Book of Abstracts

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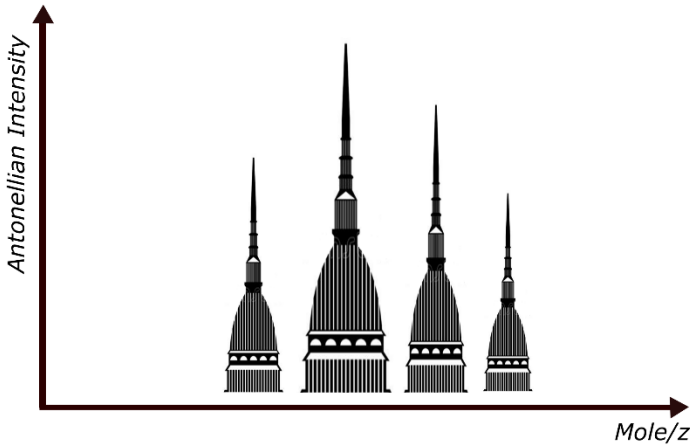

Giovani e Spettrometria di Massa

Una giornata di scambio di conoscenze e competenze aperta a tutti i giovani ricercatori.

15 Giugno 2022

Oral presentations program:

10:00-10:20	*Mass spectrometry-based proteomics of human tears for noninvasive biomarker discovery and precision medicine* ***Erika Ponzini**, Dipartimento di Scienza dei Materiali, Università degli Studi di Milano-Bicocca*
10:20-10:40	*An original MS-based method for the analysis of CBD and metabolites in whole blood microsamples* ***Marco Cirrincione**, Research Group of Pharmaco-Toxicological Analysis (PTA Lab), Department of Pharmacy and Biotechnology (FaBiT)*
10:40-11:00	*Polar x, True innovation for your analysis* ***Emanuele Ceccon**, Restek Italia, 20063 Cernusco sul Naviglio Milano (MI)*
11:00-11:20	*Coffee Break*
11:40-12:00	*EASY FIA a readably usable standalone tool for high resolution mass spectrometry metabolomics data preprocessing* ***Giulia De Simone**, Laboratory of Mass Spectrometry, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy*
12:00-12:20	*Mass Spectrometry Histochemistry: a novel spatial multi omics technology with single cell resolution* ***Peter Verhaert**, Proteoformix, Beerse (Belgium)*
12:20-12:40	*Development of an innovative GC-MS method for the determination of 3-monochloropropanediol, 2-monochloropropanediol and glycidol without derivatization* ***Alessia Lanno**, Laboratory of Mass Spectrometry, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy*
12:40-13:00	*The effect of Carnosine on Scaffold Free Human Dermis Spheroids Model investigated by proteomics* ***Gilda Aiello,** Department of Human Science and Quality of Life Promotion, Telematic University San Raffaele,00166 Rome, Italy;*

Abstracts Index

Mass spectrometry-based proteomics of human tears for non-invasive biomarker discovery and precision medicine

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In the last decades, the lacrimal fluid has gained increasing interest as a potential source of biomarkers, thanks to its accessibility, moderate complexity, and responsiveness to physio-pathological conditions. Despite the still limited number of studies, tear investigation, in particular single-tear analysis, could offer unique contributions to the identification of noninvasive and easily accessible biomarkers, and to development of a feasible approach to precision medicine.

High-performance liquid chromatography-mass spectrometry (LC-MS) has led to promising results in tear proteomics, despite the intrinsic limitations in sample amounts that can be collected. By ultrahigh-resolution, shotgun proteomics, we developed and effective analytical pipeline for single-tear analysis and we analyzed the tear fluid of 23 healthy volunteers, achieving high-confidence identification of 890 proteins. This study demonstrates feasibility of single-tear quantitative proteomics, highlighting the unique contributions that this unconventional body fluid can offer to personalized approaches in biomedicine.

Multi-Omics investigation of primary bronchial epithelial cells derived from CF and non-CF subjects

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Cystic fibrosis (CF) is an autosomal recessive genetic disorder caused by a defect in the transmembrane conductance regulator (CFTR) gene. The gene codifies for a 1480 amino acid cAMP-dependent chloride channel (CFTR) located at the apical surface of epithelial cells whose main function is to regulate transmembrane ionic exchanges. The disease usually begins in early childhood damaging, in particular, the respiratory systems.

The most common mutation is the deletion of a phenylalanine at position 508 leading to a reduction in the transport of new CFTR proteins from the ER to the Golgi, and loss of function at the cell surface. Structural and functional defects are pharmacologically treated with small molecules (modulators) acting differently depending on whether the mutation affects the synthesis, processing or functionality of the protein.

Omics technologies, in particular mass spectrometry, greatly increased the possibility of exploring the molecular mechanisms involved in the CFTR pathology and rescue, raising the possibility of getting new therapeutic strategies for rare and nonsense mutations still ineligible for pharmacological therapies.

Besides the relevance of the proteomics, the investigation of alterations in lipid profile caused by pathological and pharmacological conditions can improve the understanding of the biochemical mechanisms in which CFTR is involved.

Lipids play a key role in several processes related to protein homeostasis, including the maturation of CFTR as well as its stabilization on the cell membrane: molecules, such as phosphatidylserine (among the main components of the plasma membrane), are known to be of primary importance in the stabilization processes of CFTR on the membrane; CFTR is also known to form clusters on the membrane involving molecules such as ceramides (CER) and cholesterol. Moreover, there is evidence of the

interaction of some modulators, such as VX770 (a potentiator able to stimulate the opening of mutant CFTR with gating defects) with the membrane lipids.
In a previous study, we investigated the expression and localization changes in the proteome of immortalized human bronchial epithelial cells derived from a cystic fibrosis patient homozygous for the F508del CFTR mutation (CFBE41o- cells) after treatment with the corrector VX-809 (capable of stabilizing the CFTR structure and promoting their maturation).
In another study, we evaluated the alteration of the lipid profile in the same model by treating the cells with currently available drugs involved in CF treatments.
In this study, we aim at performing a combined proteomic and lipidomic investigation on a much more relevant biological model, represented by primary bronchial epithelial cells (HBE) derived from CF and non-CF patients, to characterize the bronchial epithelium of CF and non-CF subjects. We will study the bronchial epithelium of 10 subjects F508del +/+ and 4 CF subjects with minimal function (MF/MF) mutations and we will compare them with 10 non-FC subjects (control), treated and untreated with Kaftrio (a triple combination of elexacaftor/tezacaftor/ivacaftor found to be safe and effective on subjects with at least one F508del aged 12 years or older).
By comparing CF subjects with non-CF we will get an extended picture of both lipidomic and proteomic alterations caused by the disease, or related to drug treatment (including possible off-target effects) and we will identify pathways altered in patients.
By comparing CF F508del and MF/MF subjects we will identify signatures specific for protein absence and the effect of modulators on rescuable and non-rescuable CFTR.
With this approach, we will also explore the role of ceramides in the metabolism and rescue of CFTR by monitoring both the precursors and the enzymes involved in their synthesis and degradation pathways (De novo, Smase and Gcase pathways).
This will help to understand if CF (or the drug treatment) alters CER levels in HBE. By comparing the data from patients F508del and MF/MF we will try to understand if alterations are related to the presence of CFTR in the plasma membrane.
Finally, we will study the regulation of complex glycosphingolipids, such as GM1, found to be decreased in CFTR-deficient cells, by monitoring their levels in all experimental conditions to assess the role played by GM1 in CFTR stabilization on the plasma membrane.

EASY FIA a readably usable standalone tool for high resolution mass spectrometry metabolomics data preprocessing

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Flow injection analysis coupled with high-resolution mass spectrometry (FIA-HRMS) is a reliable trade-off between resolution and speed for metabolomics analysis. However, free software available for data pre-processing are few, web-based, and often requiring high user specialization. Moreover, these tools rarely embedded blank and noise evaluation strategies and direct feature annotation. We developed EASY-FIA, as a free standalone application that can be employed for FIA-HRMS metabolomic data pre-processing by users with no bioinformatics/ programming skills. We validated the tool performance and applicability in two clinical metabolomics case studies. The main functions of our application are blank subtraction, alignment of the metabolites, and direct features annotation by the Human Metabolome Database (HMDB) using a minimum number of mass spectrometry parameters. In a scenario where FIA-HRMS is increasingly recognized as a reliable tool for fast metabolomics analysis, EASY-FIA can become a standardized and feasible tool easily usable by all scientists dealing with MS-based metabolomics EASY-FIA is implemented in MATLAB with the App Designer tool. It is available for download on GitHub at https://github.com/AMrbt20/ under GNU GPL v.3.0 license.

Structural Refinement of the Tumor Suppressor p53 by Mass Spectrometry-Guided Computational Modeling

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The tumor suppressor p53 is a multidomain transcription factor that plays a central role in DNA repair, cell cycle control, and apoptosis. It binds to specific DNA response elements, is integrated in various signaling networks by a multitude of protein-protein interactions, and is controlled by extensive posttranslational modifications. p53 is a homotetramer, with each monomer consisting of two folded domains, the DNA-binding domain (DBD, aa 94–294) and the tetramerization domain (aa 323–360). In addition, p53 contains four intrinsically disordered regions (IDRs), the N-terminal transactivation domain (aa 1–67), the proline-rich region (aa 67–94), the nuclear localization signal (NLS)-containing region (aa 303–323), and the C-terminal basic regulatory domain (aa 360–393).
The DBD binds to sequence-specific response elements that are associated with p53 target gene promoters. The majority of mutations in p53 are located in the DBD and are responsible for cancer triggering. As ~40% of p53's sequence is disorded it is not amenable to X-ray crystallography. Therefore, we aim to exploit an integrative structural biology approach combining different mass spectrometry (MS)-based techniques, such as covalent labeling (CL-MS), hydrogen/deuterium exchange (HDX-MS) and cross-linking mass spectrometry (XL-MS) to study changes in p53 upon specific DNA binding in terms of solvent accessibility, conformational changes, as well as H-bond stability and protein dynamics in full-length wild-type p53.
XL-MS was applied to different full-length oligomeric p53 variants, the monomeric L344P variant, the dimeric L344A variant, and wild-type tetrameric p53. We obtained information for the p53 monomer, dimer, and tetramer using a variety of chemical cross-linking principles with different spacer lenghts and reactivities. Specifically, the homobifunctional cross-linkers DSBU and BS^2G as well as the "zero-length" cross-

linker system EDC/sulfo-NHS were employed. The distance constraints imposed by the cross-linkers then served as input for MS-driven computational modeling to generate refined p53 models in the presence and absence of DNA. These models are currently being further validated by complementary HDX-MS data.

Development of an innovative GC-MS method for the determination of 3-monochloropropanediol, 2-monochloropropanediol and glycidol without derivatization

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3-monochloro-1,2-propanediol (3MCPD), 2-monochloro-1,3-propanediol (2MCPD) and glycidol are food-born and thermally induced contaminants. They can be formed during the manufacturing process at high temperatures of different foodstuffs, such as cereal-based products and potato chips, and during the processing of vegetable oils. When present, they can be found both as free and esterified to fatty acids naturally present in foods. The esterified form is the most abundant, as reported in the literature. The International Agency for Research on Cancer classified glycidol and 3MCPD as probably and possibly carcinogenic to humans, respectively and, in 2018, the European Food Safety Authority updated the tolerable daily intake (TDI) to 2 µg/kg day for 3-MCPD and its fatty acid esters. For these reasons, there is a need for high sensitivity analytical methods for the determination of these analytes in food matrices.

Two types of analytical methods are described in the literature, the indirect and the direct methods. The indirect methods in GC-MS, some of which are standardized, can be used to determine the total amount of 3MCPD, 2MCPD, and glycidyl esters. These techniques allow the quantification of all esters as free analytes, after alkaline or acid hydrolysis. In the last few years, some direct LC-MS methods have been developed, guaranteeing the determination of single esters without the use of a transesterification reaction.

All the available indirect GC-MS methods include a derivatization reaction with different types of reagents to mask the polarity of these analytes and enhance their volatility. However, the addition of another sample preparation step may decrease the sensitivity of the method. One more disadvantage of these methods is constituted by the conversion of glycidol in 3-monobromopropanediol, necessary for the following derivatization. This reaction can lead to glycidol overestimation because the acylglycerols present in vegetable oils can also react forming the same product. Given

this basis, we propose a new indirect method that does not need the derivatization step.

We developed a new innovative GC-MS method, operating in Selected Ion Monitoring mode and we obtained the separation of the three analytes on a GC polar column within 15 minutes. Deuterated internal standards, 3MCPD-D5, 2MCPD-D5, and glycidol-D5 were used for accurate quantitation with the isotope dilution method. The developed method showed good linearity and reproducibility and the limit of detection for glycidol was 0.1 ng/µL and 0.05 ng/µL for both 3MCPD and 2MCPD.

The elimination of the conversion and following derivatization step guaranteed the absence of glycidol overestimation giving at the same time the sensitivity required to measure analytes' levels lower than the maximum concentrations allowed in food. In addition, we will test different hydrolysis conditions to find the best ones that could make the conversion of monochloropropanediol to glycidol negligible. The developed method will be fully validated and used to determine the total amount of 3MCPD, 2MCPD and glycidol esters in different edible fats and derivative products.

Mass spectrometry imaging as a tool for evaluating the pulmonary distribution of exogenous surfactant in premature lambs

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Introduction

Preterm babies with low intrapulmonary surfactant pools rapidly develop respiratory distress syndrome (RDS), which in severe cases can progress to chronic lung disease with long-term sequelae. Surfactant replacement therapy delivers surfactant directly to the lungs of RDS babies, through intratracheal administration, rapidly reversing the respiratory failure. The amount of surfactant deposited in the lungs and its overall pulmonary distribution determine the therapeutic outcome of surfactant replacement therapy. Most of the currently available methods to determine the intrapulmonary distribution of surfactant are time-consuming and require surfactant labeling. Our aim was to assess the potential of Mass Spectrometry Imaging (MSI) as a label-free technique to qualitatively and quantitatively evaluate the distribution of surfactant to the premature lamb used as animal model.

Methods:

Twelve preterm lambs (gestational age 126-127, term 150) were allocated in two experimental groups. Seven lambs were treated with an intratracheal bolus of the synthetic surfactant CHF5633, and 5 lambs were managed with mechanical ventilation for 120 min, as controls. The right lung lobes of all lambs were frozen while inflated to 20 cmH2O pressure for lung cryo-sections. A solution of 4-chloro-α-cyanocinnamic acid was used as MALDI matrix and spray

coated over the tissues, while MSI analysis was carried out on a Bruker UltraFlex III working in positive ions full scan mode. The intensity signals of SPCLeu33 and MiniB, the two synthetic peptides contained in the CHF5633 surfactant, were used to locate, map and quantify the intrapulmonary exogenous surfactant.

Results:

Surfactant treatment was associated with a significant improvement of the mean arterial oxygenation and lung compliance ($p<0.05$). Nevertheless, the physiological response to surfactant treatment was not uniform across all animals. SPCLeu33 and MiniB were successfully imaged and quantified by means of MSI in the peripheral lungs of all surfactant-treated animals. The intensity of the signal was remarkably low in untreated lambs, corresponding to background noise. The signal intensity of MiniB in each surfactant-treated animal, which represents the surfactant distributed to the peripheral right lung, correlated well with the physiologic response as assessed by the area under the curves of the individual arterial partial oxygen pressure and dynamic lung compliance curves of the lambs.

Conclusions:

Applying MSI, we were able to locate and quantify the exogenous surfactant distributed to the lung of surfactant-treated lambs. The distribution pattern of MiniB correlated well with the pulmonary physiological outcomes of the animals, suggesting a link between the pulmonary distribution of CHF5633 and the therapeutic outcomes of surfactant-deficient lambs.

AP-MALDI-MSI on ovarian carcinoma patient-derived xenografts: spatial metabolomics approach to study chemotherapy resistance

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Introduction

Ovarian cancer is one of the most common causes of death in the gynaecological field (PMID 27976698). Furthermore, due to tumor drug resistance as a result of different cellular mechanisms, pharmacological treatment becomes more complex (PMID: 32992019). Ovarian carcinoma patient-derived xenografts (O-PDXs) are *in vivo* important tools to study the mechanisms underlying chemo-resistance.
Metabolic changes can give important information on how tumors respond to therapy. We have developed a high-resolution atmospheric pressure MALDI mass spectrometry imaging (AP-MALDI-MSI) approach to visualize spatial distribution and changes of small metabolites belonging to different metabolic pathways in O-PDX cisplatin-sensitive and in the corresponding cisplatin-resistant PDX

Methods

PDXs ovarian cancer slices (10 µm thickness) frozen in liquid nitrogen were cut by cryostat and placed on an Opti-TOF insert (SCIEX®). Adjacent slices were collected for immunohistochemical staining. For the AP-MALDI imaging experiments, matrix solution of 9-aminoacridine (9-AA 10 mg/ml) and 2,5-dihydroxybenzoic acid (DHB 30 mg/ml) were sprayed separately on PDX slices using SunCollect (SunChrom®) sprayer. The AP-MALDI imaging was conducted with 50-500 *m/z* acquisition range in (+/-) ion mode with an AP-MALDI ultra-high-resolution source (MassTech Inc., MD, USA) mounted on an Orbitrap Q-Exactive instrument (Thermo Scientific®) with resolution set up at 70,000

Preliminary Data

We applied an AP-MALDI-MSI method to characterize the distribution of small metabolites in resistant and sensitive O-PDXs. Using different polarities acquisitions (+/-) through two different separated matrices, we have been able to cover a range of metabolites with functional groups that can accept or lose a proton ionizing, both in positive and/or negative ion mode.
Our method investigated the spatial distribution of a list of 41 metabolites belonging to different metabolic classes to cover the highest number of cellular pathways. These

preliminary data support the possibility to visualize the spatial distribution of metabolites belonging both to catabolic (valine, leucine, isoleucine, and lysine degradation) and cellular metabolic pathways (purine, glutathione, phenylalanine, and tyrosine metabolism), confirming the potential of this technique as a tool to identify tumor specific metabolic profiles. The ability to visualize metabolic changes can lead to a better understanding of the underlying mechanisms of chemo-resistance.

Novel Aspect

Simultaneous spatial distribution of small metabolites in ovarian PDXs slides through AP-MALDI-MSI

Towards a complete and rugged diagnostic approach for the detection of OQDS metabolic markers in olive tree leaves samples using chromatography-mass spectrometry-based metabolomics

Authors: Alberto Asteggiano (1,2), Federica Dal Bello (1), Enrica Mecarelli (1), Riccardo Aigotti (1), Claudio Medana (1)

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INTRO:

Olive trees in Puglia (Italy) of the Olea europaea specie are suffering from a terrible decrease in population since 2013. The reason for such an environmental disaster is OQDS (Olive Quick Decline Syndrome): a phytopatological condition brought by the gram-negative bacterium Xylella fastidiosa (Xf). Xf, after being transported by an insect vector, invades the plant's xylema: the vessel system used by the plant for nutrients and minerals transportation, and, by multiplication, causes its obstruction and the consequent plant death. Our work aims to find, by analysing numerous healthy and infected olive leaf samples, potential phytochemical markers for the OQDS infection to be used as an early-stage OQDS alternative detection approach to be used together with more traditional biomolecular methods.

METHODS:

Olive leaves, after being harvested from both infected and healthy trees, in different sampling months and Italian regions (Liguria and Puglia), were submitted to HPLC-HRMS analysis (Orbitrap Fusion, Thermo Scientific). For the global metabolomic approach, a pooled sample QC strategy was used. Peak picking, retention time correction and feature detection was performed on the raw data obtained with Bioconductor XCMS package and MZmine. The different classes of samples (subsetted for infection state, regionality, month of sampling and desiccation state) were compared to find discriminant features. PCA plots were used to give first visual glance of the inter-class variability. Class-discriminant features were then found and annotated by using online database (i.e. KEGG) and fragmentation pattern recognition software (MetFrag, MONA, Metlin, MzCloud).

PRELIMINARY DATA:

An early set of analyses was performed on different healthy and infected samples harvested in the regions of Puglia (16 infected, 11 healthy, 6 healthy desiccated) and Liguria (6 healthy), in order to assess those specific factors such as desiccation state

and regionality were not acting as confounding factors for the class discrimination. PCA bidimensional plots showed a good sample grouping between healthy and infected classes, while desiccation state and regionality did not influenced this two-dimensional clustering at all. Statistical analysis was used to find the infected and healthy sample discriminating features which were annotated. This step resulted in the annotation of 18 molecules, namely 14 infection markers, two healthy markers and two additional molecules reported to be phytochemical markers for olive (Olea europaea) and were able to discriminate, through their different intensity, the trees regional origin. The totality of annotated features belongs to the class of secondary plant metabolites, in particular, hydroxy-jasmonate sulphate, a jasmonic acid derivative, was found highly aboundant in most of infected plants, while, in healthy samples was undetectable or very low. Those promising results, obtained on a small number of samples, paved the way to a more systematic sampling approach lead in two different Apulian location (Latiano for infected trees and Avetrana for healthy trees) in different months, starting from March 2021 to present date. This stage of the work is, obviously, still in progress but some preliminary data show that seasonality plays very important role in the metabolomic and feature fingerprint. Results obtained in the first stage will be merged to recent ones in order to trace a more comprehensive database of healthy to infected state discriminant markers taking into account all the introduced variables: regionality, seasonality and desiccation state.

NOVEL ASPECTS:

Development of a HPLC-HRMS global metabolomics for the detection of OQDS typical markers in olive leaves

An original MS-based method for the analysis of CBD and metabolites in whole blood microsamples

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Cannabidiol (CBD) is one of the main phytocannabinoids found in *Cannabis sativa* L.. Due to the recent medical use proposed for CBD in the treatment of epilepsy, careful and frequent monitoring of patients under treatment with CBD-based drugs can play a crucial role in optimising and personalising therapies. In the study of human CBD metabolism, three main metabolites were identified: 6-OH-CBD, 7-OH-CBD and 7-COOH-CBD. However, there are a few methods in the scientific literature able to simultaneously identify and quantify these three compounds in biological matrices. The design of new analytical strategies for the assessment of CBD and its endogenous metabolites in whole blood is of great relevance for both patients and clinicians. In particular, development of fast and feasible methods featuring high throughput is of great interest in therapeutic drug monitoring (TDM), given the large number of samples to be analysed. For this purpose, chromatography-based systems coupled to mass spectrometry are among the analytical approaches mainly exploited for TDM purposes.

In this research, an original UHPLC-MS strategy was developed for the identification and quantitation of CBD and its main endogenous metabolites in whole blood dried microsamples. The use of blood microsampling techniques, such as the volumetric absorptive microsampling (VAMS), can bring many advantages to the analytical workflow such as higher stability of the analytes over time, more feasible storage of samples and a minimally invasive sampling. The analytes were separated by means of an RP C18 chromatographic column with a gradient elution program of the mobile phase composed of a mixture of water + 0.1% formic acid (FA) and acetonitrile + 0.1% FA.

MS instrumentation was equipped with an ESI source set to positive polarity and analyte identification was performed by selected ion monitoring (SIM). A fast, feasible and high-throughput VAMS-UHPLC-MS methodology was developed and validated on blank blood VAMS fortified with the target analytes. CBD and its main metabolites were identified and quantified in less than 6 minutes with satisfactory validation results in terms of extraction yield (>84%), accuracy (RSD <10.6%) and matrix effect (<12%).

In conclusion, this approach has shown to be suitable for the determination of CBD and its main metabolites in whole blood dried microsamples and may represent a valuable tool both for the monitoring of epileptic patients undergoing CBD treatment and to expand the knowledge on CBD metabolism and disposition as a promising new therapeutic agent.

Proteome profile analysis along lung development in a rabbit model

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Mammalian lung development is traditionally described as a process of five stages (embryonic, pseudoglandular, canalicular, saccular and alveolar) characterized by specific histological features, cell type organization and morphology. The complex branched structure and the associated vasculature of the respiratory system develop through the spatiotemporal coordination of more than 40 different cell types and their molecular interactions. A detailed knowledge of the respiratory system development and of the biological process involved could help in research on preterm-related diseases such as Bronchopulmonary dysplasia (BPD), chronic obstructive pulmonary disease (COPD) and respiratory distress syndrome (RDS). Differently from smaller rodents and similarly to humans, in rabbits alveolarization starts in-utero and term rabbit pups are born in the alveolar phase of lung development. The major strength of the rabbit over the smaller rodent models is the possibility to induce prematurity in order to investigate the pathophysiology of preterm birth diseases. The study of proteome in the developing lung is an expanding field, above all in murine model, but less is known about the rabbit model despite the similarity with the human lung development.

The aim of the lung development study in the rabbit model is to characterize the proteome profile of developmental stages and identify key molecular players and their modulation within time.

TMT quantitative labeling technique was applied to the characterization of lung developmental stages studied in a rabbit model. Dataset consisted of six time points (D25, D27, D28, D29, D31, D35) and three biological replicates per day. Protein groups quantified in at least 4 out of 6 samples per developmental stage were used for the quantitative and statistical analysis. The highest number of DEPs could be found between the more distant time points (25-35) and the more distant developmental stages analyzed (canalicular-alveolar). The WGCNA analysis was performed on abundance-normalized values derived from TMT data, only protein quantified in all

the 18 samples were used for module construction. The identification of pathways and processes enrichment in modules was performed using Metascape software. The WGCNA identifies four different module subgroups from the twelve modules, describing protein profiles for early development, after birth processes, middle and late development and transition.

Furthermore, within the thousands of proteins identified, the pulmonary surfactant proteins B and C deserve a specific focus because of their role in lung development. Both are processed by cathepsin H and other proteases into their mature forms that have a key role in the working respiratory system. Based on this information of surfactant proteins maturation, the bottom-up proteomics analysis performed allowed to investigate the maturation of SP-B and SP-C using peptide abundance analysis and discriminating between pro-peptides and mature peptides. The diverse forms of SP displayed different profiles, with mature peptides abundance much higher at birth and after birth.

Discovery proteomics allowed to identify and quantify thousands of proteins within the tissue during time. The strength of the approach has been completed by bioinformatic analysis that led to a deep understanding of the biological meaning and the processes involved in developing of lungs.

Novel Food and Proteomics: a bottom-up approach to identify *Acheta domesticus* allergens.

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All types of food represent a potential source of allergens and all associated risks need to be evaluated to improve quality and food safety. Food quality controls are carried out by several institutions as OMS, FAO, FDA and EFSA and their collaboration provide methods to certify food properties. Insects represent a novel food, by current European legislation definition, all those foods that have not been consumed "in significant way" before May 1997 (Regulation EU 2015/2283, 2015). Recently, they have aroused interest due to their high protein content; however proteins are often allergens and they have to be characterized and evaluated prior marketing. *Acheta domesticus* (taxonomy ACHDO), also known as house cricket, is already used as human food in Asian country and presents allergens belonging to tropomyosin and apolipoproteins family in common with crustaceous species such as shrimps and crabs.

This communication shows a gel free bottom-up proteomic approach used to identify cricket allergens. After soluble proteins extraction from homogenized cricket, a trypsin digestion was performed and peptides were analysed with a nanoHPLC coupled with HRMS (Tribrid Orbitrap Fusion) using an online concentration step and a C18 analytical column. Full mass and data dependent analysis were executed to characterize cricket extracts using bioinformatics tools and a final dedicated MS_2 was performed for tropomyosin (isoforms I and II) and apolipoprotein (isoform III) detection. This method provided a good proteins extraction protocol and allowed to identify both known and new allergens in protein based novel food.

Plant secondary metabolites profile of waste winemaking ethanolic extracts through different HPLC-MS techniques for food and cosmetic applications.

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The increasing demand by informed and susceptible costumers about environmental issues, has pushed universities and industries to research within low environmental impact products aiming to implement circular economy. Pomaces, seeds, lees and stalks - the well-known wine waste products (WWP) - are still rich in antioxidants molecules capable of anti-inflammatory, anti-hyperpigmentation, solar filter activities and antibacterial action in oral cavity. Flavonoids, anthocyanins, sphingosine and betaine figure as the main bioactive metabolites of these WWP. The Liquid Chromatography coupled with Mass Spectrometry represent a fundamental analytical resource to identify and quantify these secondary metabolites. Prior to analysis, the lyophilized ethanolic vegetal samples were extracted with organic solvents assisted by ultrasounds. After a centrifugation step, the supernatant was diluted and then injected into HPLC-MS. An *untargeted* analysis was performed by HPLC-HRMS followed by database search as to obtain many annotations as possible. An innovative *targeted* approach was used to quantify the underivatized amino acids present in the various samples, highlighting their content of free amino acids with the aim to further valorise these WWP.
A complete chemical characterisation with simple pre-treatments and fast feasibility is capable to unveil the full biopotential of these rich by-products to be used as nutraceuticals, cosmetics and in food supplement preparation.

Qualitative and quantitative analysis of oxytocin and cortisol in bonobo urine samples

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It's well known how oxytocin (OT) and cortisol, or hydrocortisone (HC), take part in communication pathways present in different animals. For instance, Pan Paniscus (bonobos), hominoid primate, is a good example for social interaction studies.
Oxytocin1 is a neuropeptide hormone, acknowledged for its role in parturition, social bonding, and stress regulation. On the other hand, cortisol2, a steroid hormone, is usually measured as biomarker of stress. The aim of this study was to quantify these two molecules, very chemically heterogeneous in bonobo urine, a suitable and non-invasive matrix, through two LC-MS methods. Sensitive and specific quantification methods are fundamental to quantify these analytes due to their low abundances in biological fluids (pg/mL and ng/mL for OT and HC respectively). A fractionated LLE (liquid-liquid extraction) with TBME (tert-butyl methyl ether) was carried out as sample pre-treatment for HC. Dried extracts were subjected to HPLC equipped with online SPE followed by C18 analytical column. HPLC system was coupled with a QTrap mass analyser used in triple quadrupole MRM (multiple reaction monitoring) mode both for HC and HC-d3 used as internal standard. Instead, an offline SPE (solid phase extraction) with a polymeric reverse phase cartridge was used both to enrich and to purify OT extracts. Dried extracts were subjected to nanoHPLC with a C18 pre-concentration cartridge. The analytical column with an integrated emitter was linked with a HRMS (Tribrid Orbitrap Fusion). The HRMS operated applying a full scan and a MS2 experiment by CID fragmentation activation mode was used to obtain b and y fragments series of OT and OT-d5 (internal standard) double charged precursor ions. A surrogate matrix was used as calibration medium to quantify analytes in urine. HC was detected in 121 samples with average level of 36.19 ng/mL; OT was detected in 25 samples with average of 0.043 pg/mL. Both methods provided efficient separation, high sensitivity, and specificity to detect hormones in bonobo urine.

La fibrillazione atriale nell'anziano: un approccio metabolomico

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La fibrillazione atriale (FA) è una patologia del cuore caratterizzata da un'attività elettrica disorganizzata a livello degli atri ed è l'aritmia cardiaca più comune nella popolazione anziana. La fisiopatologia della FA è complessa e dipende da una combinazione multifattoriale che include il rimodellamento strutturale ed elettrico del cuore. I metaboliti, rappresentando i prodotti finali dei processi cellulari, riflettono lo stato biochimico dell'organismo; pertanto, lo studio del profilo metabolico di pazienti con una determinata patologia consente di comprenderne i meccanismi biomolecolari e di identificarne potenziali biomarcatori. A questo scopo, la spettrometria di massa (MS) accoppiata alla gas cromatografia (GC) e alla cromatografia liquida (LC) è ampiamente utilizzata negli studi di metabolomica. Obiettivo di questo progetto è lo sviluppo di un metodo di metabolomica untargeted in GC-MS per studiare i profili metabolici di pazienti con FA confrontandoli con soggetti sani e l'identificazione di metaboliti che possano dare informazioni sulle disfunzioni biochimiche alla base della patologia.
A questo scopo, 50 pazienti con FA e 26 controlli sani sono stati reclutati per lo studio. I soggetti sono stati stratificati in base all'età e alle caratteristiche cliniche. Il plasma raccolto è stato processato seguendo un metodo di metabolomica untargeted in GC-MS sviluppato e adattato nel nostro laboratorio. Gli esteri metilici di acidi grassi sono stati utilizzati come standard di riferimento e l'acido succinico deuterato come standard interno. I campioni sono stati derivatizzati in condizioni anidre con metil ossima in soluzione di piridina e sililati con MSTFA. Lo strumento utilizzato per le analisi è un Agilent Technologies GC-MS con sorgente a ionizzazione elettronica e un analizzatore a singolo quadrupolo. In parallelo è stata effettuata un'analisi di metabolomica targeted misurando le concentrazioni di 35 acilcarnitine e 12 amminoacidi analizzando i campioni preparati secondo la tecnica del Dried Plasma Spot su uno spettrometro Sciex API 4000 LC-MS/MS con analizzatore a triplo quadrupolo. Le analisi statistiche sono state effettuate su software dedicati (MSDial e MetaboAnalyst).

Dall'analisi di metabolomica untargeted non sono emerse differenze significative tra i profili metabolici dei soggetti con FA e i soggetti sani. Le concentrazioni di alcune acilcarnitine e aminoacidi, invece, sono risultate significativamente differenti nei due gruppi. In particolare, due acilcarnitine a catena lunga, C14 e C18:1, sono più abbondanti nel plasma dei pazienti con FA mentre arginina e glicina sono presenti in quantità significativamente inferiori. Livelli elevati di acilcarnitine a catena lunga circolanti sono associate alla presenza di patologie cardiovascolari e studi dimostrano che esse sono coinvolte nei processi elettrofisiologici del cuore1,2. La carenza di glicina, amminoacido avente proprietà di detossificazione dell'organismo, potrebbe riflettere uno stato di stress ossidativo e metabolico nei pazienti con FA, probabilmente indotto dall'aritmia. Livelli bassi di arginina, invece, sono associati a disfunzione endoteliale3, caratteristica spesso presente nei pazienti con patologie cardiovascolari, inclusa la FA4. Le alterazioni rilevate in questo studio preliminare potrebbero rappresentare le basi per guidare gli interventi terapeutici che mirano a ridurre l'incidenza delle complicazioni della FA.

Optimization of NAFLD HepG2 cellular model for the evaluation of phytochemicals bioactivity from different plant extracts by quantitative proteomics studies

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Nonalcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease characterized by accumulation of lipids as a result of an imbalance between lipid synthesis, uptake, and export from the liver. The cellular stress caused by lipid overload can result in inflammation and cell death that contribute to the progression of the disease from simple steatosis to nonalcoholic steatohepatitis (NASH). Nonetheless, the molecular mechanisms underlying the complexity of pathological condition are still poorly described. The application of advanced quantitative mass spectrometry-based proteomic approaches in combination with network analysis could provide a depth description of changes in protein profile upon NAFLD. In this work, a label-free quantitative proteomic approach based on high-resolution mass spectrometry was applied to an in vitro cellular model of NAFLD induced by treating hepatic cells HepG2 with a complex of oleic acid and bovine albumin. The cellular model was set up after optimizing the experimental conditions to induce the specific pathological state and validated by Oil-Red O staining to confirm the presence of the lipid droplets. Besides, nanoLC MS/MS proteomic analyses resulted in the identification of 2040 proteins of which 67 significantly altered in the disease versus control cells. The functional enrichment and network analyses revealed that the 46 upregulated proteins were mainly associated with lipid metabolism, inflammatory response, apoptosis, and mitochondrial dysfunction. Among the differentially regulated proteins, it should be underlined the detected increment of Perilipin-2, a protein involved in the formation and maintenance of lipid vesicles during NAFLD progression. Overall, these acquired information confirmed the potential of the optimized proteomics analytical platform to resolve the complexity of molecular mechanisms behind the onset and progression of metabolic diseases, including NAFLD.

Once validated the cellular model, since phytochemicals from natural sources are often reported to be used in treating or preventing different pathological conditions characterized by oxidative/inflammatory states, NAFLD-HepG2 cells will be used to deeply investigate natural compounds' bioactivity, in terms of modulating specific pathways and mechanisms that lead to the observed phenotypic effects.

The effect of Carnosine on Scaffold Free Human Dermis Spheroids Model investigated by proteomics

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Carnosine is an endogenous β-alanyl-L-histidine dipeptide endowed with antioxidant and carbonyl scavenger properties, able to significantly prevent the visible signs of aging and photoaging. To deeply investigate the mechanism of action of carnosine on human skin proteome, a 3D scaffold-free spheroids model of primary dermal fibroblasts from a 50-years-old donor, was here adopted in combination with quantitative proteomics. In the present work, for the first time, a novel model of dermal spheroids of primary human fibroblasts and advanced quantitative proteomics was adopted. The label-free proteomics approach based on high-resolution mass spectrometry, integrated with network analyses provided a highly sensitive and selective method to describe human dermis spheroids model during long-term culture and upon carnosine treatment. Overall, 2171 quantified and regulated proteins allowed the in-depth characterization of 3D dermis phenotype at 14 related to 7 days of culture whereas 485 were proteins regulated by carnosine treatment at an intermediate time of culture (7 days). Among several modulated pathways, most of them are involved in the mitochondrial functionality, such as oxidative phosphorylation, and TCA cycle, extracellular matrix (ECM) reorganization and apoptosis. Specifically, regulated proteins which play a key role in the ECM assembly and remodelling such as basigin and TMSB4X, by establishing specific cell-matrix interactions with integrins and actin cytoskeleton to modulate cellular fate, architecture and proliferation, were found up-regulated. The functional modules involved in oxidative stress were upregulated in long-term culture attesting an increase of ROS species due to potential senescence of dermis model. Interestingly, carnosine induced the modulation of oxidative stress by the down-regulation of reactive oxygen species metabolism, reducing the aging process of the spheroids. The application of quantitative proteomics coupled to a new dermis spheroid model represents a new concrete tool for pre-clinical investigations and the application of a personalized care approach.

Qualitative and quantitative effects in tomato (*Solanum lycopersicum* L.) of irrigation with water containing realistic concentrations of environmental contaminants (zinc and caffeine)

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Wastewater irrigation can be considered a promising alternative to meet the growing demand for water in arid and semi-arid regions. However, it is necessary to verify whether the presence of possible organic and inorganic contaminants that may have negative effects on the crop irrigated. The effects on tomato plants (*Solanum lycopersicum* L. cv. Panarea F1), fruit production and quality were analyzed in relation to treatment with water containing realistic concentrations of zinc and caffeine. Plants were grown in pots in a greenhouse and treated weekly with uncontaminated water (control) or water contaminated with $ZnCl_2$ (1 mM Zn), caffeine-(trimethyl-^{13}C) (1 mg L^{-1} Caffeine) and with a mixture of $ZnCl_2$ and caffeine-(trimethyl-^{13}C) (Mix).
After 52 days, plants were sampled and the concentrations of Zn, Fe, Cu, Mn, Na, Ca and Mg in the fruits were measured by microwave plasma atomic emission spectrometry (MP-AES). Caffeine and polyphenol concentrations in the fruits were determined by LC-MS/MS mass spectrometry (Sciex 5500 QTrap+), using an Information Dependent Acquisition (IDA) method with one Selected Reaction Monitoring (SRM) transition per component as survey scan and Enhanced Product Ions (EPI) for full MS-MS spectrum acquisition.
The results showed that the concentration of Zn followed the order: roots> stem> leaves> tomato with average values from 20.16 to 231.79 (mg kg^{-1} dry weight). Caffeine is concentrated at the root (2.12 ng g^{-1} fresh weight) and leaf level (2.05 ng g^{-1} fresh weight) and only a small part remains in the fruit (0.2 ng g^{-1} fresh weight). In plants treated with Mix, the number of leaves was lower, while no changes were observed for Fe, Cu, Mn, Na, Ca and Mg. The presence of catechin is detected only in the fruit of plants treated with Mix while the Rosmarinic acid it is present only in control plants. Data suggest that, under our experimental condition, plants could be watered with water containing realistic concentrations of environmental contaminants (zinc and caffeine).

Se-biofortified tomato fruit: a nutraceutical perspective under MS disclosure.

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Selenium (Se) is an essential element and plays several important functions in both humans and plants. One billion people on the Earth are facing insufficient Se dietary uptake, therefore strategies to improve Se uptake are of paramount importance. In plants, Se is well known to affect antioxidant capacity, fruit technological parameters, and biochemical composition (phenols, sugars, amino acids). However, the physiological bases of such effects on metabolic processes are still poorly understood. To investigate these aspects, the tomato, the second most important vegetable crop, and a well-studied model plant have been selected for the application of different Se enrichment protocols to assess fruit physiological responses.

Tomato plants were hydroponically grown in a greenhouse and sprayed with Se solutions at a dose of 0, 5, and 10 mg Se L^{-1} before fruit entered the Mature green stage. Both sodium selenate and chemically synthesized Se nanoparticles (SeNPs) have been tested. Volatile Organic Compounds (VOCs) and polyphenols profiles have been analyzed via GC-MS and LC-MS/MS, respectively, to investigate Se effect on volatilome and metabolome. The expression level of genes involved in the biosynthesis of altered compounds has been also studied by RT-qPCR.

Se was absorbed by aerial plant parts and re-allocated in different organs, including fruit, where it accumulated. Spraying plants with 10 mg Se L^{-1}, with both Se salt and SeNPs solutions, influenced the accumulation of several terpenoids (β-ionone, citral, geranyl acetone, and linalool) and core phenols (coumaric acid, chlorogenic acid, naringenin, phloridzin). The identified biochemical alterations have been confirmed by the results from gene expression analysis. Both Se nanoparticles and sodium selenate have been found suitable for the biofortification of tomatoes. The proposed Se delivering protocols increased the nutraceutical properties of tomato fruit, without exceeding the recommended dose for the human diet, and, possibly, improved their organoleptic characteristics.

Allergens identification in food: the MaxQuant setting

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The experiment workflow to identify the immunoreactive food allergens is well established. After extraction of allergens from the food matrix, the proteins are separated by electrophoretic technique, blotted on nitrocellulose membranes and immunorecognised by the allergic patient's IgEs. The immunoreactive bands are cut, digested by trypsin and identified by MS technique. In our set-up, after LC-MS/MS analysis, protein identification is performed by using MaxQuant software. In order to discover which is the "allergen" among all the proteins present in the band, we have to: i) identify all the band-contained proteins, ii) evaluate their relative abundance and iii) find out which is the protein involved in patient's IgE binding. Considering that MaxQuant default setting is designed for off-gel comparative proteomics, to extend its applicability to our purposes we modified some parameters (i.e. peptide to be included in the identification, the score value of modified and unmodified peptides, iBAQ calculation). The comparison between default and our setting will be presented.

Permeability of Quorum Sensing Signal Molecules through a mucus model: an LC-MS/MS Quantitative Analysis

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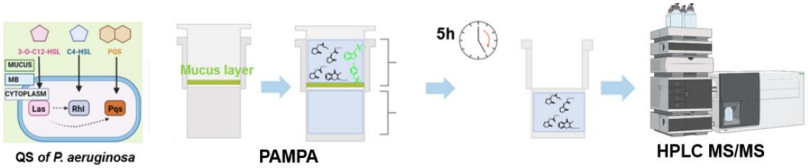

The majority of the human microbiome is hosted in the mucus layer, a 3D hydrogel that plays a key role in mitigating bacterial virulence, particularly interfering with Quorum sensing (QS). QS is one of the major density-dependent bacterial communication systems, involved in regulation of gene expression and virulence factors of many pathogens, such as Pseudomonas aeruginosa. Understanding the interaction between microbiota metabolites and mucus may provide opportunities to investigate the unknown etiologies of many diseases and display promise in developing strategies against the QS circuits.

The aim of this study was the investigation of QS signalling molecules permeability in the presence or absence of mucus, establishing an analytical method based on liquid chromatography-mass spectrometry.

For this purpose, a pathological cystic fibrosis mucus model coupled to a 96-well permeable support precoated with structured layers of phospholipids (parallel artificial membrane permeability assay, PAMPA) was used. The tested molecules, including N-acyl homoserine lactones (AHL) and hydroxyquinolone (HQ) of P. aeruginosa, were quantified by HPLC MS/MS, using a multiple-reaction-monitoring (MRM) method previously developed.

Mass Spectrometry Histochemistry: a novel spatial multi omics technology with single cell resolution

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Introduction

We developed a workflow for label-free top-down MS neuropeptide analysis in sections of formalin-fixed paraffin-embedded (FFPE) tissue. In analogy with immunohistochemistry (IHC), the method was designated mass spectrometry histochemistry (MSHC). MSHC paves the way for innovative biomarker research in pathology, as biopsy material from human clinical samples is rendered accessible for in-situ (neuro)peptide and metabolite studies. In addition, the vast collections of well-conserved human biopsy and autopsy samples documented in tissue banks such as at hospital pathology departments and research institutes, can now be incorporated in this type of biomolecular discovery work.

We are currently mapping all molecular forms of FFPE-detectable endogenous peptides and metabolites throughout the human body, complementing existing human molecular atlases with novel biomolecular information.

Methods

Five-micron sections of human tissues, FFPE-processed according to hospital standard operating procedures, are mounted on regular (non-ITO) histological glass slides. After deparaffinization, sections are thoroughly dried and coated with DHB (MALDI matrix; HTX TM-Sprayer). Subsequently, MSHC is performed on a platform consisting of an atmospheric pressure MALDI source (MassTech AP MALDI UHR) fitted to a HRMS system (Thermo Fisher Scientific LTQ Orbitrap Velos).

Data are acquired at a lateral resolution between 10 and 20 µm defined by MSTarget (MassTech). Data files are analyzed by HistoSnap (ProteoFormiX) and further processed by Mozaic (Spectroswiss). This includes mass recalibration, MS-based feature filtering, mass spectra plotting and MSHC image generation.

Metabolomics data exported to .imzML files are annotated through METASPACE (EMBL, Heidelberg).

Results

MSHC of a 5 x 12.5 mm 2 surface at 20 µm pixel size (scanning at m/z 50-200 and 150-2000 mass ranges) takes more than 68 h continuous MS acquisition time, generating .raw files of nearly 4 GB (full profile data), and corresponding .xml files of ~11 MB.

In human neurosecretory tissues relevant neuropeptides are unambiguously detected. The preliminary detection of neuropeptides at the level of single neuronal

cell bodies in various hypothalamic regions suggests that MSHC is a genuine single-cell spatial -omics technology.
In human patient cancer tissues MSHC yields several biomolecular images in the metabolite and/or peptide molecular class which are correlated with the disease, and even with disease subclasses like aggressive versus non-aggressive tumours, which today cannot yet be discriminated at the molecular level.
Various modes of MSHC acquisition are discussed which can be optimized for more efficient detection of (low abundant) (neuro)peptide signals, as well as all the different soft- and hardware features of our platform which can be exploited to increase the performance of our MSHC workflow.
These include MS image data acquisition in full profile mode, with on-the-fly (re)calibration, as well as sensitivity increases by time-domain transient data acquisition such as with FTMS Booster (Spectroswiss).

Novel Aspect

Single cell resolved spatial multi-omics imaging of human FFPE tissues in health and disease.