels of leucine and isoleucine, arginine, and lysine, while late PMI samples (>1000 min) show high levels of taurine, succinate, and choline. Moreover, the metabolomic approach suggested a picture of the mechanisms underlying the post-mortem biological modifications, highlighting the role played by taurine, choline, and succinate. The time-related modifications of the ^{1}H NMR AH metabolomic profile in the first 24 hours since death seem to be encouraging in addressing the issue of a reproducible and robust model to be useful employed in the estimation of the time since death.

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From actinic keratosis to squamous cell carcinoma: NMR analysis with clinical and histological aspects

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The most common lesion found on sun-damaged skin is Actinic Keratosis (AK). AKs are considered pre-malignant cutaneous lesions that may progress to Squamous Cell Carcinoma (SCC).[1] The development of new AKs lesions, the recurrence of treated AKs and the progression to SCC is frequently observed in clinical practice, suggesting the hypothesis that AK is the first manifestation of a disease continuum that may lead to SCC. [2] The reason behind this hypothesis can be explained by the concept of field cancerization, either cutaneous or in other organs may be defined in molecular terms, in which the presence of mutated cells forms the groundwork that permits the progression of epithelial carcinogenesis with its relevant clinical consequences.[3] Exploring the metabolome of cancer and precancerous lesions seems to be a parallel and effective way to understand the phenotypic changes associated with cancer progression. Metabolomics could reveal novel cancer biomarkers that might expand our current understanding of the multi-factorial disease. With Nuclear Magnetic Resonance (NMR) spectroscopy, we obtained an accurate description of the molecular composition of the tissue and provide a "fingerprint" of the whole metabolome. We found correlations between some metabolites and proliferative markers that allow gaining insight into the relationship between cellular proliferation and metabolic changes associated with the presence of tumor and its aggressiveness.

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An integrated software platform to improve the identification of metabolites, on the untargeted LC-MS metabolomics

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Untargeted metabolomics, using high-throughput techniques like liquid chromatography coupled to mass spectrometry (LC/MS) [1], allows monitoring of the whole metabolic composition of a specimen, without a preliminary selection of specific metabolites, producing a large amount of data. A well-defined workflow is essential to guarantee accurate and high quality data [2], however comprehensive software solutions for untargeted metabolomics data management are limited. Currently, different software platforms are used to implement individual workflow steps [3]. The main challenge is to integrate them in a unique, user-friendly architecture, where automatic procedures for data processing are applied to ensure data stability. This work aims to create a customized solution where different software platforms are integrated to implement our workflow for untargeted metabolomics. Specifically, MS data and sample information are collected and stored in a single database based on ACD/Spectrus technology. The generated database must be continuously updated every time a new metabolite is identified, in order to have a ever-grown knowledge of compounds. The designed data model allows a simple integration with Progenesis, aiming to perform a faster component identification process with high confidence, reducing workflow time and interpretation errors in the metabolic pathways involved. Importantly, in-house data were merged with the available databases to improve the quality of data annotation/identification.

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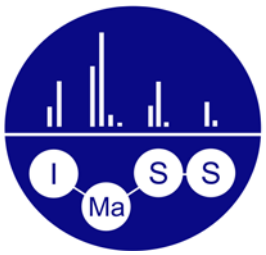

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Poster Communications

From beans to brew: NMR based metabolomic approach to assess traceability of coffee producers within a restricted geographical area of Colombia

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The precise identification of the origin of the coffee plantation is a really challenging task. Colombia is the world's third coffee-producing country, after Brazil and Vietnam. We think that the identification of the origin of coffee beans coming from small, geographically localized Columbian plantations is the ideal case to proof the power of the NMR fingerprint approach. Our preliminary results are based on the collection of 51 batches of green coffee beans (*arabica*) coming from different Columbian producers. After setting the most suitable protocol for the analysis, from each batch we prepared 7 different samples undergoing NMR analysis. OPLS-DA model was built on 357 green coffee bean spectra acquired using NOESYGPPS pulse sequence. The model, validated using leave-one-out cross-validation scheme, shows an overall of 90.7% in predictive accuracy. In parallel, 18 of those batch, were sent for roasting to a professional coffee trader. The statistical model built on roasted coffee beans shows an overall predictive accuracy of 95% in identifying the farms (origin). We correlated metabolites with batch characteristic (e.g. altitude of the plantation, time of fermentation etc.) and organoleptic properties finding weak correlations (alanine/altitude = R 0.56; $p<0.001$; sucrose/altitude = R 0.53; $p<0.001$; maleate/bitterness = R 0.60; $p<0.05$), using green coffee fingerprints we correctly predict the coffees with a quality score above 70 (i.e. of remarkable quality).

NMR-based metabolite profiles in mytilus galloprovincialis: experimental set-up and preliminary data

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Metabolomics studies founded on mass spectrometry (MS) or nuclear magnetic resonance (NMR) have rapidly expanded in the last decade. This and other current 'omics (genomics, transcriptomics, proteomics) complement each other and contribute to a holistic view of organism responses to stimuli. In fact, the profiles of all small molecules detectable in biological fluids or tissues capture a further phenotype level, that of condition-dependent and time-dependent metabolome changes.
We have evaluated different protocols in order to process the whole haemolymph, its acellular fraction and other soft tissues of the Mediterranean mussel *Mytilus galloprovincialis*, relying on available metabolite databases and suitable software for the identification and representation of mussel metabolite profiles. Mussels maintained in standard conditions or kept in air at 4 °C were used in the procedural set-up. The NMR profiles resulting from the analysed tissue matrices allowed the identification of amino acids, fatty acids, organic acids and typical osmolytes. These profiles represent the starting point for hypothesis-driven basic and applied research, such as the quality assessment of marketable stocks.

KEYWORDS

Mytilus, metabolomics, NMR

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The importance of bucketing procedure for NMR-based metabolomic fingerprinting

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NMR spectroscopy is a powerful technique used for structure elucidation and for the analysis of complex biological matrices. However, parameters such as temperature, pH, concentration of salts and ionic strength can influence peak location leading to frequent misalignments [1] which could strongly affect chemometric classification models.
Bucketing represents a useful procedure to compensate for these misalignments and it is also used to reduce the total number of variables [2] by dividing NMR spectra into small "buckets" typically spanning 0.02 – 0.04 ppm. [3]
Although there are different bucketing techniques [3], the equidistant binning is the most commonly applied method and works quite well despite its simplicity.
This approach is particularly useful to perform untargeted NMR-metabolomics via fingerprinting. [2]
How can NMR-based metabolomic fingerprinting be affected if we use different ways to perform the bucketing procedure? Can sample classification and patter recognition change?
In this study, we address these questions using large cohorts of urine and serum NMR spectra to evaluate firstly, the change of the bucket width going from considering the full resolution spectra to bins of 1 ppm and secondly, the effect of shifting the starting point of the bucket integration.
The results achieved suggest the best compromise to choose among bucket width values and starting point of binning integration to perform NMR-based metabolomic fingerprinting. They also confirm the use of the bucketing technique as the best and the easiest method to keep all the necessary information to perform sample classification.

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Blood microsampling for untargeted lipidomics

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Lipidomics has become a good bioanalytical tool for biomarker identification in a wide range of diseases [1]. To this aim, whole blood could be promisingly exploited to obtain as much information as possible, despite the well-known intrinsic problems of this biological matrix, mainly related to sampling invasiveness, pre-analytical manipulation and processing, when compared to plasma and serum [2]. To overcome the disadvantages of classic whole blood analysis, two microsampling approaches are proposed: Dried Blood Spot (DBS) and Volumetric Absorptive Microsampling (VAMS). Both techniques allow the collection of small amounts of matrix (20 µL) in a minimally invasive way, directly by fingerprick followed by drying and storage at room temperature. After the loss of water, often responsible for degradation reactions, dried microsamples ensure analyte stability. In addition, VAMS device can absorb a fixed and highly reproducible whole blood volume by means of a hydrophilic polymer tip, regardless of haematocrit value [3]. Fast and feasible original pretreatment procedures have been developed and optimised by testing different pure and mixture solvents and extraction means. To evaluate the best performances for each lipid class and sampling mode, 15 benchmark lipids have been chosen, being representative for the most abundant lipid categories. Quali-quantitative results by means of an originally developed high-resolution UHPLC-MS/MS method were processed and compared by using a multivariate data analysis approach. This allowed to define the best extraction protocol for DBS and VAMS and, after comparison with fluid blood, to suggest VAMS strategy as a promising alternative procedure for blood sampling for untargeted lipidomics.

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MS metabolomic overview of chemical interactions from leaf cutting ants symbionts

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Attine ants are part of a quite complex symbiosis in nature: the mutualistic fungus with their garden fungus, *Leucoagaricus gongylophorus* that constitutes their main food source [1]. Since the garden fungus is threatened by pathogenic fungi from the genus *Escovopsis*, and the ants need to defend their nest and to do so they joined alliance with symbiotic actinobacterial symbiont *Pseudonocardia and Streptomyces* that produce antibiotics compounds against the parasites [2]. There are others microbes in the microcosmos of leaf cutting ants, for example, the black yeasts from the genus *Phialophora* that its ecological role remains understudied. [3,4]. The leafcutter ant symbiosis has been particularly well studied of the biological perspective, however recently the chemical signals involved in this communication have been studied as well [5]. This work shows how to improve the data management from different interactions of leaf cutting ants` symbionts through the use of MS molecular networking and quimiometric analysis. Ten different chemical interactions (five symbiont microbes of ants) were analyzed and compared the metabolic induction in different cocultures. Different secondary metabolites were identified using library GNPS such as terpestacin, ginsenoside Rh1, shearinines D and F and others. MS network approach can be used together to metabolomic tools to detect some m/z ratio that only induced only in the cocultures. In conclusion, a combination of molecular networks and metabolomic approaches can advance the MS/MS data analysis of secondary metabolites in microbial interactions and it can be possible to understand how it works the communication in complex environments as the leaf cutter ants garden.

Key-words: Microbial interactions, leaf cutting ants symbionts, metabolomic, molecular network.

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Development of a green method to extract lipids from human plasma

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Bligh and Dyer (B & D) and Folch procedures for the extraction and separation of lipids from microorganisms and biological tissues using chloroform/methanol/water are considered as "gold standards" for the analysis of extracted lipids.[1,2] However, these methods have serious disadvantages in terms of safety, especially with chloroform, which is highly toxic and carcinogenic, making it inappropriate for large-scale use in metabolomics.[3] Here we present the application of a green B & D extraction on human plasma. Lipid extraction was tested in mono- and biphasic condition (respectively using ethylacetate/ethanol/KCl_{acq} 0.8% 40/40/40 and 60/18.8/21.2 w/w/w). Using the Folch method as reference, three aliquots of the same plasma sample were extracted in order to assess the extraction efficiency and reproducibility of each method. Our results highlight higher extraction efficiency of biphasic B & D with respect to Folch. Moreover, the use of green solvents and the easier recovery of the organic phase containing the lipids, make this method more feasible to be automated than other extraction procedures.

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Non canonical Cyclic Nucleotides Monophosphates in Aphanizomenon flos-aquae: nuclear magnetic resonance and mass spectrometry

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Aphanizomenon flos-aquae (AFA) cyanobacteria from Klamath Lake (Oregon) are considered a "superfood", due to their complete nutritional profile that has proved to have health-enhancing properties. AFA metabolome is quite complex. Here, we present a study that, combining multinuclear ^{1}H, ^{31}P and ^{13}C NMR spectroscopy and high-resolution mass spectrometry, allows the detection of rather unusual phosphorylated metabolites in AFA [1,2]. In this study we focused our attention on AFA phosphorylated metabolites giving ^{31}P NMR signals at 20 ppm, a chemical shift that pointed to phosphonates. They instead revealed to be nucleoside 2',3'-cyclic monophosphates (cNMPs), that were characterized by multinuclear ^{1}H, ^{31}P and ^{13}C NMR spectroscopy and high-resolution mass spectrometry. Our data are fully consistent with the proposed structures and hence demonstrate the presence of cNMPs in AFA, for the first time. The most studied of these biomolecules is cAMP that activates a protective mechanism in the case of brain tissue injury, whereas it inhibits mitophagy of damaged mitochondria in the kidney [3]. The role of the other cNMPs there is much to be discovered.

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Lipidomic data analysis for non-alcoholic fatty liver disease

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Non-alcoholic fatty liver disease (NAFLD) is a complex metabolic disease, which currently may be diagnosed by liver biopsy. The outbreak and the progression of NAFLD vary widely from one subject to another, depending on a huge number of factors, as genetics, environment or lifestyle. Biomarkers of altered hepatic metabolism are therefore needed.
It is known that alterations in lipid metabolism and insulin resistance (IR) are related to the development and the progression of NAFLD. Moreover, previous lipidomic analyses in subjects with NAFLD have found that having a high prevalence of saturated fatty acids (FA) in hepatic triglycerides (TAGs) was related to the severity of the liver disease.
The aim of this research was to investigate *how* the lipid profile is affected by the progression of NAFLD.

We considered 44 subjects with biopsy proven NAFLD, 29 non obese NAFLD-NO and 15 obese NAFLD-Ob (BMI= 25.6±0.5 and 32.4±2.2) and 9 non-obese CT without fatty liver. We distinguished NAFLD subjects according to their fibrosis stage rate (from F0 to F4).
In all the subjects, we evaluated TAG composition by LC/MS-QTOF and FFA composition by GC/MS. We also detect the composition of Phosphatidylcholines (PC), lyso-PC, Phosphatidylethanolamines (PE) and ceramides. We measured also several metabolic parameters: IR in adipose tissue (AT-IR= FFA x insulin) and in liver (Hep-IR=EGP x Insulin where EGP is the hepatic glucose production measured using stable isotope tracers), HOMA-IR, plasma concentration of Monocyte, Chemoattractant Protein-1 (MCP-1), hepatic fat in biopsy.
We also calculated the ratio of saturated to unsaturated FFAs (SFA/PUFA), SCD-1 activity (palmitoleate/palmitate). We considered low plasma unsaturated FA if there were less than 2 db in each fatty acyl chain.

The statistical analyses were conducted in order to highlight which parts of the lipid profile are mainly altered at each stage (F0-F4) of the disease. Comparing with the control cases and by means of correlation analysis, we evaluated how the interrelationships in the lipid metabolism were altered. With the auxilium of discriminant analysis techniques, we tried to detect those compounds which better may reveal the severity of the disease.
Our study confirms the relation between an increased presence of saturated FA and the progression's degree of NAFLD, even in non-obese subjects with NAFLD.

Metabolic profile of hemp flowers from Lazio: an NMR study

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Cannabis sativa L. (industrial hemp) is an important raw material in different fields and for centuries it was cultivated all over the world. However, because of the association with narcotic Cannabis (*Cannabis indica*) in the 20th century the cultivation of industrial hemp has almost disappeared. Recently, the EU published a regulation for the reintroduction of selected varieties of this crop in the Europe [1]. The present study, developed in the frame of the Regione Lazio project "*Industrial Hemp: development and valorization of a sustainable new food chain"* [2] aimed at the identification of the *Cannabis sativa* L. cultivars best suited to the pedoclimatic condition present in well-defined Lazio areas. In this attempt, both agronomical and chemical-nutritional aspects related to the monoecious Ferimon variety were investigated. Moreover, samples from different dioecious cultivars, cultivated in selected Northern Lazio areas were analyzed. The raw material (inflorescences) of samples from all varieties collected over the season were characterized through a multi-methodological protocol, involving different advanced techniques (NMR, HPLC, spectrophotometry). NMR analyses were carried out on each sample, using hydroalcoholic and organic extracts obtained by Bligh-Dyer procedure. *One dimensional (^{1}H) and 2D (*^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC) experiments were performed in order to define the metabolic profile and monitor the trend of primary and secondary metabolites.

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Effects of sample preparation procedures on non-targeted NMR analysis of tomatoes

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In the last years the application of nuclear magnetic resonance spectroscopy (NMR) to the metabolomics has being experienced an increasing number of applications [1], due to the development of new mathematical algorithms for the statistical analysis, and more sensitive and highly reproducible spectrometers. In metabolomic analysis the sample stability is a crucial aspect, being the prerequisite for assuring high reproducibility of the results obtained by different laboratories and, thus, a valuable inter-laboratory comparison. The stability of a sample is largely affected by its preparation protocol, which should include all the operations aimed at ensuring an efficient extraction of the metabolites and a lasting stability of the metabolic profile over the time [2].
As part of a collaborative research project (Istituto Poligrafico Zecca dello Stato – Politecnico di Bari) aimed at discriminating the geographical origin of Italian tomatoes, we disclosed the crucial aspects of the sample preparation affecting the metabolic profiles of the tomato extracts, and, importantly, we shed light on the practical procedures able to reveal the presence of some unknown metabolites and to control their transformations over the time. Moreover, we demonstrated that depending on the sample preparation the metabolic profile can suffer a significant variation.
The results reported in this communication should be of general interest for the scientific community involved in food analysis.

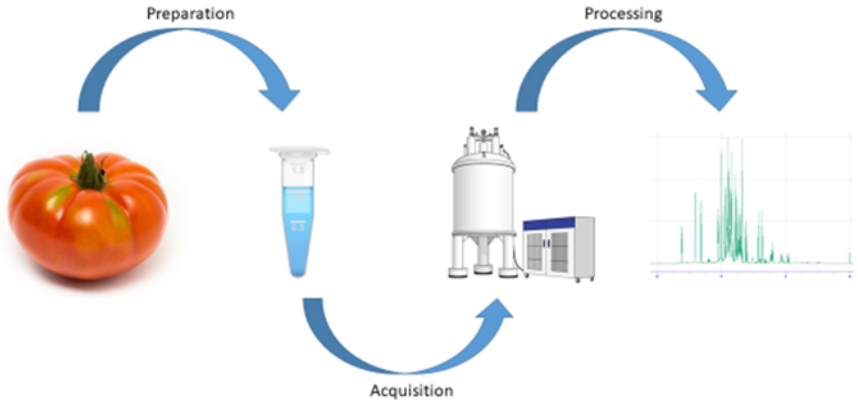

Fig. 1. ^{1}H-NMR metabolomic workflow.

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An NMR-MS metabolomic study of brain tissue from D-aspartate oxidase knock-in mouse model

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The presence of D-amino acids, which until few years ago was believed to appear only in bacteria and invertebrates, is nowadays known to occur also in mammalian. In particular, high levels of free D-serine (D-Ser) and D-aspartate (D-Asp) are found in the brain. While the functions of the first are well known,[1] many questions remain unclear about the role of D-Asp in the central nervous system. D-Asp is very abundant at the embryonal stage, while it highly decreases after birth[2]-[3] due to the expression of D-aspartate oxidase (DDO), which catalyzes its oxidation.

D-Asp acts as an endogenous agonist of N-methyl D-Aspartate (NMDA) and mGlu5 receptors, having beneficial effects at physiological concentrations on brain development, synaptic plasticity, spatial memory and cognition.[4] However studies on DDO knock-out (DDO-/-) mice demonstrated that uncontrolled high levels of D-Asp bring to overstimulation of NMDARs, causing neuronal cells death, in a condition that is very similar to the one caused by neurodegenerative diseases.[5]

Very recently a knock-in mouse model has been generated for the first time by Usiello and coworkers (manuscript submitted for publication). In this model, DDO is expressed starting from the zygotic stage, to enable the almost complete removal of D-aspartate in prenatal and postnatal life.

The aim of the present report is an NMR and MS metabolomic study of brain tissue derived from DDO knock-in mouse model. Brain tissue samples of DDO-/- were analyzed in comparison with brain tissue of wild type mice, at different stages of embryonic and post-natal brain development. The study, pointing to a metabolomic signature associated with the different levels of D-Asp and DDO, provides a useful contribution to unveiling the role of D-aspartate as signaling molecule involved in neural development, influencing brain morphology, and behaviors.

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Real time metabolomics analysis of breath volatile organic compounds (VOCs) by selected ion flow tube mass spectrometry (SIFT-MS) in cancer patients

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The VOCs in the exhaled breath represent promising biomarkers able to give useful information on the individual physio-pathological status. The real-time breath analysis of VOCs is an attractive and non-invasive approach for cancer diagnosis and monitoring. Here we report a SIFT-MS feasibility study for real time breath VOCs monitoring of cancer patients in a clinical setting. The analysis has been performed by Voice 200 Ultra™ targeting 32 VOCs: ammonia, acetone, isoprene, acetonitrile, dimethyl ether, aliphatic alcohols (n=5, C1-C5), cyanuric acid, low chain aliphatic acids and their corresponding aldehydes (n=12, C1-C6) including benzaldehyde, surfer ethers (n=5) and phenol compounds (n=3). The study involved 12 healthy controls and 17 metastatic cancer patients. Twenty-two (72%) out of 32 VOCs were detectable by SIFT-MS and among the analysed compounds, ammonia ($p<0.001$), acetone ($p<0.05$), carbon disulfide ($p<0.01$) and phenol ($p<0.05$) showed a significant lower breath level in cancer patients compared to healthy individuals. Within the investigated series, qualitative and quantitative individual VOCs signatures were recognized for both studied groups and specific VOCs profile associated to disease progression was observed in a patient with sarcoma.

Such observations support the application of breath metabolomics analysis for monitoring cancer progression and the use of SIFT-MS for its feasibility, may represent a non-invasive and cost-effective diagnostic tool in clinical investigations.

Metabolomic investigation of the effects of nutraceuticals and potential drugs in murine inflammatory models

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The metabolome can be considered as a snapshot of the physiological state of an organism, being the downstream product of the *omics'* cascade, as well as the ultimate response to disease or environmental influences. Metabolomics allows the analysis of biochemical pathways and their perturbations resulting from diseases, drugs, diet, or life style [1,2]. Currently, there are two major analytical techniques employed in the metabolomic field: Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS). The most common data analysis approaches available to extrapolate information from metabolomics datasets are chemometrics tools such as Principal Component Analysis and Partial Least Square Discriminant Analysis.
Herein, we present the results obtained from a couple of metabolomics investigations on murine inflammatory models. The first one focuses on the effects of phytosterols on mice affected by intestinal inflammation [3], while the second aims to understand the impact of an interleukin (IL-17) neutralizing antibody on amyloid-β-induced neuroinflammation and memory impairment in mice.

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NMR and MS-based metabolomic study on the effects of structurally different mixed linkage β-glucan in hypercholesterolaemic rats

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Mixed linkage (1→3),(1→4)-β-D-glucan (BG) is a soluble fiber available from oat and barley grains that has been gaining interest due to its health-promoting role in cardiovascular [1].
In this study we investigated the effect of three purified barley BGs, with different molecular weight and block structure, on plasma metabolome and on fecal bile acids excretion in hypercholesterolaemic rats.
An untargeted NMR-based analysis of plasma samples followed by Principal Component Analysis revealed no differences among the control and the different BG-treated groups. Interestingly, a metabolite profiling mass spectrometry-based investigation on fecal samples revealed that all three BG-enriched diets increased bile acid fecal excretion compared to the control group. Moreover, the bile acids excretion was found to be different in all three BG diets and, in particular, the medium molecular weight BG group showed a significantly higher level of secondary bile acids, including deoxycholic acid, hyodeoxycholic acid, and lithocholic acid. It is hypothesized that the secondary bile acids, which are known to cause colon cancer, have a hydrophobic surface that has high affinity to the hydrophobic surfaces of cellulosic blocks of that particular BG group. This study demonstrates that the molecular weight and/or block structures of BGs can modulate the excretion of carcinogenic secondary bile acids. This suggests that developing diets with designed BGs characterized by an optimal molecular structure to trap carcinogenic bile acids, can have a significant impact on counteracting cancer and other lifestyle associated diseases.

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Poster P15

Identification of *Citrus* metabolites associated with the defense against *Phyllosticta citricarpa* using NMR and GC-MS profiling techniques

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Citrus black spot caused by the fungus *Phyllosticta citricarpa* is one of the most serious citrus disease, which affects most *Citrus* varieties. Therefore, the disease causes economical damage in the production and has prompted scientists to investigate the disease for long time. Nowadays, metabolomics has been applied to a wide range of life sciences, especially to plant-pathogen interactions. In this study, we aimed to uncover metabolic reflection of the fungus on *Citrus* plants. Two *Citrus* species were used, *Citrus limon* and *Citrus latifolia*, susceptible and resistant species, respectively. The two species were infected by the fungus, separately. ^{1}H NMR and GC-MS were employed for both targeted and untarged approach. All the analytical data were analyzed by multivariate data analysis to investigate metabolic changes in the leaves. As untargeted analysis, ^{1}H NMR was used for the overall metabolic profiling. Sugars, amino acids and organic acids were the most detected metabolites. Additionally, a wide range of flavonoids and coumarins also were identified from ^{1}H NMR spectra. In the loading plot of partical least square (PLS) modeling, many phenolics including flavonoids and coumarins were found to be correlated with the resistance. Besides the metabolites, *Citrus* plants are also well known as a rich source of volatile organic compounds, which might presumably play important roles in defense responses, GC-MS-headspace was used for the volatile analysis. The PCA score plot showed a trend in the susceptible sample separation. On the other hand, the resistant species did not show metabolic difference, even after the infection. This may be related with to the resistance of this plant to the fungus, inversely observed in the susceptible plant.

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Metabolomic profiling of human saliva

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Saliva is a complex biofluid essential to maintain normal oral functions and health. Whole saliva is a mixture of different fluids secreted from minor and major salivary glands such as parotids and submandibular/sublingual glands. It represents an informative body fluid offering many insights into health and disease of the oral cavity, as well as into systemic alterations. Since saliva is collectable by non-invasive procedures, a growing number of studies has exploited this fluid to detect biomarkers for different diseases[1,2].

We collected three different types of saliva (whole - WS; parotid – PS, and submandibular – SMS) from 20 healthy volunteers and produced their metabolite profiles using ^{1}H-NMR spectroscopy. For each saliva sample, eukaryotic and prokaryotic cell counts were performed.[3] Metabolites identification and quantification were carried out using Chenomx software and Principal Component Analysis (PCA) was performed using MestReNova software.

PCA plot displayed a distinct cluster for WS; PS and SMS resulted more dispersed and showed a partial overlap, thus suggesting a certain degree of similarity.

Metabolomics profiling of WS, PS and SMS allowed the identification of metabolites originated both from eukaryotic cells and from bacterial flora.

This approach, allowing the comparison of the metabolic profile of different salivas, provides hints for a deeper understanding of the oral cavity environment, including the contribution of oral microbiota to saliva metabolome.

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Mass spectrometry imaging as a tool to visualize the plant metabolome changes in response to mycotoxin accumulation

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Mass spectrometry-based metabolomics has been applied to understand the molecular interaction between plant and pathogens, essential to develop possible strategies to counteract mycotoxin accumulation. Nevertheless, this approach results in a loss of the spatial information of the metabolites due to the extraction process. In this regard, mass spectrometry imaging (MSI) has become a powerful tool capable of achieving the spatial distributions and chemical specificity, useful to assign the metabolites' functional role.

Here, we aimed to visualize the distribution of metabolites involved in the plant response after mycotoxin administration using an *in-vitro* plant model. To address this challenge, transversal cross-sections were obtained from wheat roots, stems, leaves and were analyzed using atmospheric-pressure (AP)-scanning microprobe matrix-assisted laser desorption/ionization MSI ion source (AP-SMALDI5 AF, TransMIT GmbH, Giessen, Germany) coupled to a Q-Exactive HF orbital trapping mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany).

The data obtained were processed using LipostarMSI (Molecular Horizon srl, Italy), novel comprehensive software to assist MSI-based untargeted metabolomics. Segmentation and region of interests (ROI) were created to perform multivariate statistical analysis, both unsupervised and supervised techniques.

Our results demonstrated the analytical potential of innovative high-throughput technique for gaining insight into the plant resistance mechanism. MSI holds a unique potential for untargeted detection and spatial localization of metabolites from intact plant samples without need for extraction or extensive sample preparation.

‘Functional Microbiomics’ – Assessing nutrition-microbiome-host interaction in blood and feces

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In recent years, microbiome research has dramatically reshaped our understanding of how microbes impact on a multitude of (patho-)physiological processes in the host. However, causal links are still lacking to a large extent. Metabolomics allows the investigation of microbial metabolic activities, and is thus the ideal technology to assess functional nutrition-microbiota-host crosstalk.

Here, we discuss the application of a newly developed targeted assay for the quantification of endogenous and microbiota-derived metabolites. 10 µl human plasma and fecal samples were analyzed by a standardized, quantitative assay in kit format allowing for the analysis of 630 metabolites 106 small molecules from 13 compound classes are analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), hexoses and 523 lipids from 12 lipid classes by flow injection analysis-tandem mass spectrometry (FIA-MS/MS) on an Agilent 1290 Infinity UHPLC – SCIEX QTRAP® 5500 MetIDQ™ software was used for the entire automated workflow.

In plasma, more than 455 metabolites were quantified above LOD with high, and more than 120 metabolites in fecal samples. To a large extent, the small molecules and lipids quantified in feces overlap with those in plasma. A higher number of lipids, especially phosphatidylcholines and triglycerides, were quantified in plasma compared to fecal samples. In addition to endogenous metabolites, a multitude of microbiota-derived metabolites were quantified. The capability to quantify microbiota-derived metabolites in blood and fecal samples allows for correlation studies also with data from other omics technologies to investigate functional nutrition-microbiome-host interplay for uncovering causal links to pathophysiological processes, disease development, and response to drug treatment.

A targeted LC-MS/MS analysis of circulatory lipid profile to highlight biomarkers for patient stratification according to coronary artery disease severity

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Traditional risk factors play a pivotal role in predicting coronary artery disease (CAD) presence/severity and correlation with standard bio-humoral parameters may contribute to improve predictive models of coronary heart disease (CHD).
Recently, the use of molecular markers has been proposed as a tool for an adequate risk assessment of CHD [1] and discovery of new potential biomarkers is considered useful for early diagnosis and prognosis of CAD.
The study of plasma lipidomics through mass spectrometry is gaining an increasing clinical relevance and lipids are emerging as promising biomarkers of plaque development, composition and vulnerability in patients with CAD [2-7].
In the frame of the "SMARTool Project" we developed a targeted LC-MS/MS method to perform fast, single run evaluation of lipid fingerprint in CAD patients. Our method allowed quantification of 152 lipid species distributed among 9 lipid classes.
Principal component analysis evidenced a remarkable separation between obstructive and minimal/absent coronary disease (> 50% vs < 30% degree of stenosis of major vessels). Nine lipids belonging to triglycerides (TG), phosphatidylethanolamine (PE) and Ceramides (Cer), were found significantly upregulated in obstructive patients (TG(48:1), TG(50:2), TG(52:2), PE(34:1), PE(34:2), PE(36:3), PE(36:4), PE(38:5), Cer(d18:1/23:0)). Among them, TG(48:1), PE(36:4) and PE(38:5) [3,5] have been already reported as prognostic markers of future adverse cardiovascular outcomes.
Our results suggest that this LC-MS/MS based plasma lipid profile represents a source of potentially useful biomarkers for CAD patient stratification according to disease severity, thus paving the way for future clinical applications.

Acknowledgments
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Quantification of twelve neurotransmitters in mouse cerebrospinal fluid

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So far, analytical investigation of neurotransmitters in cerebrospinal fluid (CSF) of rodent models has been limited to rats, given the intrinsic anatomic difficulties related to mice sampling and the corresponding tiny amounts of CSF obtained. This poses a challenge for the research in neuroscience, where many, if not most, animal models for neuronal disorders rely on mice. We here introduce a new, sensitive and robust method to quantify a panel of 12 neurotransmitters from mouse CSF. The paper describes the sampling procedure that allows the collection of 1-2 microliters of pure CSF from individual mouse specimens. With a conservative approach, the amount of CSF is sacrificed to obtain samples devoid of blood contaminations. The procedure does not involve any extraction or derivatization procedure: samples are simply diluted and analysed as such by LC-MS/MS, using a dedicated ion pairing agent in the chromatographic setup. To test its applicability, we challenged our method by sampling 37 individual animals, thus demonstrating its strength and reliability. Given the number of mouse models used in the neuroscience research, we believe that our work will pave new ways to more advanced research in this field.

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NMR-driven identification of potential antitumoral and antiamyloidogenic activity of Cinnamon extracts

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Cinnamon is one of the most commonly used spices, obtained from the evergreen trees growing in Southeast Asia, China and Australia, belonging to genus *Cinnamomum* (*Lauraceae* family).
The aim of this work is to determine and compare the metabolic profiles of two different species (*Cinnamomum zeylanicum* Blume and *Cinnamomum cassia* J. Presl) in two different forms (bark for *C. zeylanicum* and *C. cassia* and buds only for *C. cassia*) and three extraction methods (alcoholic extraction, hydroalcoholic extraction and water extraction). An NMR-based protocol has been developed to this purpose. The metabolic profiling was aimed at revealing differences in the chemical composition between species, allowing the identification of potential biologically active molecules.
In addition, we measured the antioxidant activity of these extracts and two potential biological activities were tested. In particular, the ability to reduce the viability of three colon cancer cell lines (Caco-2, E705, SW480) in comparison with healthy colon cell line (CCD841) and the ability to inhibit the Aβ-induced toxicity on neuronal cell lines were assayed.

Poster P22

Metabolomic profiling of beers: combining ^{1}H-NMR spectroscopy and chemometrics approaches to discriminate craft and industrial products

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^{1}H-NMR analysis of 31 beer samples, differing for beer style and brewing method (craft or industrial) was combined with multivariate statistical analysis, according to both an untargeted and a targeted approach, revealing the possibility to discriminate craft and industrial beers. In addition, the metabolites mainly responsible for craft and industrial beers' segregation were identified. Samples were prepared by lyophilization or lyophilization followed by an ultracentrifugation step to remove solvents and high molecular weight components optimizing spectra' analysis and small metabolites' identification and quantification. NMR-based analysis of beer samples was speeded through the development of a specific protocol enabling the automatic identification and quantification of metabolites in approximately thirty seconds per spectrum.

Atrial fibrillation in the elderly: a metabolomic approach with GC-MS

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Atrial fibrillation (AF) is a cardiac condition characterized by a disordered electrical activity of the atria and it is the most common type of arrhythmia among the elderly. The pathogenesis of AF is still poorly understood and available treatments are limited in their effectiveness and rarely definitive [1]. AF is often asymptomatic and, in several cases, it remains clinically undetected [2]. Metabolites are suitable candidates to be utilized as biomarkers as they represent the end products of cellular processes and their presence and abundance reflect the biochemical status of an organism [3]. GC-MS is the most standardized and widely employed method in metabolomics [4]. The aim of the project is to identify metabolic markers characterizing AF patients with different clinical features. For this purpose, 45 plasma samples of elderly patients affected by AF, stored at -80°C, will be utilized for a metabolomic study using GC-MS. A pool of these samples have been employed to develop and test a method (adapted from Fiehn 2016), using fatty acid methyl esters (FAMEs) as internal reference standards and succinic acid-d4 as internal standard. Plasma samples have been derivatized under anhydrous conditions with methyl oxime in a pyridine solution and tert-butyldimethylsilyl chloride (TBDMS). The instrument used for the analysis is an Agilent Technologies GC-MS equipped with an electron ionization source and a quadrupole mass analyzer. In the first step, the metabolomic analysis will be carried out with an untargeted approach. Statistical analyses will be performed using MSDial and R. Subsequently, an in-depth metabolomic analysis will follow according to the results obtained from the untargeted approach.

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Mapping of elements in MeLiM tissues by LA-ICP-MS

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Melanoma remains one of the most aggressive forms of cancer, being responsible for 1.2 % of all cancer deaths in the European Union. Cancerous growth develops when unrepaired DNA damage to skin cells triggers mutations that lead the skin cells to multiply rapidly and form malignant tumours [1]. The most widely accepted criteria for the prognostic evaluation of melanoma are histopathological and clinical parameters, and the identification of additional tumour markers is thus of paramount importance. Zn, the most abundant transition metal in most cells, plays important complex role during the transformation of normal tissue to cancerous one [2,3]. In order to better understand the processes involved in the carcenogenesis, we apllied laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) for analyses of four elements Zn, Cu, Fe and Ca in histologically characterised melanoma tissues from the MeLiM (*Me*lanoma-bearing *Li*bechov *Mi*nipig) strain of miniature pigs. In MeLiM tissue sections, the Zn and Fe levels were elevated in the majority of melanomas in comparison with the skin of healthy controls. As a type of physiological defence, cells can over-express Zn-containing antioxidant molecules or transport and accumulate them from adjacent tissues. Furthermore a content of Cu and Ca declines as a result of advancing spontaneous regression (due to destruction of melanoma cells by anti-tumour immune reaction). Expression of metal and calcium-binding proteins might be directly linked to the changes of elements decribed here. Neverthless this statement needs further examination applying LC-MS and MALDI-MSI techniques.

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