International Proteomics and Metabolomics Conference:

MS-based omics in ageing and age-related diseases

BOOK of ABSTRACTS

NOVARA | 2-3 October 2023
Campus Perrone,
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PLENARY
& KEYNOTE
ORAL COMMUNICATIONS

Uncommon post-translational modifications in neurodegenerative and neuromuscular diseases

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Life depends on a vast repertoire of chemical reactions we call metabolism. This activity is generally represented by well-defined biochemical pathways where enzymes catalyze specific reactions. However, this represents only part of the complexity of cellular metabolism. While many enzymes are highly substrate-specific, they can often also act on related compounds, albeit with less efficiency. What's more, some metabolites undergo chemical reactions that are not accounted for in these biochemical pathways. Thus, at low rates, our cells form non-canonical metabolites that are not represented in biochemical pathways.
We seek to understand how cells deal with these non-canonical metabolites.

Recently, we discovered that, in some hereditary cases of Parkinson's disease, damage is caused by a metabolite derived from glycolysis, the central pathway of sugar metabolism. This metabolite spontaneously forms a reactive metabolite which damages both proteins and other metabolites. The DJ1 enzyme destroys the reactive metabolite before it can cause any damage, thus preventing such damage. We are currently trying to understand how this type of damage leads to disease, and whether similar processes may contribute to other neurodegenerative diseases and to aging. Proteomic and metabomic approaches play a key role in this quest, but are limited by our capacity to anticipate the unexpected.

Oxidized PUFAs are more promising for health: the case of F_4-neuroprostanes and F_2-isoprostanes

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Isoprostanoids are cyclic oxygenated metabolites, commonly known as isoprostanes (IsoP) derived from non-enzymatic oxidation of n-6 and n-3 polyunsaturated fatty acids (NEO-PUFA) (Galano *et al.* 2018) such as arachidonic acid (AA, C20:4 n-6); adrenic acid (AdA, C22:4 n-6) and eicosapentaenoic acid (EPA, C20:5 n-3). α-linolenic acid (ALA, C18:3 n-3) produced phytoprostanes (PhytoP), and docosahexaenoic acid (DHA, C22:6 n-3) led to neuroprostanes (NeuroP) (Morrow *et al.* 1990; Jahn *et al.* 2008; Milne *et al.* 2015; Galano *et al.* 2017). Evidences have emerged for their use as biomarkers of oxidative stress and more recently as bioactive lipids acting at molecular level as secondary messengers; the latter ones are mostly related to n-3 PUFAs. Collectively, the existence of these NEO-PUFAs are not limited to mammalian specimens, they are found as well in our food such as nuts, seeds, cocoa, and algae depending on the type of PUFA (Ahmed *et al.* 2020). This lecture will focus on the total synthesis of Dihomo-IsoP and NeuroP generated from AdA and DHA, and precisely their role in neuronal, cardiovascular and muscular diseases.

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Is label-free analysis of 6000 protein groups with a CV approaching 10% from a single cell proteomics standard within reach?

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Introduction:
Sample preparation and analysis methods for bottom-up proteomics of single cells have become feasible in recent years. Measurement of sub-nanogram peptide samples on LC-MS systems poses challenges with regard to sensitivity, precision and accuracy of quantification. Methods optimization is a multi-dimensional task. Using Data Independent Acquisition (DIA) on a timsTOF instrument, we analyzed the impact of an alteration of factors such as sample preparation, liquid chromatography (LC) and mass spectrometry (MS) settings and instrumentation on results.

Method:
We measured 250pg digests of HeLa & K562 digests on an LC-MS system using a Bruker timsTOF instrument dedicated to single-cell proteomics data acquisition.

Preliminary result:
Using an IonOpticks Aurora25cm column and DIA-NN 1.8.2 with isoform IDs as protein infertence method and heuristic protein inference (to reduce the number of protein groups for fair benchmarking purposes), we were able to 5878 +/-61 protein groups from 250pg K562 digests. The number of identifications at 1% FDR was 51531 +/-535.
Coefficient of variation for technical replicates was 10.4% for K562 on the protein group level. This suggests that single cell proteomics with figures of merit that can compete with RNAseq is within reach.

Decoding the protein dance

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Significant progress has been recently achieved in the prediction of protein structures, due to advances such as AlphaFold, RosettaFold and in general deep-learning based methods. These tools are revolutionizing the field of structural biology by providing reliable predictions of protein 3D structures. Despite these developments, predicting protein dynamics remains a formidable challenge due to the inherent complexity of dynamic motions of proteins and the scarcity of experimental data on alternative protein conformational states. Detecting and characterizing protein conformational changes on a large scale would be however very relevant as they can profoundly alter protein function and activity. In my talk, I will present a mass spectrometric method that enables in situ analyses of protein conformational changes for thousands of proteins simultaneously and across conditions. The approach probes dynamic alterations of protein structures due to post-translational modifications, binding of other molecules, cleavages and protein aggregation events. Such a structural 'omics readout, combined with predicted or experimental protein structural data, enables screening for pathological or physiological protein conformations from cells and tissues, supports the generation of mechanistic hypotheses and bridges system and molecular views. I will present applications of this approach to the in situ detection of drug-target interactions and the identification of a novel class of structural disease biomarkers.

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Exploring the epi-proteomic landscape of pancreatic cancer stem cells by super SILAC-based mass spectrometry

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Summary: *PCSCs drive tumorigenesis, chemoresistance, and recurrence in PDAC. Histone PTMs (hPTMs) play a pivotal role in cancer stem cell biology. Here, an assessment of hPTMs in PCSCs through epiproteomic was performed to deepen the understanding of PCSC biology and detect potential therapeutic targets of PDAC.*
Keywords: *mass spectrometry; super SILAC; histone profiling.*

Introduction

Pancreatic ductal adenocarcinoma (PDAC) stands as the seventh major contributor to cancer-related fatality, typically eluding detection until its proliferation extends beyond the confines of the pancreas itself. Several types of research underscore that the propagation of tumours and the development of resistance to treatments predominantly derive from the presence of pancreatic cancer stem cells (PCSCs) [1]. The orchestration of gene expression, often resulting in pathological and tumorigenic outcomes, is intricately linked to epigenetic marks, including DNA methylation and histone post-translational modifications (PTMs) [2].

There is a growing body of evidence indicating that irregular epigenetic changes could play a pivotal role in the transformation process of normal stem cells into cancer stem cells; moreover, a multitude of epigenetic modulators are implicated and potentially targetable for intervention [3]. Consequently, conducting a precise quantification of histone PTMs through an epi-proteomic analysis emerges as a valuable approach to gain deeper insights into PCSC biology and to propose potential novel therapeutic targets against PDAC [4]. Considering this, we undertook a comprehensive MS-based profiling of histone acetylation and methylation in two distinct PDAC cell lines, PaCa3 and PANC-1, each characterized by its own set of genetic alterations.

Experimental

In this research, both the parental and the cancer stem cells from two distinct PDAC cell lines (PaCa3 and PANC-1) underwent epi-proteomic analysis concerning histone acetylation and methylation patterns.

The MS-based analysis of histone PTMs was performed by using the super-SILAC approach which involved the mixing of light (L) and (H) heavy histones and a separation through a 17% SDS-PAGE gel, followed by in gel trypsin digestion of bands corresponding to H3 and H4 histones. The resultant peptide mixtures were subject to analysis using an ultra-nanoflow HPLC system, coupled to a Q Exactive HF MS (Thermo Fisher Scientific). LC-MS/MS analysis and quantification of the L and H forms of the modified peptides were carried out, from which L/H ratios corrected for mixing errors were obtained [4].

The resulting epi-proteomic data were then integrated with both the complete proteome analysis and immunoblotting investigations, affording a comprehensive understanding of the PCSCs landscape.

Results

In this investigation, a total of 55 modified histone peptides were found in the PaCa3 cell line, while 48 were identified in the PANC-1 cell line. The epi-proteomics revealed a distinct histone PTMs profile that set apart PCSCs from their more differentiated counterparts. These modifications are linked to cellular quiescence, apoptosis, chemoresistance, and the epithelial-mesenchymal transition.

Noteworthy instances encompass H4K20me3, recognized as a repressive mark impacting key drivers of the epithelial state, and H3K9me3, a

marker linked to the suppression of pro-apoptotic genes in chemoresistant PDAC cells [5]. These marks exhibited higher levels within PCSCs across both cell lines in comparison to parental cells. Additional histone peptides undergoing modified states within PCSCs included H3K27me3, H3K4me3, acetylated H4K5K8K12K16, and acetylated H3K9K14.

Validation of the increasing presence of H4K20me3 in PCSCs was confirmed through immunoblotting while the band patterns detected for the other histone marks posed challenges in interpretation. Interestingly, the comparative investigation of the PANC-1 cell proteome demonstrates the presence of aberrantly regulated proteins, including certain histone methyltransferases that exert influence over distinct histone modifications. Additionally, epigenetic regulators and enzymes involved in 1C-metabolism, which also impacts histone methylation, were identified within PCSCs.

Conclusions

In summary, comprehensive epi-proteomic profiles of pancreatic cancer stem cells were delineated: the histone-focused super-SILAC approach represents a robust method to accurately profile hPTMs across PDAC cell lines. These results hold the potential to drive the advancement of enhanced PDAC anticancer therapies, addressing the current challenge of their limited efficacy against PCSCs.

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Assessing Adherence to Healthy Dietary Habits: the plasma nutrimetabolomics fingerprints in frail elderly

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***Summary**: Adherence to a healthy diet is associated with a lower risk of frailty. Food frequency questionnaires cannot account for food metabolism, which can impact the diet's effects. Therefore, the measurement of food intake biomarkers helps in the definition of the relationship between diet and frailty onset.*
***Keywords**: Frailty, food metabolism, mass spectrometry*

Introduction

Frailty is a geriatric syndrome characterized by a decline in physiological functions, leading to higher vulnerability to stressors. Although frailty is closely related to aging, its degree differs among people of the same age [1]. The crucial role of diet in frailty onset is well recognized [2]. Food intake biomarkers (FIBs), which are molecules derived from food digestion and metabolism, can provide objective information on dietary habits [3].

Experimental approach

This study aimed to assess the role of diet on frailty by a mass spectrometry (MS) workflow to identify plasma FIBs. We built a FIBs database (>950 compounds) by literature search, considering potential biotransformation after food digestion. FIBs database included phytochemicals, microbiota-derived metabolites, glucosinolates, methylxanthines, alkaloids, and miscellaneous compounds (fish- and meat-derived). Food additives (e.g. artificial sweeteners, preservatives) were also included.

Results

FIBs were identified by an untargeted high-resolution MS approach on plasma sample of 130 elderly subjects (76-78 years, InveCe.Ab cohort) classified as frail or fit according to the frailty index. MS/MS analysis confirmed 88 FIBs in plasma.
A high prevalence (FIBs in > 50% of subjects) of consumption of plant foods, meat, coffee, tea, cereal, legumes and a low prevalence (FIBs in <50% of subjects) of fish and seafood, citrus fruits, artificial sweaters, alcoholic beverages, and olive oil were observed in all subjects. The identified FIBs were used to compute a Mediterranean Diet Score (MDS) that incorporates nine key components of the MD food groups. The MDS score was divided into tertiles of abundance. Tertiles did not show differences between frail and fit subjects (Cochran–Armitage test-for-trend). FIBs were individually tested (Wilkoxon-Mann-Withney test, linear regression) and four FIBS showed significant alteration and negative association with frailty.

Conclusions

Overall, our nutrimetabolomics approch was crucial in identifying circulating compounds derived from food associated with the frailty onset, with potential impact for counteracting frailty development.

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Analysis of lipidomic profile in Parkinson's disease patients carrying TMEM175 gene mutations to define new potential diagnostic markers

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Summary: *To better explore the role of lipids in Parkinson's disease (PD), in this preliminary study we performed lipidomic analysis in plasma sample and in Human dermal fibroblasts (Hdf) derived from PD patients carrying TMEM175 gene mutations. These results could elucidate possible novel deregulated pathways and druggable targets associated to TMEM175 mutations.*
Keywords: *Lipids, Parkinson, dysregulation*

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by a complex genetic background [1]. Large-scale sequencing studies identified several risk genes including *TMEM175* encoding for a lysosomal K^+ channel. We recently demonstrated that mutations in this gene occur in a wide number of Italian PD patients (about 6 %) and are associated with altered channel activity, increased $Ca2^+$ release from endoplasmic reticulum, impaired lysosomal-autophagic pathway, ER stress and activation of Unfolded Protein Response [2]

Experimental

To better investigate the impact of *TMEM175* mutations on cellular metabolism we performed a multi-omics approach including genomic, metabolomics, lipidomic and proteomics in dermal fibroblasts and circulating biofluids from 40 PD patients and 16 healthy subjects, matched for sex and age. The group of PD patients was matched for sex, age, therapy, and clinical features (age at onset, motor symptoms, cognitive impairment, and non-motor symptoms). At genetic level PD patients were classified in two sub-groups, one carrying *TMEM175* mutation (n=26) and one carrying mutations in genes not related to the lysosomal pathway (n=14). Omics data and meta-data were first analysed as independent omics layer.

Results

Preliminary data showed a general deregulation of lipids and metabolites in PD patients compared to controls. Interestingly, we observed a specific lipidomic and metabolomic signature associated with *TMEM175* mutations both in dermal fibroblasts and in plasma. Particularly, we found a dysregulation of Sphingomyelin, Phosphatidylcholine and Acylcarnitine lipid classes which are closely related to lysosomal and mitochondrial functioning.

Conclusions

Systems biology approaches is ongoing to integrate data from different omics levels to improve our understanding of their interrelation and combined influence. These results might lead to identify novel deregulated pathways and druggable targets associated to *TMEM175* mutations to better address therapeutic strategy.

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Comprehensive characterization of protein therapeutics using electron activated dissociation (EAD)

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The growth of protein therapeutics has largely been due to advances in recombinant DNA technology that have provided the means to produce protein therapeutics in the discovery of novel protein drugs. However, the characterization of these drugs brings with it numerous analytical challenges due to the structural complexity of these molecules but above all to the production complexity underlying these new therapies.

As one of the most highly utilized analytical techniques in pharmaceutical research and development, mass spectrometry (MS) has been widely used in the characterization of protein therapeutics because of its analytical sensitivity, selectivity, and specificity [1].

In this scenario, the search for new tools to better characterize therapeutic proteins is always current. For this purpose, the electron-activated dissociation (EAD) offered by the ZenoTOF 7600 system is a new powerful fragmentation tool (complementary to that classically used in ESI-LC-MS/MS systems) to ensure a better characterization of protein therapies [2]. Moreover, compared to traditional low-energy approaches, the Zeno Trap-enhanced EAD offers faster scan speed and higher sensitivity, making this analytical approach suitable for routine biopharmaceutical characterization.

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Thermo Scientific Orbitrap Exploris GC 240 for metabolomics

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Thermo Fisher Scientific

Keywords: *Orbitrap Exploris GC 240, Compound Discoverer software*

For laboratories whose primary focus is metabolomic studies, the capability to comprehensively and accurately characterize and quantify the metabolome is critical. Gas chromatography high-resolution accurate mass (HRAM) mass spectrometry provides supportive information on volatile, semi-volatile, and derivatized polar metabolites across a range of biological, environmental, and clinical sample matrices. This capability in conjunction with liquid chromatography-mass spectrometry provides the ultimate coverage into the metabolome. A complete process to generate meaningful conclusions also includes careful experimental design and conduct prior to sample preparation. The Thermo Scientific™ Orbitrap Exploris™ GC 240 mass spectrometer delivers the very highest data quality for metabolomics to enable researchers to gain unprecedented depth of analysis and to have the flexibility to adapt to evolving research demands, such as biomarker discovery or metabolic pathway identification. With this analytical versatility, the system can provide the deepest insight into metabolomic studies.

High quality raw data is important, but equally so, are informatics solutions to take that data and make clear discoveries between sample groups and to follow up with confident compound identifications resulting in actionable outcomes in an easy-to-use platform. The unrivalled combination of high-sensitivity full-scan HRAM data and intelligent identification using Thermo Scientific™ Compound Discoverer™ software addresses these challenges to advance scientific understanding. In this presentation, we take a closer look at metabolomics with the Orbitrap Exploris GC 240 MS [1] and Compound Discoverer software.

Effects of pre- and/or post-biotics on circulating neurotransmitters and gut-brain axis modulation in Rett syndrome

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Summary: *Evidence indicates a key interplay between gut microbiota and central nervous system. Here, we investigated the effects of two pre-/post-biotics products on circulating neurotransmitters using targeted metabolomics. Improvements in behavior and gastrointestinal function in the challenge phase and transient worsening in the washout phase were related to the neurotransmitter levels.*
Keywords: *Neurodevelopmental disorders, Gut microbiota, Innovative nutraceuticals*

Introduction

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder characterized by methyl-CpG-binding protein 2 gene (MECP2) mutations [1]; however, the mechanism by which the mutation causes the RTT phenotype is largely unknown which makes it difficult to treat [2]. RTT is increasingly recognized as a multi-systemic condition including epilepsy, sleep disturbances, gastrointestinal dysfunction (GID), and dysbiosis [3]. Emerging evidence indicates a key interplay between gut microbiota and central nervous system (CNS) function [4]. Gut microbiota can regulate neurodevelopment through at least three pathways, i.e., immune, neuronal, and endocrine [5,6]. Pre- and post-biotics have been shown to regulate the gut-microbiota both directly and indirectly [7]. In the present research we investigated the impact of nutraceuticals (pre- and/or post-biotics) on circulating neurotransmitters using a targeted metabolomics approach. Correlations between neurotransmitters levels and disease severity, sleep quality and epileptogenesis were explored.

Materials and Methods

The design study was a pilot, randomized, cross-over trial (LALBIZETA_RTT, ClinicalTrials.gov #NCT05420805) with two assigned nutraceutical interventions (1: pre- and post-biotic product containing alpha-lattoalbumin (ALAC), inulin, fructo-oligosaccharides (FOS) and sodium butyrate (NaB) (Kolfarma srl, Italy); 2: post-biotic product containing NaB and zinc oxide (Kolfarma srl, Italy). The design study was a 29-week pilot, single site, randomized, cross-over trial with a 12-wks treatment period and a 4-week wash-out time. A total of n=28 female patients (age range 3.9 - 45.2 years) with a clinical diagnosis of typical RTT and pathogenic proven *MECP2* gene mutation were recruited. Clinical severity was evaluated according to standardized severity scales (RCSS, MBAS, RSBQ) [8-10]. Gastrointestinal health status was examined by Gastrointestinal Health Questionnaire (GHQ) [11], Bristol stool scale [12] and fecal calprotectin [13]. Sleep quality was assessed using by Sleep Disturbance Scale for Children (SDSC) [14]. Quality of life of patients was evaluated according to the Quality of Life Inventory-Disability questionnaire [15-16]. Dietary intakes were evaluated through dietary history and 7-day food diary [17-19], and analyzed using the WinFood software (Medimatica, Teramo, Italy) as compared to the recommended dietary allowances (RDA). The study was approved by the Ethical Committee of Regione Toscana Area Vasta Sud Est (Approval number 21577 and approval date 02/21/2022). Informed consent was provided by the parents/legal guardians. Fifty neurotransmitters and precursors were analyzed in serum samples before and after the treatments using high-resolution LC-MS/MS analysis. Two complimentary chromatographic columns were used, including reverse phase C18 and HILIC. For data analysis, a two-tailed $p < 0.05$ was considered to indicate statistical significance.

Results

Both the treatments were associated with an improvement of GI symptoms, attention/vigilance, mood/behavior and respiratory dysfunction. Interestingly, the observed clinical improvements

were linked to a significant decrease of tryptophan, phenylalanine and 2-pyrrolidinone. Tryptophan is a precursor of serotonin and melatonin, phenylalanine is a precursor for tyrosine which is used for the synthesis of catecholamines (dopamine, norepinephrine, epinephrine), while 2-pyrrolidinone is a product of GABA metabolism. In contrast, the washout phase in both treatments was associated with marked worsening of behavioral and gastrointestinal symptoms. Intriguingly, clinical changes were associated with a decrease of GABA and an increase in choline (product 1). Likewise, a decrease of indole-3-acetonitrile followed the washout phase in RTT patients treated with product 2.

Conclusions

The data, for the first time, indicate that a specific combination of pre- and/or post- biotics is able to improve RTT disease severity and hallmark symptoms without significant adverse effects. Remarkably, observed clinical changes were mirrored and/or guided by host neurotransmitters and gut microbiota metabolites.

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Advanced Proteomic and Bioinformatic Approaches Revealed New Potential Therapeutic Targets for Soft Tissue Sarcoma

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Keywords: *Soft tissue sarcoma, proteomic characterization, druggable targets*

Introduction

Soft-tissue sarcoma is an aggressive cancer that originates from mesenchymal tissue. It is a heterogeneous disease since comprises several histotypes and can arise in different areas of the body (1). Its aggressiveness is mostly related to its high metastasis rate (40-50%) and the unsatisfactory efficacy of traditional therapies such as surgery, radiation and chemotherapy (2). Given this, it is mandatory to deepen the biological knowledge of this group of malignancies and to find out new therapeutic strategies based on their specific molecular patterns. To this aim, drug repurposing is one of the most powerful tool for therapeutic discovery because it allows the reduction of time and costs. In this research, we used an advanced proteomic and bioinformatic approach on sarcoma and healthy tissue, to identify new potential therapeutic targets.

Experimental

We performed proteomic analysis of 26 tumors and healthy samples from the same sarcoma patient, including dedifferentiated liposarcoma, dedifferentiated liposarcoma/MSF, myxoid liposarcoma, epithelioid sarcoma, leiomyosarcoma, rhabdomyosarcoma, spindle cells sarcoma, synovial sarcoma, and undifferentiated pleomorphic sarcoma. Tissue biopsies were lysed, digested, and analyzed on an Ultimate 3000 RSLC nano coupled to an Orbitrap Exploris 480 with a High-Field Asymmetric Waveform Ion Mobility Spectrometry System. The acquired raw MS data files were processed and analyzed using Proteome Discoverer. Bioinformatic analysis were performed on modulated proteins using DAVID and IPA software. Druggable proteome was also investigated using the Drug Bank database. Potential drug targets were further mapped to UniProt, Therapeutic Target Database (TTD) and Cancer/testis antigens database. In addition, investigation of clinical trials' status on ClinicalTrials.gov was performed.

Results

The proteomic analysis allowed the identification of almost 10000 unique proteins and the quantification of more than 8000 proteins. Sarcoma cancer tissue was characterized by a specific proteomic signature: an enrichment of pathways related to PPAR signaling, carbon metabolism and antigen processing and presentation was identified. Regarding molecular functions, an enrichment of proteins related to RNA binding, chromatin and ribosome was found. The comparison of cancer/healthy proteome allowed the identification of specific over-expressed/under-expressed drug targets for each patient / histotype. Among all the drug targets, 370 were present in at least 88% of the samples, of which 314 were upregulated and 56 were down regulated. A more in-depth analysis allowed the identification of six drugs potentially effective against sarcoma, and that were already employed in the treatment of other diseases.

Conclusions

In the presented work we exploited mass spectrometry to create a proteomic mapping of 26 sarcoma biopsies of different soft tissue sarcomas. We were able to characterize the biological signature of soft tissue sarcoma and to obtain a list of reliable new drug targets to develop new therapeutic options. Further *in vitro* and *in vivo* validations will be performed soon.

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Investigating the Role of Circulating small extracellular vesicles Lipid in Prostate Cancer

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Introduction

Cell-cell communication is crucial to the development and proliferation of prostate cancer. Small extracellular vesicles (sEVs) are composed of a lipid bilayer, and are key mediators of this communication because they carry various signaling molecules secreted by cells.

Experimental

In the present study, we investigated whether lipids from circulating sEVs in prostate cancer (PCa) and chronic prostate inflammation (CPI) patients might have a different composition and contribution in the development of the disease. sEVs from PCa, CPI, and healthy subjects (HS) were obtained from plasma samples, and lipids were extracted and then analyzed using mass spectrometry.

Results

The sEVs lipid content of PCa patients was largely different from HS, while CPI patients presented a similar content to PCa. Among most different lipid classes, we identified phospholipids (phosphatidylethanolamines and phosphatidylinositols), but also lipids such as lysophosphatidylinositols (LPIs), which play an important signaling role. In agreement with sEVs content from literature in vitro experiments, higher levels of LPI 18:1 were found in sEVs from PCa and CPI patients, thus supporting the important role of LPIs in the development of the disease. CPI sEVs were also characterized by higher levels of N-acyl ethanolamines (NAEs), which not only play an important signaling role on the endocannabinoid system, a well-known tumor target in prostate cancer, but are also involved in the production of pro-inflammatory cytokines.

Conclusion

Overall, the presence of higher concentrations of those lipids that act on membrane receptors may indicate that tumor and inflammatory cells tend to transmit pro-tumor and pro-inflammatory signals to neighbor cells.

Finally, a significant structural-level change in phosphatidylcholines was detected in PCa. These changes, in the number of double bonds and the length of lipid chains, might result in the remodeling of the sEVs membrane which leads to increased fluidity, increased permissiveness of exchanges, and a change in the curvature of the membrane itself, supporting an active role of sEVs in cancer signaling. Overall, sEVs from CPI and PCa patients were similar, suggesting, at least at the level of lipid metabolism and signaling, a potentially active role of inflammation in the development PCa.

Proteome landscape of pancreatic cancer-derived extracellular vesicle reveals diverse oncogenic variance and identifies SLC5A3 as a novel player in pancreatic cancer progression

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Summary: *Pancreatic ductal adenocarcinoma is a highly aggressive, metastatic, asymptomatic cancer which relies profoundly upon crosstalk with the stroma and microenvironment. Small extracellular vesicles (sEV) or exosomes, display a proteome cargo essential to their biology and function. Understanding EVs' proteome composition is a crucial step towards defeating this disease.*
Keywords: *extracellular vesicles, pancreatic cancer, inositol metabolism*

Introduction

Pancreatic cancer remains difficult to detect and diagnose and is broadly considered an incurable disease, with a 5-year survival rate of about 11% [1]. This disease is molecularly heterogeneous, making targeted therapy difficult. Future identification of biomarkers demonstrating the subtype of pancreatic cancer could determine decisions within the clinic tailored to the genomic phenotype, which may lead to improved patient outcomes. Tumour-derived small extracellular vesicles (sEVs) are heavily implicated in PDAC's metastatic ability, holding proteins known to regulate pre-metastatic niche formation, proliferation, invasion and metastasis [2]. Extracellular vesicles (EVs), play a significant role in maintaining homeostasis, and range between 30-1000 nm in diameter [3]. Vesicles are diverse, depending on the cell type and origin, and hold macromolecules and smaller molecules like salts and cofactors which get secreted into the extracellular space [4]. It is known that some sEVs are enriched in specific proteins, while other proteins and lipids are omnipresent in all sEVs.

In the present study, we performed a comprehensive investigation of the proteome of sEVs from a range of pancreatic patient-derived models of distinct morphology and characterised genetic phenotypes [5]. We hypothesised that the cargo of these sEVs will reflect the phenotypes of the donor cells, with specific proteins being identified as potential novel biomarkers for pancreatic cancer.

Experimental

We characterised sEVs from human non-malignant pancreatic duct cells, PC cells and patient-derived xenograft (PDX) PC cell lines. Plasma samples (60 PC patients and 30 healthy controls) have been also collected. Comprehensive proteomic and lipidomic analyses of sEVs have been performed.

Results

Our data revealed that specific oncoproteins and lipids are only present in PC-derived sEVs and not in non-malignant pancreatic duct cells. In addition, we have found that sEVs from human PC cell lines and PDX cells contained metastatic regulatory factors and signalling molecules fundamental to PC progression as cargo unique to PC sEVs. Interestingly, proteomic analysis of sEVs-derived from human PC cell lines reveals the enrichment of key enzymes involved in inositol synthesis and metabolism such as the transporter SLC5A3. SLC5A3 is involved in inositol transport and the formation of key signalling molecules such as phosphatidylinositol 4,5-bisphosphate. SLC5A3 is an unfavourable prognostic factor for PC and correlates with a lower survival probability. Our findings have been validated in sEVs obtained from blood samples from PC patients.

Conclusions

We propose that the combination of lipids and proteins could be a potential PC biomarker, which offers the opportunity for an early PC diagnosis. In

addition, we identify how sEV-derived lipids and proteins mediate signalling between PC and recipient cells in the tumour microenvironment.

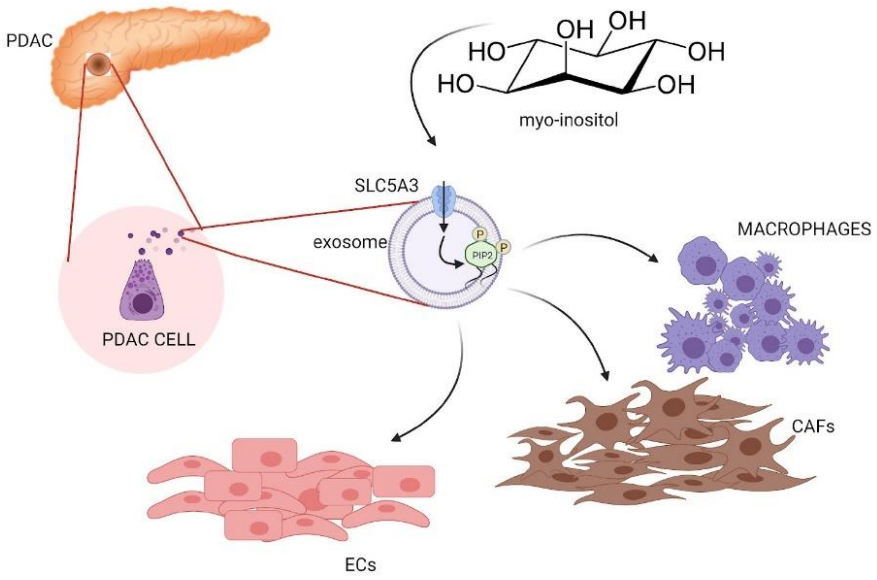

Fig. 1. Understanding the role of SLC5A3 in pancreatic cancer

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An integrated novel approach to artificial intelligence-omics strategies for precision medicine

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***Keywords**: Omics, Artificial Intelligence, Precision medicine, Mass spectrometry*

Introduction

Today artificial intelligence is widely used in the mass spectrometry omics field. For example, several studies have shown that machine learning can use omics data to help with disease detection, prognosis, and treatment. Although many algorithms and tools are available, little is known about their performances and software that allows the use multiple features selection and classification algorithms for mass spectrometry data elaboration is still missing. In addition, there are still many concerns on the minimum amount of data required for training a reliable model, on the effect of imbalanced class distributions on model performances, and on the consistency of biological features used in classifier models.

Methods

We developed a software platform called AI-POSTOMICS (AIPO) on KNIME platform (version 5.1.0) that allows the automatic elaboration of mass spectrometry data in order to classify samples. AIPO uses several feature selection algorithms including filter methods (Gain ratio, Relief-f) and wrapper methods (Boruta and Recursive feature elimination with random forest) for dimension reduction and to obtain top features for biological interpretations, while for classification tools we included Random forest, Support vector machine with radial and polynomial kernels, Logistic regression, K-nearest neighbor (KNN) and ranger to build robust models and to classify unknown samples. The software is particularly suitable for biomarkers discovery studies and to identify the most important variables in a biological experiment. AIPO is an open-source software and will be soon made available online.

Results

Through AIPO, the user can upload omics data to determine which is the most effective combination of algorithms. The AIPO platform has the capacity to independently choose the combination of algorithms included in the platform for their specific dataset; user could also predefine the algorithms. The optimal selection of algorithm combinations is achieved by considering additional aspects that influence the efficacy of machine learning models. The parameters encompass the split ratio for the training and validation subsets, together with the impact of feature selection stability on the classifiers' performance. Users are also allowed to preserve the trained model for making predictions on new, unknown samples later. Additionally, users can retain the results of the model's performance, together with the features used during the model's training to aid in future investigations for biological interpretations.

AIPO was evaluated on different omics datasets, including metabolomics data of COVID-19, as well as proteomics data of tumor and healthy subjects. A comparative analysis was conducted between AIPO and the current AutoML tool, focusing on their respective performance in classification tasks. The results revealed that AIPO exhibited higher performance in terms of classification performance scores.

Conclusion

The importance of artificial intelligence lies in its ability to analyze big dimensional datasets and assist in the diagnosing process. Here we showed a promising strategy for improving MS-based omics data analysis. AIPO tool achieved higher performance score in each scenario data, as well outperformed existing tools, indicating that AIPO can be useful for MS-based data analysis investigations.

Mass spectrometry, a strong weapon against Alzheimer disease. From early diagnosis to disease biomarkers discovery and metabolomic studies

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Summary: *Alzheimer's disease is a progressive dementia associated with loss of memory and cognitive dysfunction. Triple quadrupole mass spectrometry (MS) can represent a powerful tool for quantification of blood biomarkers that can lead to an early diagnosis. High resolution-MS coupled with imaging can help on the understanding the disease behavior and find new biomarkers.*
Keywords: *Alzheimer, mass spectrometry, imaging*

1 Development of a multiplex ultra-sensitive LC-MS method for the quantification of Alzheimer's disease main blood biomarkers

Current routine clinical method for detecting and monitoring of Alzheimer's disease (AD) biomarkers are performed using cerebrospinal fluid (CSF). However, CSF sampling through lumbar puncture can be both tricky for the clinician and invasive for the patient.
Therefore, the quantification of plasma-based biomarkers such as phosphorylated Tau (p-Tau), Tau, APOE and Aβ1-42 and alpha-synuclein would allow for a large-scale population screening and would facilitate patient monitoring through a non-invasive sampling procedure.
The collection of genetic and physiological data would enable easy and early diagnosis, would improve patient care, and would support the development of new therapeutic drugs. Besides, LC-MRM method, in comparison with commercial immunoassays, allows multiplex analysis and detection of structural modifications such as post-translational modifications (PTMs).
Primary development was carried out with specific immunocapture targeting several phosphorylation sites on Tau protein in CSF before further implementation in plasma.

The objective of this work is the development of a unique and innovative LC-MRM method using a LCMS-8060NX (Shimadzu) triple quadrupole to detect Alzheimer's blood biomarkers involved in diagnosis and prognosis: Tau, pTau, Apolipoprotein E, Aβ peptide and alpha-synuclein. The first part of this project was dedicated to the development of a workflow enabling the detection of Tau and pTau (T217, T205, T181) which are major biomarkers correlating with AD progression.

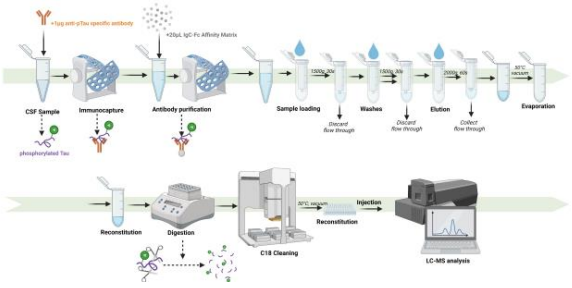

Fig. 1. Sample preparation workflow

The preliminary development of this innovative method allowed the detection of phosphorylated peptides corresponding to specific phosphorylation sites correlated with Alzheimer's disease progression as previously reported. Among which, pTau217, pTau231 and pTau181.
Further optimization of the sample preparation would be performed to increase the performances of the method and combine with other biomarkers for multiplexing. This would be carried on using the method with plasma samples and implement the latter on triple quadrupole systems.

2 Coupling High Resolution Mass Spectrometry and imaging techniques for research and biomarkers discovery

Alzheimer's disease (AD) is a progressive dementia associated with loss of memory and cognitive dysfunction. Is demonstrated a decrease in b-series gangliosides along with a change in ganglioside molecular species in the hippocampal grey matter of patients with AD.
The present study demonstrates the use of imaging mass spectrometry for analyzing the spatial arrangement of ganglioside GM1 (GM1) molecular species in the hippocampus. In AD patients, we found a decrease in the ratio of GM1(d20:1/C18:0)

to GM1 d18:1/C18:0) in the outer molecular layer (ML) of the dentate gyrus. Because the outer ML is the region of main input into the hippocampus, our findings may have a direct relationship to the mechanism of dysfunction in AD.

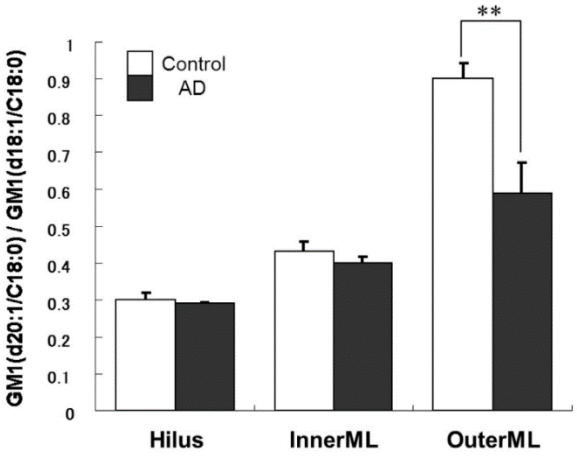

Fig. 2. The ratio of GM1(d20:1/C18:0) to GM1(d18:1/C18:0) in the hilus, inner ML and outer ML

To evaluate the distribution of GM1(d18:1/C18:0) and GM1 (d20:1/C18:0) and differences between control and AD, we compared the ratio of GM1(d20:1/C18:0) to GM1(d18:1/C18:0). The hilus, granule cell layer (GL), inner ML, and outer ML were analyzed.

Mass spectra were extracted from each selected area, and the average mass spectra of the selected areas were used for comparison.

While gangliosides were clearly detected in the hilus, inner ML and outer ML, they were hardly detected in the GL. For control samples, the ratio of GM1(d20:1/C18:0) to GM1(d18:1/C18:0) in the hilus, inner ML and outer ML was 0.3, 0.4, and 0.9, respectively (Fig. 2). In AD samples compared to controls, the ratio of GM1(d20:1/C18:0) to GM1(d18:1/C18:0) was significantly decreased only in the outer ML ($P < 0.01$, Student's t-test).

These results strongly suggest that the AD-related decrease in the GM1(d20:1/18:0) to GM1(d18:1/18:0) ratio in the outer ML may play a causal role in AD or correlate with the progression of the disease [1].

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Mucosa-associated microbiota and metabolome signatures discriminate between low-grade and high-grade dysplastic colon polyps

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***Summary**: Based on the driver–passenger model for colorectal cancer, we characterized the mucosa-associated microbiota and metabolome in low- vs. high-grade dysplastic colon polyps from 78 patients and we identified different mucosa-associated microbiota and metabolome signatures able to distinguish between low-grade and high-grade dysplastic colon polyps.*
***Keywords**: mucosa-associated microbiota, mucosa-associated metabolome, colorectal polyps*

Introduction

Colorectal cancer (CRC) is a multifactorial pathology, involving genetic, environmental, life-style factors, gut microbiota and metabolites. According to the driver-passenger model, driver bacteria may be involved in CRC initiation, while passenger in progression.
Our aim was to characterize the mucosa-associated microbiota (MAM) and its metabolome in patients with colon adenomas to define the role of driver and passenger bacteria and metabolites in tumor development.

Methods

We recruited 78 patients and collected MAM, mucosa-associated metabolome and adenomas histology. MAM was analyzed by 16S ribosomal DNA sequencing. Metabolome adherent to the polyps was analyzed through a high-throughput metabolomics approach recently described by our group [1] by bidimensional gas chromatography mass spectrometry. Patients were divided according to the grade of dysplasia into low- or high-grade dysplasia groups.

Results

MAM analysis showed an enrichment of candidate driver and passenger taxa in low- and high-grade group, respectively ($p<0.05$).
By metabolome analysis of 59 (34 low- vs. 25 high-grade) out of 78 patients, we identified 41 metabolites able to distinguish between high and low-grade dysplastic polyps ($p<0.05$). More specifically, we found L-serine, reported to be required for CRC cell proliferation [2], enriched in high-grade group. Moreover, two other metabolites found enriched in the high-grade dysplasia group were lactic acid and butyric acid, which may both derive from the aerobic glycolysis occurring in CRC cells and/or the metabolism of gut bacteria (e.g. Anaerococcus that we found enriched in high-grade group).
The integrated microbiota-metabolome analyses showed a negative correlation of Pelomonas and Phascolarctobacterium, enriched in low-grade, with organonitrogen compounds, whose reduced levels have been shown to increase CRC risk [3].

Conclusions

These preliminary data support our hypothesis that driver and passenger bacteria and associated metabolites are involved in CRC in a tumor-stage specific manner.
This project was supported by AIRC-25886.

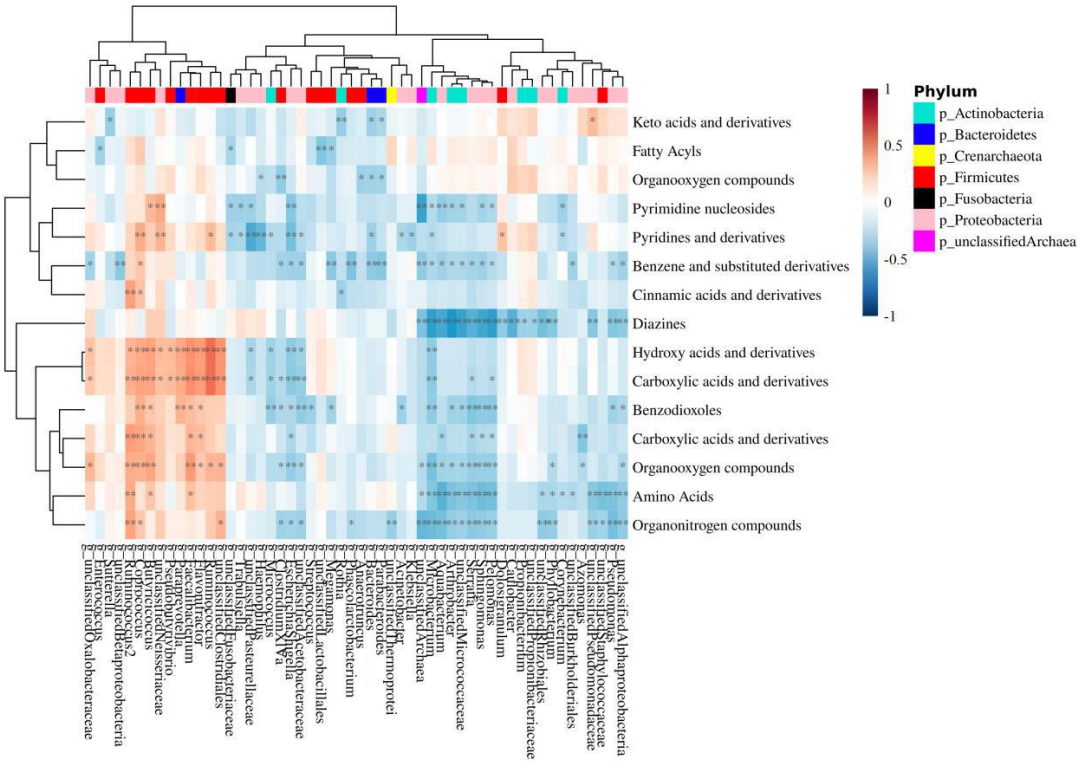

*Fig. 1. Correlation heat map between MAM genus and mucosa-associated metabolite class. The red color indicates a positive correlation, while the blue color indicates a negative correlation. The darker color indicates the most highly correlated variables. The asterisks indicate the correlation coefficient p-value: * $p < 0.05$ and ** $p < 0.01$.*

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Proteomics reveals mitochondrial targeting by a tri-phenyl-phosphonium substituted fatty acid in pancreatic cancer

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Summary: *PCSCs are the major biological source of PDAC recurrence and chemoresistance. Recent studies demonstrated increased CSC mitochondrial mass, reflecting their dependence on mitochondrial function and OXPHOS. We explored the effects of TPP-based compound SS4 on a PDAC cell line by LCESI-MS/MS. Omics data were deepened by biochemical and cellular assays.*
Keywords: *pancreatic cancer stem cells (PCSCs); tri-phenyl-phosphonium (TPP); LC-ESI-MS/MS*

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a leading cause of cancer-related death, with its aggressiveness and resistance to therapies attributed mainly to the presence of pancreatic cancer stem cells (PCSCs). Interestingly, recent studies demonstrated that energetic metabolism and mitochondrial function play a fundamental role in phenotype maintenance and spreading of PCSCs [1]. Mitochondria-impairing agents can be used to hamper PCSC propagation [2].
Among them, tri-phenyl-phosphonium (TPP)-based compounds are small non-toxic, and biologically active molecules that accumulate in mitochondria and act as potent inhibitors of mitochondrial function in cancer cells and CSCs [3]. Considering the strategic importance of TPP-based compounds to target CSCs, this study aimed to investigate for the first time the effect of a TPP-substituted fatty acid (SS4) on an *in vitro* model of PCSCs and related parental cells (i.e., PANC-1).

Experimental

The effect of SS4 on the proliferative activity of parental (P) PANC-1 cells and derived PCSCs was assessed and the IC_{50} was calculated using a Cell Titer Fluor Cell Viability Assay (Promega). The possible effect on the morphology of treated cells after 24 hours was also evaluated by the EVOS FL Imaging System (ThermoFisher Scientific).
To obtain information about the modulation at the proteomic level, proteins from three biological replicates of both untreated and treated P and PCSCs were analyzed by LC-ESI-MS/MS utilizing an Ultimate 3000 nano-UHPLC system coupled to an Orbitrap Fusion Lumos Tribrid MS (ThermoFisher Scientific) and identified dysregulated proteins were subjected to bioinformatic to detect correlated pathways. To further investigate SS4 effects, the modulation of proteins involved in ER stress, unfolded protein response (UPR), and autophagy in P and PCSCs treated at different time points (i.e., 0, 12, and 24 hours), was assessed by immunoblotting. Lastly, total and lipid ROS levels were determined using ROS assay kits, based on detecting 2',7'-dichlorofluorescein (DCF) diacetate and C11-BODIPYò 581/591 fluorescence, respectively. The DCF and C-11 BODIPY fluorescence intensities were analyzed using a microplate reader (Tecan Infinite PRO 200) or a flow cytometer (LSRFortessa X-20, Becton Dickinson, USA), respectively.

Results

PCSCs and P cells treated for 24 hours with SS4 showed inhibition of their growth at μM doses, with an IC50 value for PCSCs equal to 18 μM.
The LC-MS-based proteomics allowed the identification of a total of 65 and 45 modulated proteins in the comparison between untreated vs. SS4-treated P and PCSCs, respectively.
Functional GO analysis revealed that in SS4-treated P cells, a considerable portion of dysregulated proteins were mitochondrial, mainly involved in the mitochondrial electron transport and mitochondrial ATP synthesis coupled with proton transport. On the other hand, in SS4-treated PCSCs, differentially expressed proteins were mainly nuclear or cytosolic implicated in response to heat, to unfolded proteins, and in the chaperone-mediated protein folding.
These results were further deepened by immunoblotting of proteins involved in ER stress (i.e., PERK, CHOP, XBP1, ATF6, and p-eIF2α) and autophagy (i.e., ATG7, LC3B), indicating the modulation of these pathways in SS4-treated P and

CSCs at different time points (i.e., 0, 12, and 24 hours).
Moreover, since some of the dysregulated proteins seemed to suggest the induction of ferroptosis, the effects of the SS4 treatment on total ROS and lipid peroxidation were evaluated. The data obtained suggested that in both P and PCSC of PANC-1 cells, the SS4 treatment induced a general increase in total ROS and, particularly after 24h of SS4 treatment, an enhanced lipid peroxidation that could lead to the activation of ferroptosis.

Conclusions

In conclusion, the results achieved revealed mitochondrial targeting and cell killing by the newly synthesized triphenyl-phosphonium substituted fatty acid on an *in vitro* model of pancreatic cancer cells. These findings deserve further experiments to complete the characterization of the SS4 anticancer mechanism of action. However, the data obtained suggest that this TPP-based compound could provide a novel chemical strategy for effectively killing both "bulk" cancer cells and PCSCs, underlining the potential use of TPP-based compounds as anti-cancer drugs in PDAC.

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Improving analytical and annotation robustness in small molecule metabolomics using GCxGC-HRTOFMS

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Summary: *This presentation focus on the enhanced annotation and detectability capacity provided by high resolution information contained in multidimensional chromatography data. The presentation will focus on the combination of untargeted screening of small metabolites and small lipids in human blood serum samples from different form of colorectal cancers.*
Keywords: *Metabolomics, GCxGC-HRMS, Chemometrics*

1 Introduction

According to the world health organization (WHO), colorectal cancer (CRC) ranks as the third most frequently diagnosed cancer and the second leading cause of cancer-related deaths. The current endoscopic-based or stool-based diagnostic techniques are either highly invasive or lack sufficient sensitivity. Thus, there is a need for better screening approaches.

The rapid advancement of high throughput "omics" approaches, such as meta-genomics, transcriptomics, proteomics, metabolomics, lipidomics, microbiomics, and volatolomics, offer a potentially less invasive alternative than available techniques to develop novel biomarkers for CRC screening that could contribute to its clinical management.

For small molecules "omics" screening, gas chromatography coupled to mass spectrometry (GC-MS) represents a method of choice to screen biofluid samples. However, GC-MS is mostly used for targeted analysis due to the high complexity of the matrices. To tackle this limitation, comprehensive two-dimensional gas chromatography coupled to mass spectrometry provided increased separation capabilities, making it a method of choice for untargeted small molecule metabolomics.

In this study, we analyzed 64 human serum samples representing three different groups of colorectal cancer using cutting edge GC×GC–LR/HR-TOFMS techniques. We analyzed samples with two different specifically tailored sample preparation approaches for lipidomics (fatty acids) (25 µL serum) and metabolomics (50 µL serum). In-depth chemometric screening with supervised and unsupervised approaches and metabolic pathway analysis were applied on both datasets. This study was published and is available in open access [1].

2 Methods

In this study, we focused on three sample classes: advanced cancer – adenocarcinoma; cancer – adenoma; and control samples. The demographic information is included in the table below (Table 1).

Table 1: main demographic parameters for the study population.

	Adenocarcinoma	Adenoma	Control
Total no of participants (Female/male)	20 (9/11)	23 (12/11)	21 (9/12)
Female age (Mean ± SD)	66.50 ± 7.99	64.70 ± 8.69	65.81 ± 8.73
Male age (Mean ± SD)	69.45± 8.54	64.70 ± 8.69	65.81 ± 8.93
Location[1] (A/T/D/S/R/recto-sigmoid/splenic flexure)	(7/0/3/3/6/0/1)	(7/1/4/3/7/1/0)	-
BMI (Mean ± SD)	25.39± 3.66* missing 4 data	25.63± 4.28* missing 5 data	27.40± 3.89* missi 5 data
pTNM staging[2]			
Stage-0 (pTisN0M0)	1		
Stage -I (pT1N0M0 or pT1NxMx)	5	-	-
Stage -II (pT2N0Mx or pT2NxMx)	8		
Stage -III (pT3N0Mx or ypT3N0Mx)	6		
Alcohol (Yes/No)	10/10	10/13	12/9
Smoking (Yes/No)	5/15	4/19	3/18

Each serum was analyzed using two different analytical approaches. First, a lipidomics method focused on specifically on fatty acids and derivatives. Next, a more polyvalent MSTFA derivatization approach was developed to screen for more general metabolite families. The protocols are presented in Figure 1.

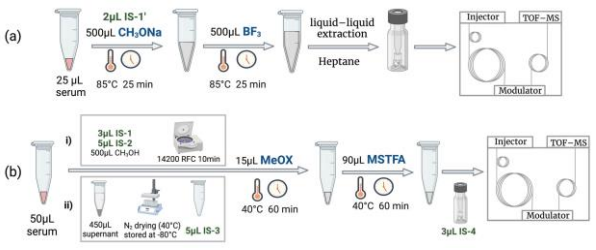

Fig 1. Sample preparation workflows

A general instrumental set up was used for both analytical workflows. The analysis was performed using a normal column set

configuration on a Pegasus 4D (LECO corp). Each workflow has an optimized temperature program. For the identification confirmation, the same set up was installed in a GC-HRT 4D (LECO corp). The data acquisition was conducted following the mQACC guidelines.

For the data processing, the chromatograms were aligned and pre-processed. A PLS-DA model was created to identify group-specific features for both analytical workflows. All the method detailed can be found in Bhatt et al. [1].

3 Results and discussion

Using the lipidomics approach, we obtained a semi-targeted screening of 30 fatty acids compounds. From those compounds, 8 displayed statistically significant differences between the groups (Table 2). Based on these 8 compounds, we obtained a clear stratification based on cancer status. PUFA (ω-3) molecules are inversely associated with increased odds of CRC, while some PUFA (ω-6) analytes show a positive correlation.

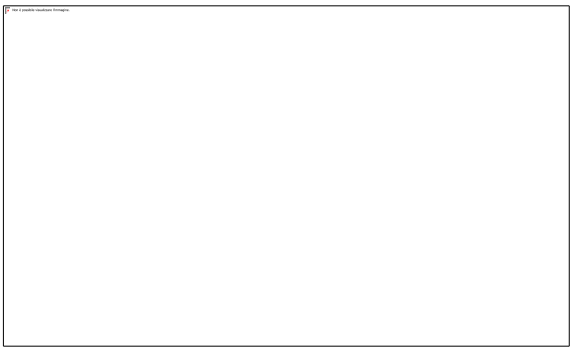

Fig 2: Normalized area for the 8 significant compounds listed in Table 2.

Moreover, these 8 markers were also providing clustering trends for adenocarcinoma stages (Figure 3). These results will require future validation.

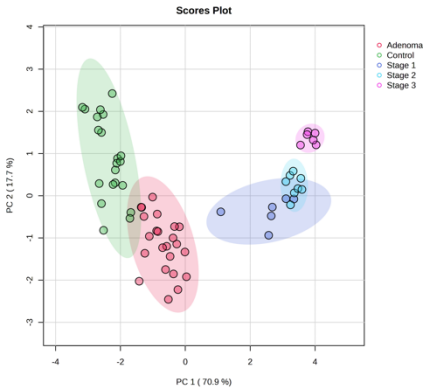

Fig 3: PCA score plot showing group clustering and including adenocarcinoma staging.

For the metabolomics profiling, 8 compounds were also identified as significant using the same data processing approach. However, the fold change and the resulting clustering were not as clear as for the lipidomics workflow.

4 Conclusions and perspectives

A Lipidomics study revealed that specific PUFA (ω-3) molecules are inversely associated with increased odds of CRC, while some PUFA (ω-6) analytes show a positive correlation. With the metabolomics approach, some proteogenic amino acids were identified but they do not lead to a clear stratification between groups. This unique study provides a more comprehensive insight into molecular-level changes associated with CRC and allows for a comparison of the efficiency of two different analytical approaches for CRC screening using the same serum samples and a single instrumentation. Moreover, method translation between large screening on LRMS system and targeted injection on HRMS provided an added value for specific annotation of the compounds of interest.

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Pushing the boundaries for Automated End-to-End, high-throughput and standardized single cell proteomics

Erik Verschuuren

Evosep

Proteomics has evolved at a rapid pace with recent advances relating to liquid chromatography mass spectrometry (LCMS) sensitivity, robustness and throughput enabling the transition from basic to clinical research. The application of standardized Evosep One methods have been instrumental in this shift due to the ability to routinely process hundreds of samples per day in a robust and reproducible manner. The emphasis is thus on automated sample preparation to facilitate standardization and reproducibility. At the same time the field of Single Cell Proteomics has evolved rapidly, enabling true single cell proteome analysis at increasing speed and automation.

We'll present an end-to-end pipeline that enables sample processing from protein lysate to loaded Evotip in a fully automated manner. The workflow, implemented on an open-source and cost-effective Opentrons OT2-2 liquid handling system. Peptides are seamlessly loaded on Evotips in a fully automated manner and can be further stored for weeks bypassing the requirement for additional transfer and storage in tubes or vials that typically leads to increased sample loss.

Secondly we'll present the use of Evosep One in Single Cell Analysis workflows. The use of Evotips as sample input devices allows for a seamless transfer of peptides from an automated cell selection and preparation device like the Cellenion CellenONE. Transferring these minute amounts of peptides onto Evotips will prevent loss of valuable peptides in the workflow, resulting in more peptides that remain available for analysis.

LC-MS/MS targeted metabolomics of Kynurenine Pathway Catabolites: Insights from Selective Indoleamine or Tryptophan 2,3-Dioxygenase Inhibition and IDO1 Inhibitor Potency

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Summary: *This study investigates the kynurenine pathway's response to selective TDO or IDO1 inhibition. Validating an LC-MS/MS method for quantifying kynurenines in cell culture media, it explores metabolic differences, cell responses to IFNγ, and novel IDO1 inhibitor selectivity. These findings have implications for cancer immunotherapy and preclinical drug research.*
Keywords: *Kynurenine Pathway, LC-MS/MS, Cancer Immunotherapy*

Introduction

The kynurenine pathway (KP) is crucial for tryptophan catabolism, impacting immune regulation and cancer. It involves heme-oxidoreductases, like tryptophan or indoleamine 2,3-dioxygenases (TDO, IDO1, IDO2), yielding immunosuppressive kynurenines in tumors.[1]
Research centers on inhibiting human indoleamine 2,3-dioxygenase 1 (IDO1) for cancer immunotherapy. IDO1 exists in two forms: holo-IDO1 (active) and apo-IDO1 (Fig. 1). Noteworthy inhibitors like epacadostat (EP) and linrodostat (LIN) have progressed in clinical trials, but results have prompted further exploration.[2,3]

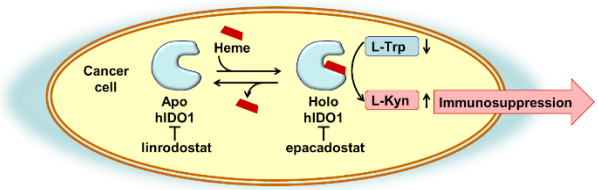


Fig. 1. Apo and Holo Inhibition of IDO1: A Pharmaceutical Perspective on Heme-Driven Balance

Aim of the work

The primary objective of this study is to investigate changes in the kynurenine pathway following selective inhibition of TDO or IDO1. Our specific goals include:

1. Identifying metabolic differences post selective inhibition of IDO1 and TDO.
2. Evaluating the pertinence of the cell lines involved in the inhibition assays.
3. Determining the half-maximal inhibitory concentrations (IC_{50}) for known and synthesized compounds in preclinical drug research.

To achieve these objectives, we have optimized an LC-MS/MS method for the rapid quantification of KP catabolites in the supernatants of three cancer cell lines: U87 (glioblastoma), MDA-MB-231 (breast cancer), and A375 (melanoma).

Experimental

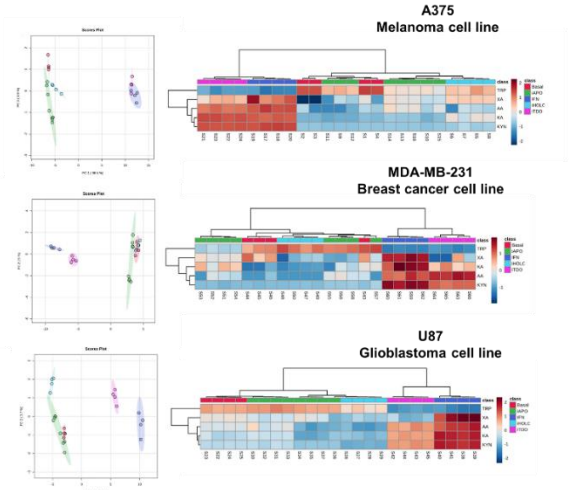
External calibration utilized standard solutions of Tryptophan (TRP), L-kynurenine (KYN), xanthurenic acid (XA), 3-hydroxykynurenine (3OHKYN), kynurenic acid (KA), 3-hydroxyanthranilic acid (3OHAA), anthranilic acid (AA), 5-hydroxytryptamine (serotonin, 5HT), tryptamine (TRYP). L-tryptophan-(indole-d_5) (d_5T) served as the internal standard due to its structural similarity with the other kynurenines. Method validation followed FDA, EMA, and the ICH M10 guidelines. Instrumental settings summarized in Table 1.
U87, MDA-MB-231, A375 cell lines were cultured, treated with IFNγ, LIN, BL5, EP inhibitors, or TDO selective inhibitor 680C91.[3] IC_{50} values were determined using dose-response curves of Graphpad 9.1.0. Data underwent statistical analyses, including PCA (Principal Component Analysis), biplot, correlation, heatmaps via Metaboanalyst 5.0.

Table 1. LC-MS/MS instrumental settings

Instrument: Q-Exactive Plus UHMR Hybrid Quadrupole Orbitrap™ Mass Spectrometer equipped with a Vanquish™ Duo UHPLC system (Waltham, MA, USA)	
MS tune settings – ESI source	**LC conditions**
ESI^+ ionization	**Column:** Synergi 4 µm Polar-RP 80 Å, LC 150 x 2 mm
Spray voltage: 3.50 kV	**Column guard:** Polar-RP Security Guard precolumn cartridge (4 × 2.0 mm)
Capillary temp.: 350 °C	**Column Temp.:** 45 °C
Sheath gas flow (N_2): 45.0 L/min	**Mobile phases:** A – H_2O 0.1% FA, B - MeOH 0.1% FA
Sweep gas flow (N_2): 0.0 L/min	**Flow rate:** 0.300 mL/min
Aux. gas flow (N_2): 30.0 L/min	**Gradient elution:** 5%-80% solvent B in 5 min, up to 95% solvent B from 5.0 to 8.0 min, held at 95% solvent B for 1.0 min, then column reconditioning at 5% solvent B from 9.5 to 14.0 min.
Aux gas temp.: 350 °C	**Total run time:** 14.0 min
Max Spray Current: 100.0 µA	**Injection volume:** 5 µL

Results and discussion

The study successfully validated a method comparing neat solvent and matrix-matched curves in DMEM and EMEM, demonstrating negligible matrix effects. Each analyte exhibited R-squared values above 0.99. In Figure 2, A375 cells treated with IFNγ and iTDO (680C91) displayed alignment, indicating minimal TDO expression. Conversely, MDA-MB-231 and U87 cells exhibited differences, suggesting basal TDO presence, with U87 consistently expressing TDO. This finding could be exploited for the IDO1 selectivity assessment. Multivariate analysis (MVA) explored apo/holo mechanisms differentiation. PCA revealed trends: IDO1 inhibitors correlated with higher TRP and lower downstream metabolites (blue squares), while IFN/iTDO conditions displayed elevated KYN and downstream metabolites (red squares), signifying IFNγ-



induced enhanced metabolism. Our LC-MS/MS method enables the determination of IC_{50} values for EP, LIN, and BL5 in the aforementioned cell lines. The KYN/TRP ratio served as an index of IDO1 activity (data available on request). Finally, metabolomics and RT-PCR highlighted the absence of TDO expression in the A375 cell line (Fig. 3).

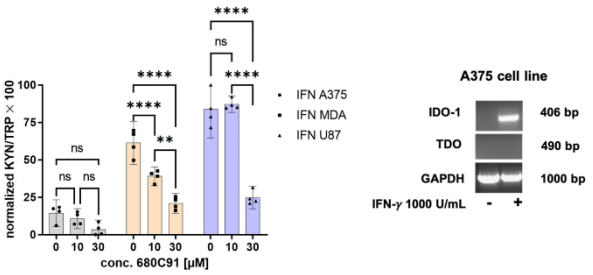


Fig. 3 Fig. 3: TDO Inhibitor Effects and IDO1/TDO RT-PCR in the A375 cell line.

Conclusions

The validated LC-MS/MS method quantified KP catabolites in A375, MDA-MB-231, and U87 culture supernatants, enabling exploration of selective IDO1 and TDO inhibition and their metabolic implications. Notably, there is a surprising lack of literature on TDO expression in the A375 cell line, commonly used for testing IDO1 inhibitors. Our paper fills this gap, providing valuable insights on this topic. The results suggest the method's potential as a screening tool for cellular models and IDO1 inhibitors.

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International Proteomics and Metabolomics Conference:

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POSTERS

The cationic amphiphilic drug penfluridol displays cytotoxicity in breast cancer cells by inducing mitochondrial damage and activating the endoplasmic reticulum stress response

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Summary: *Herein we investigated the potential of repurposing the psychotropic drug penfluridol for breast cancer treatment. Experiments revealed penfluridol's efficacy in inhibiting cancer cell growth, sensitizing cells to doxorubicin, and inducing mitochondrial and endoplasmic reticulum stress. Penfluridol emerges as a promising candidate for further research in breast cancer therapy.*
Keywords: *Breast Cancer, Drug Repurposing, Psychotropic drug*

Introduction

Breast cancer (BC) is the most diagnosed cancer worldwide, besides improvements in cancer treatment, it represents the 5th leading cause of cancer mortality. Drug repurposing consists of using an approved drug for a different application than the original one and, by reducing the time and costs required for de novo drug discovery, it represents a promising approach for cancer therapy. In this context, psychotropic drugs have been widely exploited owing to their safety and long clinical use [1,2,3].

Experimental plan

Herein, based on the screening performed in our previous work, we investigated the effect of a commercially available psychotropic drug penfluridol (PF) in BC cells using cytotoxicity, clonogenicity, chemosensitivity, mitochondrial health, and cell stress markers assays. In addition, we used PRISM repurposing in-silico analysis to characterize the antitumoral activity of PF in BC cells. Lastly, we performed a proteomic analysis to delineate the underpinning deregulated pathways involved in response to PF treatment in BC cells.

Results

Notably, PF displayed an IC50 of 5 μM in human estrogen receptor-positive MCF7 cells and estrogen receptor-negative MDA-MB-321 cells treated for 24 hours. Also, 0.4 and 0.5 μM PF treatment in mammospheres and adherent BC cells, respectively, significantly inhibited the clonogenic potential. Besides, 20 hours of pre-treatment with 5 μM PF sensitized BC cells by 50% to doxorubicin. Our *in-silico* bioinformatic analysis revealed 23 out of 26 investigated BC cell lines were sensitive to an overnight treatment with 2.5 μM PF. Aware of the previously identified cationic amphiphilic structure of PF, we investigated the mitochondrial activity of BC cells treated with PF 5 μM over time. Notably, we observed a significant hyperpolarization at 3 hours and a significant depolarization in the mitochondrial membrane potential at 20 hours, while oxygen consumption rate results revealed a compromised mitochondrial activity as well as the inhibition of nearly all mitochondrial complexes. Thus, considering the tight link between mitochondrial dysfunction and endoplasmic reticulum stress, we also investigated its activation. Western blot analysis disclosed the phosphorylation of IRE1α and eif2α already after 3 hours of treatment with 5 μM PF along with an increase in ATF4 protein levels. Likewise, mRNA analysis unveiled the increase in DDTI3/CHOP and GRP78 expression. Lastly, proteomic analysis confirmed ER stress activation in MCF7 cells and inflammation and oxidative stress in MDA-MB-231 cells along with cell cycle block and apoptosis.

Conclusions

Our presented data suggest that PF could represent a potential candidate for drug repurposing for BC treatment. Further experiments are mandatory to investigate if mitochondrial damage and ER stress are directly involved in PF cytotoxicity. *In vivo* studies are needed to define a novel therapeutic strategy for BC patients, but, considering PF localization in the central nervous system, our findings represent a promising starting point for the management of the 12 % of breast cancer patients undergoing brain metastasis.

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Understanding the Link Between Long COVID and Mental Health Symptoms Through Targeted High-Resolution Metabolomics

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Keywords: Long COVID, Neurotransmitters, Mass spectrometry, psychological symptoms

Introduction

Long COVID, which is characterized by the persistence of residual symptoms following acute SARS-CoV-2 infection, has raised considerable concerns due to its range of enduring health issues. Among these, psychophysical sequelae have emerged as a prominent issue, impacting memory, concentration, and mood regulation. More specifically, anxiety, depression, and post-traumatic symptoms have been reported in up to 30% of patients even many months after the recovery. In the present work, we investigated whether neurotransmitters levels were altered in long COVID patients with mental health symptoms.

Materials and Methods

Plasma samples from 235 patients previously admitted to Novara University Hospital for COVID-19 pneumonia from March 1 to June 29, 2020, who consented to participate in a 12-month clinical assessment, and who have been subjected to post-traumatic stress (PTS) symptoms evaluation by the Impact of Event Scale (IES) were collected. Neurotransmitters and precursors levels were quantified in all the samples through a targeted ultra-high performance liquid chromatography high-resolution mass spectrometry approach. Statistical analysis was carried out to detect neurotransmitters and tryptophan metabolites that were significantly correlated to the psychological symptoms.

Results

The concentration of 35 circulating neurotransmitters and tryptophan metabolites were obtained. Interestingly, no alteration of serotonin and GABA were detected in patients with psychological long-term effects. On the other hand, one of the most striking result was the identification of lower levels of indole 3 lactic acid (ILA) in patients with depressive symptoms. In fact, essential tryptophan can be microbially metabolized into a series of indole derivatives that help maintain the intestinal homeostasis and that have neuroprotective effects. Recent research showed that depressed mice are characterized by lower ILA concentrations than healthy mice (1), while other studies found that some Bifidobacterium and Lactobacillus spp. produce indole-related compounds, such as ILA, which might be implicated in their antidepressive properties (2).
In addition, patients with post-traumatic stress symptoms (IES) had lower concentrations of 2-pyrrolidone, which is an indicator of shunted GABA metabolisms, and acetylcholine. Decreased acetylcholine levels in the hippocampus of stressed mice were already identified (3), while other authors found that acute stress facilitates long-lasting changes in cholinergic gene expression that acts to reduce available acetylcholine and depress cholinergic neurotransmission (4).

Conclusions

These preliminary findings indicate that utilizing targeted high-resolution mass spectrometry analysis of neurotransmitters and tryptophan metabolites could be considered a potential tool to investigate the relation between psychological symptoms associated with long COVID, but also with other mental diseases. Several preclinical reports confirmed the role of indole and its derivatives associated with behavioural alterations in rodent models of anxiety and depression, and our data suggested that ILA, together with the gut-brain axis, could have a relevant role in long COVID patients with mental health symptoms (5).

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ADME investigation on new PROteolysis TArgeting Chimeras (PROTACs) based on MultiComponent Reactions approach

S. Aprile,[1,2] I.P. Bhela,[1] S. Villani,[1] E. Del Grosso,[1,2] R.M.C. Di Martino[1], T. Pirali,[1]

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Summary: *Protein degraders are representing an emerging tool to target disease-related proteins. We investigated some ADME properties of new PROTAC entities prepared exploiting MCR platforms focusing mainly on metabolic stability and metabolite profiling, using untargeted LC-HRMS approach.*
Keywords: *untargeted analysis, small molecules Met.ID, ADME optimization*

Introduction

Protein degraders represent an emerging tool to target disease-related proteins. Among them, PROTACs have drawn particular attention. Being composed of a ligand "*warhead*" for the target protein of interest (POI) and a recruiting moiety *"anchor"* for the E3 ubiquitin ligase connected through a linker, PROTACs are able to trigger polyubiquitination of POI which in turn is degraded by the proteasome. Regarding POIs, bromodomain and extraterminal domain (BET) are a family of proteins associated to different inflammatory diseases and cancer and have emerged as a suitable model for testing the activity of newly developed protein degraders (e.g. dBET1), (*Figure 1*).

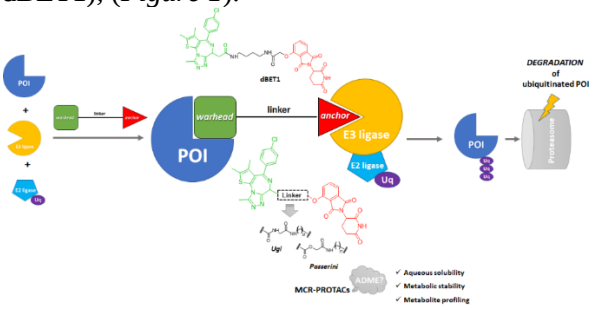

Fig.1. How PROTACs work.

From the medicinal chemistry viewpoint, in contrast with the limited room of manoeuvre on both warhead and anchor, the linker and the linkage points represent important sites of diversification to explore the chemical space of PROTACs.
Recently, as a proof of principle, a platform based on the Ugi and Passerini multicomponent reactions (MCRs) has been developed to assemble heterobifunctional efficient BET degraders bearing α-aminoacyl amides or α-acyloxy amides as a linkage [1].

Experimental

We evaluated the effect of the linkers introduced by MCRs in the synthetized MCR-PROTACs in terms of aqueous solubility and *in vitro* metabolic stability in liver microsomes to explore their drug-like properties. Besides, we studied their metabolic profile by HPLC analysis coupled to *Q-Exactive*™ *hybrid quadrupole-orbitrap* data depended ($ddMS^2$) acquisition followed by data analysis provided by the software *Compound Discoverer*™.

Results

Data analysis allowed to establish the chief metabolic pathways occurring for the assayed MRC-PROTACs. Overall (*Figure 2*), single or double hydroxylation both on the tienotriazoldiazepine (JQ1) and the thalidomide portions plus the *O*-dealkylation of the linker represented the major oxidative biotransformations occurring for the tested compounds. Talidomide hydrolysis, both at phthalimide and glutarimide levels, also contributed to the hepatic clearance of PROTACs. Additional metabolic routes were spotted for PROTACs synthetized using split-Ugi and Passerini MCRs.

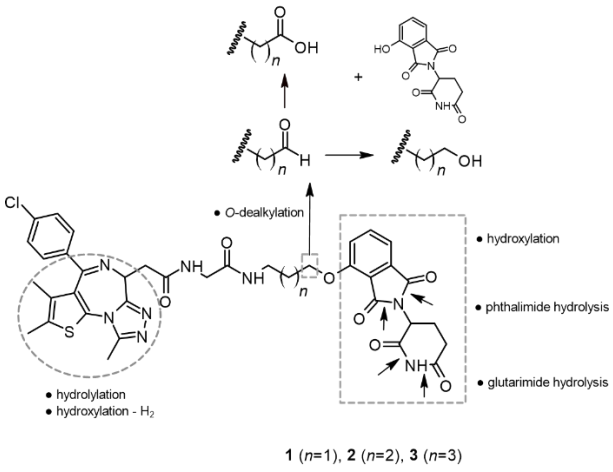

Fig.2. Example of metabolite profile for PROTACs synthetized by Ugi MCR.

Conclusions

In the present study we were interested in evaluating the behaviour, in terms of some drug-like properties, of the linkage region of new BRD4 degrading PROTACs synthetized exploiting an unprecedented MCR-based platform [1]. As regards of metabolic stability, even though the presence of different *"soft spot"* suffering from oxidation and hydrolysis, we have demonstrated the suitability of some linkage substructures accessible by MCRs to assembly metabolically stable compounds.

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Expanding the Power of Metabolomics in Human Health Using Bi-dimensional Gas Chromatography-Mass Spectrometry

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Keywords: *GCxGC-MS; complex matrices; biomedical research*

Introduction

Although two-dimensional gas-chromatography (GC×GC) –time-of-flight mass spectrometry (TOFMS) has been widely used to improve accurate molecule identification in the chemical industry, few studies were published in the biomedical field. Here, we presented and validated the performances of bidimensional GC×GC-TOFMS against that of GC-TOFMS for metabolomics analysis, which offers a great opportunity to study disease mechanisms, biomarkers, drug discovery and precision medicine.

Experimental

Clinical samples such as urine, blood, cells, feces, saliva, and tissues are rich sources of small molecules but are highly complex and very difficult to analyze. To overcome this issue, comprehensive two-dimensional GC-TOFMS has increasingly been applied to improve separation performance by using a series of two capillary GC columns with different stationary phases. GC×GC-TOFMS outperformed traditional GC-TOFMS in terms of separation performance and identification coverage. It offers the advantages of high sensitivity, robustness, and excellent chromatographic separation.

Results

Several case studies applied on different matrices will be presented including: (i) discovery of new diagnostic and prognostic biomarkers; (ii) prediction of the response to the therapy; (iii) investigation of metabolisms in vitro and ex-vivo; (iv) investigation of tumor microenvironment. This analytical comprehensive technique can also be able to deeply investigate the complex role of gut microbiota, investigating the modulation of bacteria-produced molecules. We demonstrated that GCxGC-TOFMS several metabolites found in saliva, stool, exhaled breath condensed, plasma/serum, tissues and cells were exclusively detected using GCxGC-TOFMS, and we showed the potential of the technique in the biomedical context.

Conclusions

Any advancement in technology is a challenge, and strong initial efforts are necessary. Here we demonstrated how a GCxGC approach is convenient in a research laboratory setting. Using a GCxGC system can result in time savings in sample preparation, instrument analysis, and non-target data assessment. A competitive advantage can be gained by having the capacity to simultaneously recognize targets and non-targets while analyzing a variety of complex sample. In conclusion, the comprehensive two-dimensional gas chromatography coupled to mass spectrometry can play an important role in the separation science and in the future of biomedical research.

Unraveling the Molecular Landscape of Perfluorooctane Sulfate Exposure During Pathogen-Associated Molecular Pattern Challenge in Peripheral Blood Mononuclear Cells

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Keywords: PBMC, PFOS, Proteomics

Introduction

An in increasing body of evidence suggests that per- and polyfluoroalkyl substances (PFAS) may adversely affect the immune system, potentially leading to immune dysregulation, decreased vaccination response and increased susceptibility to infections. In this study, we delve into the intricate molecular changes occurring within peripheral blood mononuclear cells when exposed to perfluorooctane sulfate (PFOS) during a lipopolysaccharide (LPS) challenge, a well-established pathogen-associated molecular pattern. PFOS is a persistent environmental contaminant with known health implications, and its interaction with immune cells remains largely undisclosed.

By employing advanced proteomic techniques, we aim to unravel the complex molecular landscape, shedding light on the cellular responses and potential biomarkers associated with PFOS exposure during an inflammatory challenge with LPS as a representative PAMP.

Methods

To this end, PBMCs were pre-treated ex vivo with increasing amounts (3-600 microM) of PFOS for 24 hours, followed by LPS challenge after an additional 12 hours. Cells were harvested, lysed, and proteins were extracted and digested before the mass spectrometry analysis. Proteomic analysis was carried out on nano chromatography coupled to high resolution mass spectrometry (Orbitrap Exploris 480) using a 40-minute gradient with a Data Independent Analysis approach. Data were analyzed using DIA-NN software.

Results

More than 4200 proteins were quantified across all the samples. The results showed that PFOS strongly impacted the PBMC proteome both at

Metabolomic Profiling of Type-2 Diabetes Plasma Utilizing GC- and GCxGC-TOFMS Workflows

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Keywords: *T2D, biomarkers, GC and GCxGC-TOFMS*

Introduction

Over 400 million adults are currently living with diabetes worldwide and this number is expected to rise to 700 million by the year 2045. Type 2 diabetes mellitus (T2D) accounts for approximately 90% of total cases. Diabetes is a major cause of blindness, kidney failure, heart attacks, strokes, and lower-limb amputations. There is a crucial need for the discovery of T2D biomarkers for early medical intervention and to avoid complications associated with this severe chronic disease. A comprehensive analytical approach is ideal for the investigation of human physiology and therefore the discovery of markers for T2D diagnosis. The objective of this study was to develop an untargeted analytical methodology for the identification of candidate T2D biomarkers in humans.

Methodology

The analytical approach included automated derivatization to minimize sample preparation errors and extend metabolite identification capabilities to include non-polar, polar, and ionic compounds. T2D and control samples were extracted, and then derivatized in two steps: 1) Methoximation and 2) silylation. Sample data was collected using EI- and CI-TOFMS technology to not only increase the total number of compounds identified but more importantly to improve confidence in their characterization. Data were processed using untargeted peak find, and compounds were characterized through retention index filtering, mass error calculations, and spectral database searches. Differentiation of disease and control samples was accomplished using novel statistical processing software based on Fisher ratios.

Results and Discussion

The profiling methodology resulted in a significant increase (> 2x) in the total number of metabolites identified. The compounds included acids, diacids, amino acids, fatty acids, bases, monosaccharides, disaccharides, sugar phosphates, sterols, nucleosides, and others. Downstream statistical processing facilitated the identification of several T2D candidate makers including uronic acids and unbranched amino acids.

Large-scale phosphoproteomics reveals divergent kinase-activity profiles in different groups of mouse medulloblastoma.

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***Summary**: In our experiment, we aim at studying how the phosphoproteome changes in different preclinical mouse models for the different subgroups of medulloblastoma to define key regulators of the acquisition of highly malignant features.*
***Keywords**: Medulloblastoma, phosphoproteomics.*

Introduction

Medulloblastoma (MB) is the most malignant brain tumor in children. Despite the significant clinical progresses of the last decades, the long-term outlook and survival of patients remain very poor. To date, four main groups of MB have been recognized (wingless (WNT)-activated, sonic hedgehog (SHH)-activated, group 3 and group 4) and have been further subclassified into 7 to 12 different subtypes. While the four main MB groups have been extensively characterized at both genome and transcriptome level, the proteome remain to be comprehensively explored. In particular, phosphoproteomics can provide insight on active oncogenic signalling that could help to elucidate specifically activated and therapeutically actionable pathways. Quantitative phosphoproteomics analysis has already described distinct post-translational profiles in specific groups of human MB. In our experiment, we aim at studying how the phosphoproteome changes in different preclinical mouse models for the different subgroups of MB in order to define key regulators of the acquisition of highly malignant features.

Experimental procedure

We characterized the phosphoproteome of three subtypes of MB murine models: Ptch1+/-p53+/+ MBs (henceforth referred to as HetWT, representing a subgroup of SHH-activated group); Ptch1+/-p53-/- MBs (indicated as HetNULL, mimicking the high-risk subgroup of SHH-activated group) and Myc amplified/p53 mutant MBs (referred as MYC, corresponding to the group 3 of MB). Due to the limited amount of tissue available, we developed a sample preparation protocol based on two phospho-enrichment strategies (MOAC, Metal oxide affinity-chromatography and IMAC, Immobilized metal affinity-chromatography) that enabled us to use only 1 mg of proteins without any need of labeling.

Results and conclusions

By this protocol, we were able to identify and quantify 4570 phosphosites in the three studied groups. In combination with a recently-developed R package (called PhosR), we were also able to predict the activity of the kinases responsible for the phosphosites dysregulated in the different groups of MB. Our study describes distinctive protein phosphorylation landscapes of different MB groups that could be helpful in clinical decision-making and treatment.

Identification of aging and Frailty biomarkers in Disease and aging cohort studies

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Rationale for the Study:

This study is part of a research project with the overarching goal of identifying individual or combined biomarkers for multiple purposes, including: i) gaining insights into the biological processes linked to aging; ii) early detection of accelerated aging in individuals; iii) assessing the risk of chronic disease onset and functional decline; iv) monitoring the effectiveness of interventions aimed at mitigating biological aging. The primary aim of this work is to establish a proof of concept for identifying molecular signatures in blood that can be applied for predicting frailty and, more broadly, forecasting aging trajectories. Additionally, this research serves as a pilot study for characterizing samples obtained from participants in the Novara Cohort Study (NCS), a comprehensive longitudinal study investigating the determinants of aging and longevity in the Novara area population.

Study Design:

This study encompassed three distinct cohorts: young participants (N=35; <35 years), participants from the NCS (N=56), and patients who experienced acute kidney injury (AKI; N=29), serving as a model of accelerated aging. Plasma samples were examined for potential biochemical markers associated with frailty. The Frailty Index based on laboratory tests (FI-Lab) was computed, and the distribution of frailty markers among the three groups was analyzed. Comparative analyses were conducted to explore the relationship between the identified significant markers, age, and health status. Additionally, a metabolomics analysis was conducted on a subset of AKI patients and young individuals, establishing standard operating procedures (SOPs) and collecting preliminary data for future applications in aging studies.

Results:

Appropriate SOPs were established for determining frailty biomarkers in samples from these cohorts. A panel of 24 biochemical markers was selected and measured across these cohorts. The FI-Lab effectively stratified AKI patients when compared to the healthy young group. Within the NCS group, despite the relatively small sample size, interleukin-6, albumin, and TSH exhibited statistical significance in distinguishing healthy individuals aged >55 from the young group. When comparing the unhealthy NCS group (≥ 2 diseases) with the young group, C-reactive protein, albumin, and triglycerides displayed significant differences. Untargeted MS-GS and NMR metabolomics analyses of AKI and young control group serum revealed significant disparities in metabolite profiles, indicating disruptions in metabolic processes associated with AKI and implicating these metabolites in accelerated aging.

Conclusion:

These preliminary findings support the utilization of laboratory markers of frailty for defining the clinical syndrome of frailty. This approach is not limited to the elderly population but can also be applied to patients with various diseases. By incorporating these markers, we can predict adverse outcomes, such as reduced responses to disease-targeted therapies, a higher risk of surgical complications, and delayed recovery from illness. Future research should focus on further exploring frailty markers and their association with clinical frailty assessments in age-related diseases. By investigating the potential clinical applications of the FI-Lab, including screening, diagnosis, prognosis, and treatment decisions, we can enhance healthcare practices for older individuals. Additionally, the exploration of metabolite profiles may unveil new predictive biomarkers and intervention targets for aging and aging-related diseases.

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Building a Food-Intake Biomarkers Database for Mass Spectrometry based Nutrimetabolomics Profiling: A Novel Workflow to Enhanced Nutritional Assessment

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Summary: *Aging is a natural biological event that has some negative aspects, including the development of frailty. The crucial role of diet in frailty onset is recognized. In this scenario Food-Intake Biomarkers, molecules derived from food intake and metabolisms, may be able to provide objective information on the humans' nutritional status.*

Keywords: *Food-intake biomarkers, Mass spectrometry, Nutrimetabolomics*

Introduction

Frailty is a geriatric syndrome characterized by a decrease in the physiological reserves, leading to higher vulnerability to stressors. Food-intake biomarkers (FIBs) cast light on food metabolism, to establish the association between dietary habits and the risk of developing frailty [1]. The aim of this work is the construction of an *in-house* FIBs database to evaluate the interaction between diet, FIBs, and the development of frailty.

Methods

FIBs database was built by curating and integrating literature-known molecules with established associations to specific foods [1,2] also including phase I and II metabolites.

Monoisotopic masses of each FIBs were queried against three different databases: the Human Metabolome Database (HMDB), FoodDB and Phenol Explorer, obtaining more than 1500 possible FIBs. The database was cleaned and reduced by considering only FIBs with validated MS/MS spectra.

Results

Our *in-house developed* database included 976 FIBs. The database structure was designed to accommodate monoisotopic mass and the most common adducts (+Na, +K), the HMDB ID, the molecular classes, and food species. Table 1 shows the food categories and the number of analytes in each categories that are included in the database. To test the performance of the database we extracted and analysed metabolites from plasma samples of 130 elderly subjects using flow-injection analysis-mass spectrometry (FIA-MS). EASY-FIA [3] was used to pre-process and annotate the obtained data using the *in-house* FIBs database and MS/MS fragmentation patterns were used to confirm the FIBs identity.

Food categories	
Alcoholic beverages (N=39)	Heat-treated foods (N=11)
Artificial Sweeteners (N=12)	Legumes (N=59)
Cereals (N=125)	Meat (N=7)
Citrus fruits (N=38)	Olive oil (N=10)
Coffee, tea (N=21)	Plant foods (329)
Curcuma (N=15)	Preservatives (N=17)
Fish&Seafood (N=4)	Tuber or cereals (N=16)
Fruit (N=74)	Ultra-processed food (N=21)
Fruit&Vegetables (N=128)	Vegetables (N=50)

Table 1: Food categories included into the FIBs database

Conclusions

We developed an *in-house* FIBs database ready-to-use on our free software EASY-FIA, for FIA-MS pre-processing and annotation.

We tested our database on a small population without dietary intervention (130 elderly subjects, 65 Fit and 65 Frail) confirming the presence of 83 plasma FIBs that capture a picture of individual dietary habits.

The FIBs approach must be validated in further Italian Longitudinal cohorts to assess the role of diet in the development of frailty.

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Metabolomic profiling in differential diagnosis for Parkinson's disease and atypical parkinsonisms

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Summary: *MS based untargeted metabolomics was applied to support early differential diagnosis of patients with Parkinson disease (PD), Multiple System Atrophy (MSA) and Progressive Supranuclear Palsy (PSP). Metabolomic profile of 96 plasma samples was performed using micro liquid chromatography coupled with high-resolution mass spectrometry (microLC-HRMS).*
Keywords: *untargeted metabolomics, microLC-HRMS, Parkinson's disease*

Introduction and aim

Parkinson's disease (PD) is the second most frequent progressive neurodegenerative disease. PD development depends on an intricate combination of genetic and environmental factors [1]. The main motor symptoms of PD (tremor, rigidity, bradykinesia) can also be observed in complex neurodegenerative pathologies, namely Multiple System Atrophy (MSA) and Progressive Supranuclear Palsy (PSP), grouped as Atypical Parkinsonisms (APs).

AP prognosis, evolution, and therapeutic treatment are deeply different from PD's. Currently, PD and AP (i.e. MSA and PSP) diagnoses rely on clinical evidence, due to the lack of specific biochemical and neuroradiological markers, so an improper diagnosis could lead to ineffective therapeutic interventions. This study aimed to identify specific biomarkers for PD and AP classification by untargeted metabolomic analysis using microLC-HRMS (Fig. 1).

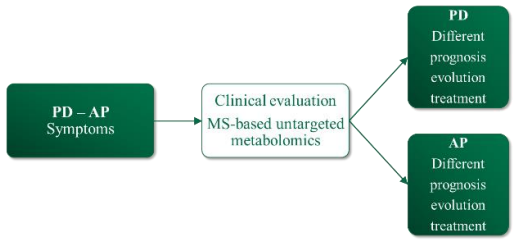

Figure 1. *Rationale of the study.*

Metabolomics workflow using microLC-HRMS

- Plasma samples (51 PD, 19 MSA, 26 PSP) were prepared via protein precipitation by adding cold acetonitrile/0,1% formic acid (v/v) to a final plasma/solvent ratio of 1:4 (v/v) [2].
- The extract was analysed in Data Independent mode (SWATH®-MS) [3], by microLC - TripleTOF 6600+ (Sciex, Concord, ON, Canada) equipped with a Luna Omega Polar C18 100x1mm 1,6 µm.
- Metabolites were identified by SCIEX OS Software using Accurate Mass Metabolite HR-MSMS Spectral Library 2.0 (Sciex, Concord, ON, Canada).
- Data were processed using MarkerView™ Software (Sciex, Concord, ON, Canada) for simultaneous feature finding, alignment, and statistical analysis to highlight metabolites of interest [4].
- Confidence in identification of metabolites of interest were further confirmed reprocessing the MS and MS/MS data with SCIEX OS (Sciex, Concord, ON, Canada).

Results and discussion

Hundreds of metabolites were confidently identified with a single run, generating a peculiar metabolic profile for each biological sample. A smaller group of patients (20 MP, 4 MSA, 6 PSP over a total of 96) was selected for a preliminary multivariate statistical analysis to identify disease-specific metabolic alterations (Fig. 2). Metabolites involved in tyrosine, tryptophan, purines, and fatty

acids metabolism result to be discriminant for PD, MSA, and PSP (p-value < 0.05, Fig. 2). Further targeted analyses on some of the discriminant metabolites may help to identify a biologically relevant subset of readily measurable markers for PD and AP differential diagnosis.

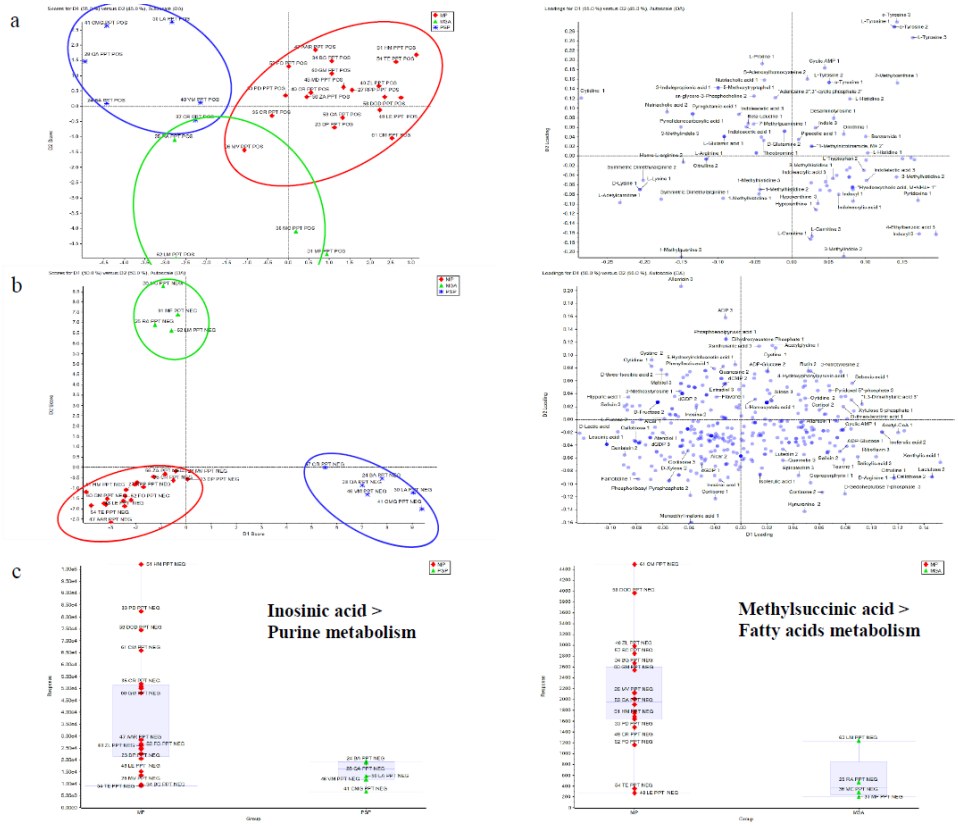

***Figure 2**. PCA-DA (supervised) scores plot and loadings plot for sample analysed in positive (a) and negative polarity (b). Representative metabolites (p-value < 0,05) response for each sample group for negative polarity (c). Red squares for MP and green triangles for PSP or MSA. Box plots depict the range between the 25th and 75th percentiles of the data. The horizontal line marks the median value; capped bars indicate min and max values*

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Metabolomics and lipidomics in cardiometabolic risk and disease: a focus on Resveratrol effects on sphingolipid metabolism and signaling

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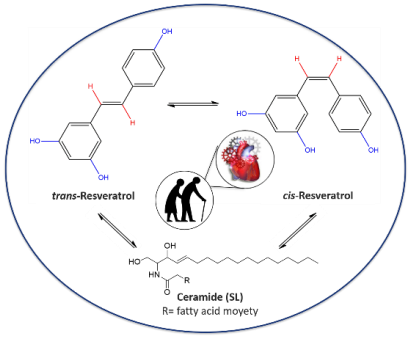
Summary: *Metabolomics and lipidomics are largely used to discover novel therapeutic approaches. Natural products play a pivotal role in human health: the recent discovery of Resveratrol, natural polyphenol, has received attention due to its beneficial roles on human health. This work aimed to discuss the effects of RSV and sphingolipids in human health.*
Keywords: *resveratrol; ceramides; cardiometabolic risk.*

Background

In the era of precision medicine, ''omics'' techniques allow a universal detection and identification of early biomarkers of alterations. Metabolomics and lipidomics are largely used to study cellular pathways affecting cardiometabolic (CM) health and to discover novel related therapeutic approaches. Natural products play a pivotal role in maintenance of human health: the recent discovery of Resveratrol (RSV), a natural polyphenol produced by plants, has received great attention during the past few years due to its beneficial roles, through sphingolipid modulation, on human longevity and cardiometabolic health. This work aimed to discuss the available data on the effects of RSV on sphingolipids regulation in CM disease and human health.

Resveratrol: chemical and physiological properties

Polyphenolic molecule that can be found in different plant species, especially red grapes.
Two isomers of RSV are available, both rather unstable, thus the clinical applications of RSV are limited, also because of its biotransformation, in the liver, in polar derivatives, excreted by kidneys.
However, anti-inflammatory, anti-carcinogenic, and cardioprotective effects are linked to this natural product [1].



Resveratrol effects on sphingolipid metabolism and cardiometabolic risk

Ceramide (Cer), Sphingosine (SPH) and Sphingosine-1-Phosphate (S1P) are among the most functionally active SL. Higher levels of Cer and S1P are associated with the pathogenesis of several age-related diseases and CM diseases. Effects of RSV administration have been considered, but results are controversial and difficult to address:
-in RSV-treated hepatocytes from old rats, lipidomic and metabolomic analysis showed a significant reduction in ceramide content in plasma membranes of senescent;
-in cancer cells, RSV contributed to Cer accumulation and induced apoptosis [1].

However, concentration- and time-dependent mechanisms are involved in RSV effects.

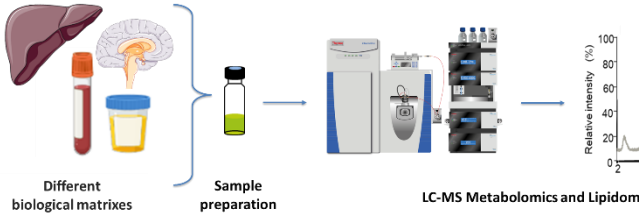
Indeed:

-RSV concentrations < 50 µM increase SL and Cer, without inducing apoptosis;

-higher RSV concentrations (> 50µM) increase pro-apoptotic Cer and reduce pro-survival S1P

levels, suppressing cell growth and inducing apoptosis.

Analytical workflow for RSV and SL detection in biological samples [2]



Conclusions and Outlook

RSV exhibits a number of pleiotropic actions, generally beneficial in various pathophysiological conditions. One key mechanism through which RSV exerts its effects is represented by the modulation of sphingolipid metabolism and signaling. Thus, RSV represents an exciting adjuvant tool to be further studied as a possibility for the development of complex therapeutic strategies that may be used to prevent and address CM risk and several pathological conditions.

However, RSV-related effects are often dose- and time-dependent, and its main limit is represented by its low bioavailability. Moreover, many other aspects of this molecules are still unknown and unclear. The study of novel drug delivery systems may help to overcome this difficulty and better understand its complexity, together with its wide range of actions, especially with regard to its differential actions on sphingolipids.

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Oxidative stress-induced senescence in visceral adipose stem cells: a promising model to investigate visceral adipose tissue aging

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Summary: *In the last few decades, the increase in life expectancy has been correlated with an increased incidence of numerous age-related diseases. The role of visceral adipose tissue (VAT) senescence in metabolic disorders development has been extensively demonstrated. However, the molecular mechanisms involved in VAT senescence are still broadly unknown.*
Keywords: *Aging, Cellular senescence, Adipose tissue*

Introduction

In the last decades, it has been observed an increase in the expectancy of life, especially in developing countries; however, this extended lifespan remains associated with the development of many age-related diseases [1,2]. At molecular level, aging is characterized by an impaired fitness of cells, and this is accompanied by the establishment of a chronic status of inflammation [3] Senescence is an aged status of cells characterized by an irreversible cell cycle arrest, molecular alterations, and displaying of a senescence-associated secretory phenotype (SASP) [4]. The accumulation of senescent cells promotes inflammation and tissue dysfunction, leading to the onset and progression of several diseases [5,6,7] Adipose tissue (AT), and in particular the visceral one (VAT), is considered a very active and highly dynamic tissue that is able to secrete a broad type of molecules, such as cytokines, chemokines, interleukins, growth factors, miRNAs, and extracellular vesicles (EVs), exerting its activity at both paracrine and endocrine levels [8]. However, during aging, an accumulation of senescent cells in VAT is observed, mainly due to reactive oxygen species (ROS) accumulation [9], leading to impaired tissue function, with hypertrophic adipocyte and dysfunctional staminal and immune compartments. Dysregulated VAT promotes and sustains tissue inflammation, which contributes to the development of Type II Diabetes (T2D), Metabolic Syndrome (MetS), and other diseases [10,11].

Although VAT senescence has been described as a crucial risk factor for the development of many pathologies, the molecular alterations occurring still remain poorly described, since VAT samples' retrieval results challenging. For these reasons, after the isolation of visceral adipose stem cells (vASC) from VAT samples, we investigated several molecular and phenotypic alterations occurring during senescence, by comparing an oxidative stress-induced senescence, achieved by using hydrogen peroxide (H_2O_2), with a replicative one.

Experimental

For defining senescence induction, some wide-spread senescence markers were evaluated, such as impaired proliferation, the expression of senescence-associated β-Galactosidase (SA-β-Gal), DNA damage induction, the expression of cell cycle inhibitors such as p53, p21, and $p16^{INK4A}$, and SASP production. Moreover, the functional status of senescent vASCs was assessed by evaluating differentiation capacity, autophagy, mitochondrial function, and secretome content.

Results

vASCs were isolated from human visceral adipose tissue biopsies from collaborating patients (n=2). To characterize the isolation of vASCs, morphology, differentiation capacity, and stemness marker expression were evaluated.

Consequently, we decided to establish an oxidative stress-induced model of senescence by treating cells with hydrogen peroxide (H_2O_2); in addition, replicative senescence was also achieved by extensive culturing (P>20). A dose-response assay with scalar doses of H_2O_2 (50-600µM) defined 100 and 200µM as the most suitable doses for investigating senescence since they were able to impair vASCs viability without inducing cell death after 48h from treatment. Moreover, both replicative and induced senescent cells showed a reduction in long-term proliferation (7 days)

compared to untreated control cells (P<8). To evaluate senescence establishment, the senescence-associated β Galactosidase (SA- β-Gal) expression was evaluated. After 48h from H_2O_2 treatment, an increase in the % of cells expressing SA-β-Gal was observed. Furthermore, this increase in β-Gal expression was accompanied by several morphological alterations, with senescent cells displaying flattened morphology and higher cell and nuclei size.

Independently from the senescence inducer, the impaired proliferation observed in senescent cells usually occurs as a consequence of double-strand break (DSB) and DNA damage response (DDR) induction. For this reason, we evaluated, through immunofluorescence, the nuclear foci accumulation of pH2A.X and 53BP1, respectively markers of DNA damage and repair. Moreover, PML and p-ATM foci accumulation was also evaluated since both play a pivotal role in DDR during senescence. An accumulation of nuclear foci of all markers tested was observed in both oxidative stress-induced and replicative senescent cells, confirming as induction of DNA damage in senescent vASCs. In addition, Western Blot analysis showed an increase in the protein expression of several markers downstream DDR involved in cell cycle inhibition, such as p53, p21, and p16.

vASCs undergoing senescence displayed several alterations in their phenotype. Senescent vASCs showed impaired differentiation capability and altered mitochondrial status, characterized by mitochondrial membrane depolarization. Furthermore, an induction of autophagy was confirmed, since it was observed an increase in several autophagic markers expression, such as LC3B, BECN1, and p62. Lastly, inflammation establishment was confirmed by Nf-kB nuclear localization and increased gene expression, evaluated through qRT-PCR, of several inflammatory factors, including IL-6 and TNFα. To deeply investigate the paracrine role of senescent vASCs, we performed an untargeted proteomic analysis on cells supernatant. Among the 345 proteins identified, 139 resulted differentially expressed in oxidative stress-induced senescence, while 167 proteins were differently expressed in replicative senescent cells compared to control ($p<0.05$). Furthermore, 62 deregulated proteins were expressed in both replicative and induced senescent cells. In particular, 38 proteins were upregulated (FC>1.25) and 24 proteins were downregulated (FC<0.75). Among the upregulated proteins we identified several enzymes involved in glycolytic and glucose metabolism pathways such as phosphoglycerate kinase (PGK1), lactate dehydrogenase (LDH), and phosphoglycerate mutase 1 (PGAM1). Instead, within the downregulated proteins several collagen-related proteins such as COL15A1, COL6A3, and COL6A2 were identified, suggesting a role of vASCs senescence in the modification of the extracellular matrix (ECM) structure.

Conclusions

Senescence is a relevant molecular condition that cells undergo during aging. Although VAT senescence plays a major role in the development of many pathological conditions, its characterization is still lacking. Identifying the molecular alterations occurring in VAT during aging could lead to the identification of new targetable pathways to prevent, slow down, or revert senescence. Furthermore, characterizing the secretome of senescent vASCs could underline the mechanisms through which the VAT is able to modify the microenvironment, improving the understating of the role of VAT senescence in the establishment of age-associated diseases.

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Optimization of Experimental Parameters in Data-Independent Acquisition for Serum Analysis

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Summary: *The present study was designed to optimize DIA settings to increase the number of protein identifications in non-depleted serum samples.*
Keywords: *non-depleted serum, data-independent acquisition, deep proteome coverage*

Introduction

Blood plasma and serum are the most common specimens in the clinical setting because they are easy to obtain with minimal invasiveness [1]. Among their various constituents, proteins have received increasing attention for the discovery of disease diagnosis and therapeutic monitoring [1]. However, plasma/serum proteome analysis is extremely challenging due to its high complexity and dynamic range of protein abundances and additional procedures, such as extensive prefractionation and depletion of high abundance proteins, are required [2]. Data-independent acquisition (DIA) strategy is a powerful tool that can provide a deep proteome coverage of biological samples with few missing values and good quantitative precision and accuracy [3]. The aim of this study was the optimization of DIA experimental parameters to improve protein identification in a single liquid chromatography-mass spectrometry (LC-MS/MS) analysis of serum samples, without immunodepletion or peptide fractionation.

Experimental procedure

Blood samples were obtained from healthy volunteers. The digestion of serum proteins was performed by Protein Aggregation Capture protocol using MagReSyn® Hydroxyl magnetic beads (Resyn Biosciences). Samples (300 ng) were analyzed by an LC-MS/MS system consisting of an EASY1000 chromatograph and a Q-Exactive mass spectrometer (Thermo Fisher) [1]. Peptides were separated by binary gradient using mobile phase A - 0.1% formic acid (FA), 2% acetonitrile (ACN) and mobile phase B - 0.1% FA and 80% of ACN - with a flow equal to 230 nL/minute. Peptide elution was obtained with the following gradient: from 3% to 25% B in 90 minutes, from 25% to 40% B in 30 minutes and from 40% to 100% of B in 8 minutes [3]. DIA methods combining different numbers of isolation windows, mass range, resolution, automatic gain control (AGC) target, collision energy (CE), and charge state have been constructed to evaluate the number of identifications. DIA data were processed by Spectronaut® software (16 version).

Results and Discussion

The large dynamic range of protein concentration in serum makes the identification of low abundant potential protein biomarkers difficult. Great efforts have been devoted to implement strategies that can increase the number of detectable serum proteins. Recently, DIA-based workflows made it possible to detect a high number of proteins by single-shot LC-MS/MS analysis. Within this line of research, the purpose of this work was to improve the performance of DIA analysis on serum samples by testing several DIA settings. Since the width and the number of the precursor mass segments which isolate the precursors for MS/MS fragmentation in DIA directly influence the protein depth [2], the performance of four methods employing different numbers of segments (18, 26, 50, 70) was evaluated. The DIA raw files were processed on Spectronaut® software and searched against the spectral library generated from publicly available data (4831 peptides, 415 protein groups). The 26-isolation windows method (26W) yielded the most protein identifications compared to the other settings (Fig.1).

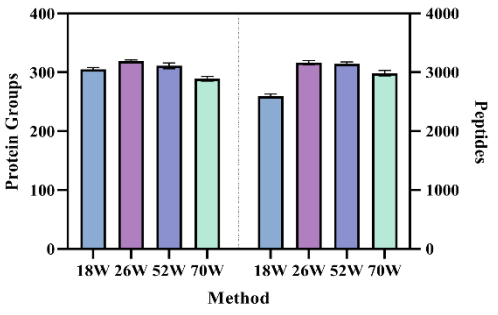

Fig1. Performance comparison between four DIA methods. Plotted are the average of identified proteins (left) and peptides (right) over quadruplicate injections.

Subsequently, to increase the protein coverage, a range of other variables were explored. First, as a narrow mass range and higher resolution enhance identifications [2], DIA method with 20 variable windows (20W-A) and the following settings was performed: scan range, 370-900 *m/z*, MS^1/MS^2 resolution, 70000. The analysis revealed a better performance of 20W-A than 26W method (Fig.2).

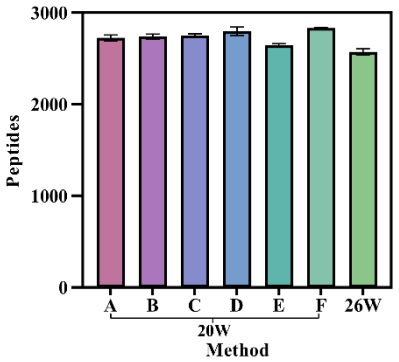

Fig2. Performance comparison of *seven different DIA methods. Plotted are the average of identified peptides over three replicates.*

Then, AGC target, CE, and charge state were evaluated (Table 1). As showed in Fig.2, the 20W-D and 20W-F methods supported a slight improvement of peptide identifications compared to the other settings assessed.

Table 1. Experimental parameters tested of different 20W - DIA methods.

Method	MS/MS Settings		
	AGC* target	CE**	Charge State
A	5 x 10^5	25	2
B	1 x 10^6	25	2
C	5 x 10^5	25	3
D	1 x 10^6	25	3
E	5 x 10^5	28	2
F	1 x 10^6	28	3

*AGC: automatic gain control; **CE: Collision Energy.

The information stored in the spectral library defines the peptide identity in a targeted DIA study [2]. Therefore, four different public source libraries (A to D) were investigated, to assess the relationship between protein coverage and size of spectral library. Specifically, library A included 15 fractions of plasma non-depleted resulting in 415 protein groups and 4831 peptides [2]; library B consisted of 24 fractions of plasma depleted leading to 2002 protein groups and 13935 peptides [4]; library C was generated with DDA analysis of fractionated serum depleted and non-depleted (215 fractions) comprising 5592 protein groups and 13935 peptides [5], and for library D, 1096 DDA raw file collected from 16 tissue biopsies and plasma samples were used, resulting in 10524 protein groups and 149066 peptides [2]. The 20W-D DIA method was acquired in triplicate and the data analysis was performed on Spectronaut®.
Results showed that a comprehensive sample-specific spectral library revealed a high number of identified proteins (Fig3).

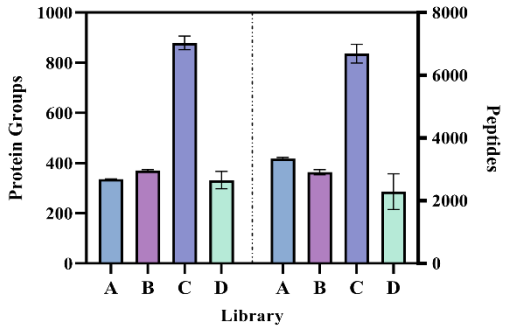

Fig3. Performance evaluation of DIA targeted analysis with four public spectral libraries (A to D). The average of identified protein groups (left) and peptides (right) over triplicate analysis were calculated.

Conclusions

A DIA-MS method for non-depleted serum samples to increase protein identification was developed. Data showed that a target analysis based on comprehensive sample-specific spectral libraries is the key to improve the proteome coverage. Additional studies are in progress to investigate the potential of DIA approach on reproducibility and quantitative precision.

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UnravelinG the proteomic signature of prostate cancer evolution from normaL to ADvanced castration-resIstAnT phenOtypes: a diffeRentIAL proteomic approach (GLADIATORIAL)

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***Summary**: To understand the evolution from healthy condition to advanced tumor in PCa, specific markers are needed. For this reason, we propose a proteomic approach to identify new possible markers, which will be useful to create a qualitative and quantitative screening panel.*
***Keywords**: Proteomic analysis, Prostate Cancer (PCa), Mass Spectrometry*

Introduction

Prostate cancer (PCa) is a heterogeneous and multifactorial disease. Today in Italy it remains the fourth cause of death (5.9% mortality, 2020). The development of the primary tumor starts from some forms of benign prostatic hypertrophy (BPH) which can evolve into an advanced phase and then in metastasis.

The main treatment for androgen-sensitive primary tumors and metastatic PCa is androgen deprivation therapy (ADT). However, cancer cells can become resistant to treatment and the disease progresses to castration-resistant PCa (CRPC) [1]. Prostate specific antigen (PSA) is the only circulating marker currently used in clinical practice to monitor resistance to mPCa therapy [2,3]. The goal of our research is the identification of new markers able to dynamically follow the evolution of phenotypic changes under

different conditions: from healthy control to the advanced stage of the tumor. This result will allow us to have a hypothetical temporal map for diagnostic and prognostic purposes, but also to act with a more appropriate therapeutic strategy for the event. The characterization of a group of proteins that may be down- or over-expressed will be the key points of the qualitative and quantitative screening panel.

Experimental

We selected nine cell lines mimicking a prostate cancer at different stages. A differential proteomics analysis has been performed by means of a bottom up approach. The Tandem mass spectrometry analysis was performed on an Ultimate 3000 nano chromatography system, equipped with a PepMap RSLC18 column (75µm x 50cm) (Thermo). After separation, the flow was directly sent to an Easyspray source connected to an Exactive Plus Orbitrap Q mass spectrometer (both Thermo). The software Xcalibur (version 4.1) (Thermo) has been used for operating the UHPLC/HR-MS. Raw data were analyzed with the Proteome Discoverer software 2.4.1.15 (Thermo). The identification of differentially expressed proteins (DEPs), was performed using the protein-wise linear models combined with the empirical Bayes statistics and Limma package.9. Finally, the DEPs were defined by the cut-off values (p <0 .05, fold change (FCR) greater than 1 or minor than −1).

Results

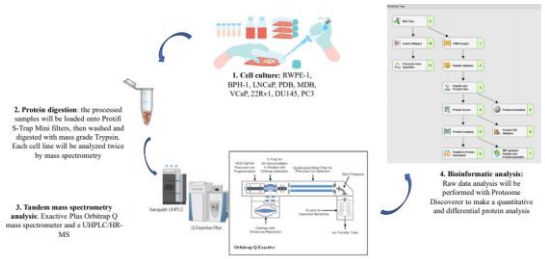

***Fig1. Workflow Analysis:** the analysis workflow starting from the biological samples. Tandem mass spectrometry analysis has been applied for peptide identification. Raw data will be analyzed with the Proteome Discoverer software. The protein identification will be performed with OriginPro 2021b software.*

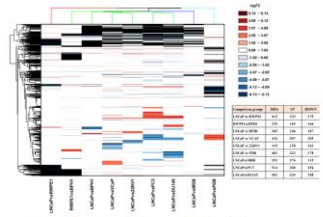

***Fig.2 DEPs total**: in the figure and in the table the proteins differentially expressed in the single cell lines with higher values, UP, or lower, DOWN, with respect to the LNCaP reference line are shown. Only proteins with Fold Change Ratio (FCR) ± 1 and p-Value < 0.05 were considered*

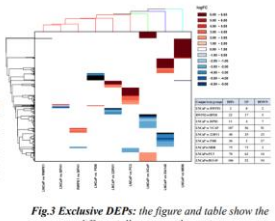

***Fig.3 Exclusive DEPs:** the figure and table show the differentially expressed proteins in the single cell lines with values higher, Up, or lower, Down, compared to the LNCaP reference line. Only proteins with Fold Change Ratio (FCR) ± 1 and p-Value 0.05 which are unique to a comparison group were considered.*

Conclusions

The construction of a qualitative and quantitative screening panel can be useful to identify the progression of prostate cancer starting from a healthy condition until malignances state.

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Lipidomic investigation in plasma of Parkinson's disease, Multiple system atrophy and progressive supranuclear Palsy diagnosed patients

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***Summary**: An MS based untargeted lipidomics investigarion was carried out to verify differences in circulating plasma lipids in patients diagnosed with Parkinson disease (PD), Multiple System Atrophy (MSA) and Progressive Supranuclear Palsy (PSP). lipidomic profile of 52 plasma samples was performed using micro liquid chromatography coupled with high-resolution mass spectrometry (microLC-HRMS).*
***Keywords**: Lipidomics, Parkinson's disease, Multiple system Atrophy, Progressive Supranuclear Palsy*

Introduction and aim

Parkinson's disease (PD), ranking as the second most prevalent global neurodegenerative disorder, presents a multifaceted clinical profile. [1]. It is primarily distinguished by motor symptoms, including characteristic tremors, bradykinesia, and akinesia, which significantly impact patients' daily lives. However, PD extends beyond these motor impairments, encompassing a diverse array of non-motor manifestations. These
encompass cognitive decline, mood disorders like depression and anxiety, sleep disturbances, and autonomic dysfunction, rendering the clinical landscape of PD intricate and challenging to navigate. The diagnostic process for PD remains a clinical endeavour, reliant on the expertise of healthcare professionals to recognize
specific motor symptoms. This lack of definitive diagnostic tests poses significant hurdles, especially during the early stages of the disease, where subtle presentations may lead to misdiagnoses, delaying crucial
interventions. Furthermore, distinguishing PD from related neurodegenerative disorders, such as Multiple System Atrophy (MSA) and Progressive Supranuclear Palsy (PSP), poses its own set of complexities. These conditions often exhibit overlapping clinical symptoms, making accurate differentiation challenging. This investigation aimed to identify lipid biomarkers for PD, MSA and PSP differential diagnosis by untargeted and semitargeted lipidomic analysis using LC-HRMS

Lipidomic workflow using LC-HRMS

• Plasma samples (27 PD, 10 MSA, 15 PSP) were prepared via a modified Folch extraction [2] by adding cold MeOH and MTBE (1:1, v/v) containing 5 ppm solution of different lipids standards (SPLASH Lipidomix).
• The extracts were analysed in Data Dependent mode in both positive and negative ionization mode on a Agilent Infinity 1290 provided by Agilent Technologies (Santa Clara, CA, USA) equipped with ACQUITY UPLC BEH C18 column and coupled with a Agilent 6545 Mass Spectrometer (quadrupole time-of-flight, Q-TOF).
• The stability and reproducibility of the system were checked with quality control (QC) samples prepared with equal aliquots of pooled plasma samples.
• The data analysis followed two complementary approaches: fully untargeted and semitargeted. For the untargeted approach feature extraction was carried out through two different software: Profinder (by Agilent Technologies) and MS DIAL 5 [3], the semitargeted approach used a locally curated database complete with retention time and accurate mass.
• Data curation was based on blank removal, signal threshold and CV >35% removal (referred to QC samples). After data curation more than 650 features were extracted for untargeted approach and more than 250 lipid entities were extracted for semitargeted approach.
• Further statistical analysis, multivariate and univariate were processed with SIMCA-P and an in-house built MATLAB script respectively. When required Probabilistic quotient (PQN) or Quality Control Samples and Support Vector Regression (QC-SVRC) based Total Useful Signal (TUS) method were employed [4,5].

Results and discussion

The lipidomics investigation provided various important insights both on the differences in results on the different investigation approaches followed as well as for clinically relevant information. Regarding the former, while comparing the results of untargeted and semitarget approaches, it became clear that analytical
signal drift had occurred. This issue was observable in the semitarget approach, potentially causing the issue to go undetected in the untargeted approach due to the intricate nature of data interpretation. (Figure 1).

Regarding clinical data, while initially we aimed to identify significant differences among the three disease lipid profiles, we encountered only limited evidence of such distinctions. Consequently, we refined our analysis to compare just two groups—Parkinson's and non-Parkinson's (NP). Employing this approach, we found 40 lipids that exhibited significant differences between these two groups and achieved an acceptable separation through PLS-DA. Further analyses and affected pathway investigation could provide more useful and discriminant information rendering a biochemical differential diagnosis finally feasible.

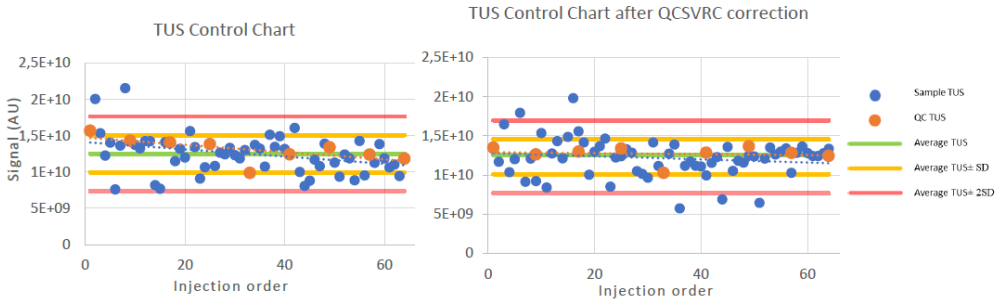

Figure 1 TUS Control chart before and after QCSVRC normalization

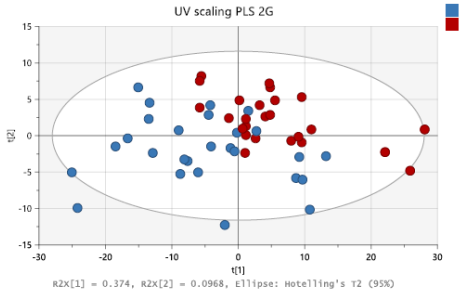

Figure 2 Partial least squares discriminant analysis (PLS-DA) between PD and NP

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Proteomic analysis of pancreatic ductal adenocarcinoma stem cells and investigation of PHGDH as a potential therapeutic target

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***Summary**: Pancreatic cancer stem cells (PCSCs) are responsible for the aggressiveness of PDAC. To detect potential therapeutic targets, we performed a proteomics analysis identifying 440 PCSC-modulated proteins. Among these, PHGDH was further studied to verify its potential as a therapeutic target by using the inhibitor BI-4916.*

***Keywords**: Pancreatic cancer stem cells (PCSCs), PHGDH, SWATH LC-MS/MS*

Introduction

Pancreatic ductal adenocarcinoma (PDAC) represents the majority (90%) of pancreatic cancers, it is the 7th most deadly malignancy in the world, and it is forecast to become the second-leading cause of cancer-related mortality by 2030 [1]. PDAC includes a small (< 1%) sub-population of undifferentiated quiescent cells named pancreatic cancer stem cells (PCSCs), whose involvement in treatment resistance and cancer recurrence has been well established [2,3]. Therefore, effectively targeting PCSCs would be a breakthrough in cancer therapy. Considering the strategic importance of the proteomics approach, this work aimed to analyze the proteomic changes in PCSCs to investigate peculiar pathways underlying PCSC biology and, at the same time, identify new potential targets. From the obtained proteomic data, the phosphoglycerate dehydrogenase (PHGDH), the first enzyme in the de novo serine biosynthetic pathway, resulted upregulated in PCSCs. Since it has been demonstrated that PHGDH overexpression is associated with higher malignancy in PDAC [4], we analyzed the effect of the specific PHGDH inhibitor, BI-4916 [5], on PANC-1 cells viability.

Experimental

Proteomics analysis has been performed on PANC-1 parental cells (P) and cancer stem cells cultured for 2, 4, and 8 weeks in the specific medium. Proteins were digested and subjected to LC-MS/MS by using a micro-LC system (Eksigent Technologies, Dublin, USA) interfaced with a 5600+ TripleTOF MS (AB SCIEX, Concord, Canada). In detail, first of all, a DDA analysis to generate the SWATH-MS spectral library was performed, followed by cyclic DIA analysis, based on a 25-Da window, to quantify the samples. After that, a KEGG enrichment pathway analysis was performed to find dysregulated pathways. Since from our analysis emerged a significant modulation of PHGDH, we assessed the efficacy of different dosages (from 2.5 to 25 μM) of BI- 4916 on cells treated twice a week for one week.

Results

The SWATH LC-MS/MS analysis identified a total of 440 modulated proteins. Following a KEGG enrichment pathway analysis, we found a regulation of oxidative phosphorylation, glyoxylate and dicarboxylate metabolism, valine, leucine, and isoleucine degradation, Krebs cycle, and cysteine and methionine metabolism in PCSCs compared to P cells. P cells have been treated every 3 days for 7 days with BI-4916 at different dosages and their viability is significantly decreased starting from a concentration of 10 μM of the inhibitor. Based on this result, the next step will be to treat PCSCs with a concentration of the inhibitor that impairs PHGDH activity and not cell viability, with the aim to study the effects of BI-4916 treatment on stemness, including the analysis of spheroids formation and stem marker expression levels.

Conclusions

In conclusion, the obtained proteomic results led us to identify a potential molecular target, such as PHGDH, in order to further investigate its effect on the aggressiveness of pancreatic cancer stem cells.

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Investigating Sarcoma Metabolism Through Untargeted GCxGC-MS Metabolomics of Cancer and Healthy Tissues

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Keywords: Metabolomics, Sarcoma, GCxGC-MS, Cancer tissue

Introduction

Sarcoma cancers are a rare and heterogenous group of neoplasms of mesenchymal origin, with a prevalence of less than 1% of all adult malignancies [1]. They comprise more than 170 different subtypes and arise in the 87% of cases from soft tissue and in the 13% from bone [2]. Sarcomas display abnormal metabolic activities but knowledge about their metabolome is quite sparse. As a matter of fact, differently from other tumors, metabolic alterations in sarcoma are difficult to be correlated with specific gene mutations and thus, are still to be characterized [3]. A more in-depth understanding of sarcoma metabolisms may allow the identification of novel potential diagnostic and therapeutic targets to treat patients. In this work, untargeted metabolomics was performed on a large cohort of sarcoma patients by comparing tumoral tissue with adjacent healthy tissue to identify differently regulated molecules and modulated pathways. The metabolomic results were then correlated with clinical and immunological data providing and an extensive mapping of sarcomas metabolism and a complete view of their biochemistry.

Experimental design

Soft tissue sarcoma samples were obtained from surgical excision and provided by the Humanitas Hospital (Rozzano, Italy). The biopsies included 7 different histotypes: liposarcoma (12 patients), leiomyosarcoma (6 patients), undifferentiated pleomorphic sarcoma (3 patients), synovial sarcoma (2 patients), rhabdomyosarcoma (1 patient), epitheliod sarcoma (1 patient), spindle cells sarcoma (1 patient). From each patient, 2 tumor tissues and 2 adjacent healthy tissues were obtained, for a total of 104 samples.

For the metabolomic analysis, after tissue homogenization, short chain fatty acids (SCFAs) and small molecules were extracted from sarcoma tissue using a liquid-liquid extraction with methyl tert-butyl ether (MTBE) and analyzed with bi-dimensional gas chromatography coupled to mass spectrometry (GCxGC-TOFMS). The remaining aqueous phase was then subjected to a second extraction and followed by derivatization with methoximation and silylation prior to the GCxGC-TOFMS analysis. The data obtained from this comprehensive untargeted metabolomic approach were then used to compare sarcoma tissues against normal tissues through a paired statistical analysis.

Results

The untargeted metabolomics analysis allowed the quantification of 158 SCFAs and small molecules showing the presence of a metabolomic signature specific for each group of samples. Among the identified SCFAs, acetate, butyrate, and lactate emerged as up-regulated in sarcoma tissues. These molecules are used as energy sources by cells and can favor tumor development. The upregulation of butyrate found in sarcoma samples suggests its role as oncometabolite and supports the emerging role of the intra-tumoral microbiome in tumor initiation.

The univariate statistical analysis of metabolites identified 212 modulated molecules out of the 415 polar metabolites identified. Among the most significantly regulated metabolites several aminoacids, organic acids like lactic acid and sugars such as glucose were found. In order to investigate the biological and biochemical significance of our results, we performed a bioinformatic analysis which showed a strong involvement of piruvate metabolism, butanoate metabolism, TCA cycle and more in general aminoacids metabolism. Of particular interest, as highlighted by the enrichment pathway analysis, is the involvement of fatty acid biosynthesis and the Warburg effect, a well-known hallmark of cancer. As a matter of fact, lactic acid was identified as up-regulated molecule in sarcoma tissues versus healthy tissues, supporting the Warburg effect.

Conclusions

The comparison between sarcoma and healthy tissues showed several modulations of molecules able to define a different metabolomic signature for each of the two groups of samples. To deepen the significance of regulated metabolites, a bioinformatic analysis was performed showing the involvement of pyruvate metabolism, TCA cycle and Warburg effect. This study showed that metabolomics is a valid approach to investigate metabolic alterations in sarcoma tissues, to map the biochemical pathways exploited by this type of tumor and that it might help to identify potential diagnostic and therapeutic targets.

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A Multi-Proteomic Approach to Unravel New Players in Metastatic Melanoma Progression

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Summary*: Specific and advanced proteomic approaches are employed to investigate protein shedding and structural perturbations within the microenvironment of metastatic melanoma, when modulating the activity of the membrane protease BACE2.*
Keywords*: microenvironment, melanoma, proteomics.*

Introduction.

Recently, amyloid-like fibrils have been observed in metastatic melanoma [1]. Although amyloids are generally harmful, fibrils of melanocyte-specific protein (PMEL) represent the physiological scaffold for melanin deposition into melanosomes. Specific proteases generate the amyloidogenic peptides. Among those, the beta-secretase BACE2 was shown to be necessary for PMEL fibrils formation. Different works have reported its elevated expression and activity in tumors, such as melanoma. This seems also to be associated with a poorer prognosis [2]. We previously found that, in metastatic melanoma, BACE2-dependent fibrils promote cancer cell growth and invasion via mechanotransduction [1]. To understand whether amylodogenesis is deregulated in high-BACE2 metastatic melanoma and to identify BACE2 targets we used different proteomics approaches.

Experimental. N-Tails degradomics [3] was applied to study the secretome of two high-BACE2 metastatic melanoma cell lines (WM266.4 and IGR-37). This proteomics technique allows to identify the cleavage sites of proteases. Differential proteomic analysis of dimethyl labeled peptides coupled to a Perl software named MaxQuant Advanced N-termini Interpreter (MANTI) has been used to elaborate and interpretate N-Tails data [4].

To gain a comprehensive understanding of BACE2 role, Limited Proteolysis coupled to Mass Spectrometry (LiP-MS) approach [5] was applied to the same cell lines. In LiP-MS, native proteins are subjected to limited proteolysis of the most exposed residues. In presence of structural modifications, cleavage site accessibility changes. The differential peptide pattern generated demonstrates which portions of proteins are involved in structural changes. The R package protti [6] was used to analyze, identify and visualize conformotypic peptides. Moreover, a R script was applied for relative abundance correction, based on method established in the MSstatsPTM package [7].

Results.

The secretome of two metastatic melanoma cell lines has been analyzed in triplicate, in normal conditions and in presence of a highly specific BACE2 inhibitor. N-Tails analysis showed more labeled N-Termini in BACE2 active samples, suggesting increased protein processing when protease is active compared to when it is inhibited (Fig.1A, B). Combining results from both cell lines, 100 N-Termini (belonging to 56 proteins) were identified (Fig.1C). As BACE2 is a transmembrane protease, it is more likely to cleave membrane associated proteins. Among those, we most identified cell adhesion molecules or cell junctions related proteins (Fig.1D). Interestingly, cleavage of molecules necessary for adhesion to the ECM and other cells can potentially facilitate cell migration and invasion, especially in the context of metastasis. Preliminary data by label free quantitative MS analysis of the secretome from the same cell lines already revealed that BACE2 inhibition caused a decreased secretion of ECM components. In addition, conformational changes were studied by LiP-MS. This analysis revealed a high number of structurally regulated proteins. Among the 317 proteins in common between the two cell lines there is also PMEL. The PMEL barcodes showed a structurally altered region in the initial part of the CAF domain, which is known to constitute the amyloidogenic core (Fig.1F). This alteration appears to be conserved in both cell lines. In addition to PMEL, there are 6 others known amyloidogenic proteins undergoing structural changes upon BACE 2 inhibition. Further, 4 transmembrane proteins cleaved in BACE2 active cells were confirmed perturbated by LiP-MS experiment (Fig.1E).

Conclusions.

N-Tails experiments identified several cell adhesion proteins as BACE2 targets and LiP-MS confirmed a structural rearrangement in some of them. Moreover, according to LiP-MS, a PMEL peptide within the amyloidogenic core is involved in structural rearrangement in two cell lines analyzed, potentially acting as seed and influencing fibrillogenesis of other proteins. Further studies will investigate whether processing of BACE2 targets sustains a pro-metastatic phenotype or the creation of a fibrillar network, that hinders drug diffusion and induces hypoxia and stress.

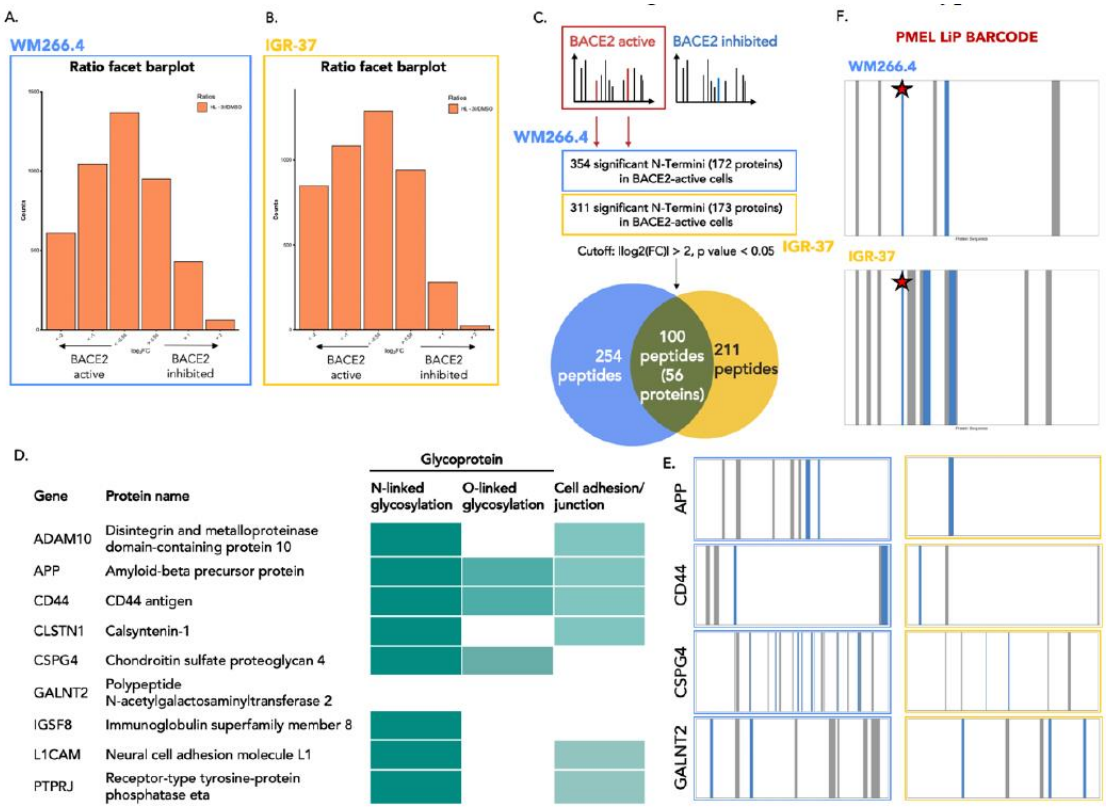

Figure 1. *A) Barplots of the high/low labelling intensity ratios at different log2FC in WM266.4 and B) IGR-37. C) Number of significant and common N-Termini identified in WM266.4 and IGR-37. D) Transmembrane proteins cleaved in high BACE2 active cells. E) LiP barcodes for WM266.4 (blue) and IGR-37 (yellow) of the proteins reported in D). Barcode represents the protein from the N- to the C-terminus. Grey bars are the identified peptides, blue bars are the significant LiP peptides. E) LiP barcodes of PMEL in WM266.4 and IGR-37. Red stars highlight the amyloidogenic peptide identified in both cell lines.*

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Proteomic profiling of breast cancer lines treated with extracellular vesicles isolated from adipocytes

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Summary: *The early diagnosis, risk stratification and monitoring of therapeutic response are of crucial importance in breast cancer. The development of sensitive mass spectrometry methods could be of great help in identifying biomarkers that may have prognostic, predictive and diagnostic value and which can be used as potential therapeutic targets.*
Keywords: *Mass Spectrometry, Breast cancer, Biomarker Discovery*

Introduction

Several epidemiological evidences showed that overweight and obesity, characterized by an excess of adipose tissue, increase the risk of developing breast cancer negatively affecting prognosis, progression and therapeutic response. The mechanisms behind the relationship obesity/breast cancer are multifactorial and among those, in recent years, extracellular vesicles are emerging as key players in intracellular communication. In particular, extracellular vesicles are nanoscale lipid-bilayer enclosed vesicles released from a variety of tissues and cells known to be involved in the pathogenesis of cancer. Differential proteomic analysis could provide valuable information on the phenotypic changes induced by extracellular vesicles on cancer cells in order to identify new potential biomarkers or therapeutic targets [2].In this work we explored the proteomic profile of breast cancer ER+ treated with extracellular vesicles isolated from adipocytes.

Materials and Methods

The MCF7 ER+ cells were treated for 48h with extracellular vesicles extracted from adipocytes. The cell lysates were digested according to the FASP protocol [3], subsequently the obtained peptide mixture was purified by SCX (Strong Cationic Exchange) [4] e analysed by LC-MS/MS with Data-Independent Acquisition (DIA) mode. Raw files were loaded into Spectronaut software and statistical analysis was performed with the software Perseus (Fig.1).

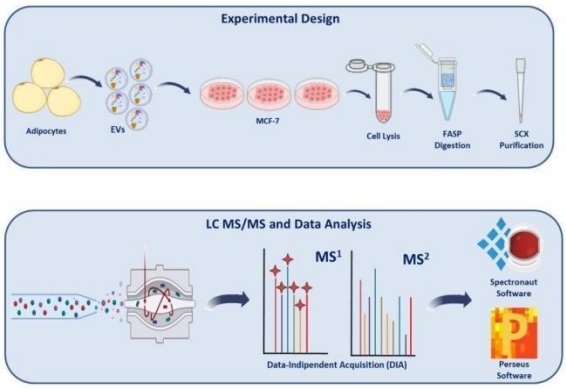

Fig. 1 Experimental design of proteomic analysis

Results and Discussion

The LC-MS/MS analysis allowed to identify 4587 total proteins obtaining both a good proteome coverage and the identification of low abundance proteins. The comparative analysis performed with the Perseus software allowed to identify 818 differentially expressed proteins (DEPs), of which 442 upregulated and 376 downregulated in treated samples compared to untreated samples. Functional gene enrichment analysis revealed that the identified proteins are mainly involved in energy pathways and RNA metabolism, and mainly perform redox activities and are structural constituents of ribosomes. Among the statistically significant proteins, attention focused on four up-regulated proteins in the treated sample: SURF4 (protein with the highest fold change), MRPL20, UBA52, BTF3 (identified as core genes). To investigate the clinical significance of the selected proteins was evaluated, by Kaplan-Meier analysis,

the relationship between the levels of expression of the relative genes and overall survival (OS), Relapse-Free Survival (RFS), Post progression survival (PPS), Distant Metastasis-Free Survival (DMFS) in breast cancer patients. The results obtained from this analysis highlighted the potential role of these proteins as negative prognostic factors in breast cancer.

Conclusions

The obtained data demonstrates how the EVs isolated from murine adipocytes can influence the proteomic profile of the cells MCF-7 ER+ breast cancer, specifically affecting mitochondrial and energy metabolism pathways. Furthermore, the clinical value of some identified deregulated proteins (UBA52, BTF3, MRLP20 and SURF4) was evaluated in order to determine their potential utility as biomarkers or therapeutic targets.

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Anomaly detection of EV-related protein expression in doped bioactive glasses

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***Summary**: Wet lab experiments focused on extracellular vesicle protein content from mesenchymal stem cells cultured on bioactive glass discs doped with different metal ions. Data analysis employed an innovative pipeline exploiting "anomaly detection" machine learning techniques to highlight a restricted set of remarkably changing proteins linked to metal ion doping of glass-ceramic biomaterials.*
***Keywords**: bioactive glasses, extracellular vesicles, anomaly detection*

Introduction

Mesenchymal stem cells are a type of adult stem cell that can uniquely differentiate into multiple cell types, including bone, cartilage, fat, and muscle cells. This differentiation potential makes MSCs promising for tissue repair, regeneration, and cell-based therapies. In addition, MSCs exhibit immunomodulatory and anti-inflammatory properties: they can secrete various bioactive molecules that influence the immune response. MSCs exert their therapeutic effects by releasing extracellular vesicles (EVs); these small membrane-bound vesicles contain bioactive molecules, including proteins, lipids, and nucleic acids (such as microRNAs). The therapeutic potential of MSC-EVs includes promoting tissue repair and regeneration by delivering growth factors and signaling molecules to damaged tissues [1]. The current investigation detected the EV protein content of MSC cells cultured on top of different bioactive glasses through mass spectrometry. In general, during proteomics analysis, statistics based on p-values are commonly used to assess the significance of differences in protein expression levels between experimental groups. Through statistics, the goal is to demonstrate whether any observed differences are statistically significant or could have occurred due to random chance. The p-value is the probability of observing a test statistic as extreme as, or more extreme than, the one calculated from the actual data, assuming the hypothesis of no real difference between the groups being compared. In other words, it informs how likely the observed differences in protein expression could have occurred due to random chance alone. In the case of small p-values, statistics suggest that the observed differences are unlikely to be due to chance and are more likely due to a natural effect. A small p-value does not prove a significant difference between the groups being compared; it simply indicates that the observed data is unlikely under the assumption of the null hypothesis (no difference in protein expression between the control and treatment groups). To propose an alternative to the classic statistical analysis, anomaly detection techniques have been evaluated to identify a limited set of extremely changing EV-related proteins. This novel analysis sequence was attempted on proteomic data derived from cells cultured on biomaterials. Anomaly detection involves identifying data points that deviate significantly from the norm or expected behavior. Proteins with an aberrant behavior inconsistent with most of the data might be potential indicators of biological phenomena occurring between experimental conditions [2].

Experimental methods

The experimental setup consisted of the culture of 5000 cells of each of the three donors independently, on top of glass-ceramic biomaterials doped with metal ions (and the respective control conditions), at 37oC, 5%CO2, for 7 days. At the endpoint, the supernatants were collected for EVs isolation through ultracentrifugation at 100000 x g for 2 hours at 4oC. The pellet enriched in EVs was then resuspended in 500uL of Phosphate Buffer Saline (PBS 1X), and the EVs protein content was evaluated through mass spectrometry. The initial data was from three donors and contained the mass spectrum peak area from the samples of each participant. The following experimental conditions were tested:

• cell cultures on bioactive glasses (i.e., control or abbreviated as “ctrl”).
• cell cultures on bioactive glasses doped with silver, copper, or tellurium metal ions (i.e.,

doped).
• cell culture on “Plastic”, a baseline condition without the presence of biomaterials (i.e., plastic)
The laboratory experiments aimed at establishing protein content modifications: those occurring between the doped glasses and the “plastic” condition could be a consequence of the presence of the bioactive glass [3]. Furthermore, protein expression altered between the doped glasses and the respective control glass should be due to the metal ion doping. The bioinformatics pipeline started from the raw mass spectrum peak area and employed the following analysis steps:
• The log-transformed values were clustered (using OPTICS), and the values of the same cluster among conditions were taken to ensure the analysis of similar data representing the same biological phenomena.
• The selected cluster values from the three donors were labeled as outliers (potential "anomaly" or extreme variation) applying Isolation Forest [4].
• Of all proteins marked as outliers computed the Mahalanobis distance to identify abnormal variations in the EV-related protein expression (an example in Fig. 1).

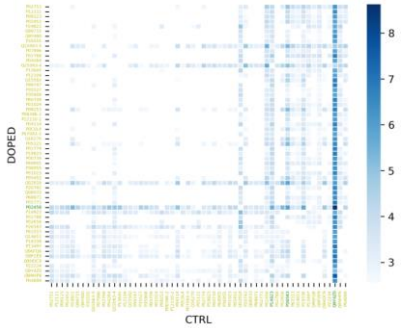

Fig 1. *Distances of Silver doped versus Control*

Results

Highly varying proteins were collected in the form of Venn diagrams for the Control versus Doped bioactive glasses (Fig. 2a, arranged as a word cloud) and Plastic versus Doped condition (Fig. 2b). The Venn diagrams were selected to illustrate the intersections between sets of data, to visually summarize the dry-lab outcomes. For example, in Fig. 2b, the protein P22413 (linked to bone mineralization) was identified in all experimental conditions as highly activated. On the other hand, Fig. 2a portrayed a peculiar set of highly changing proteins for each bioactive glass.

Fig. 2a. *Extremely changing EV-related proteins in the Control versus Doped conditions (no identified proteins in common between experimental conditions)*

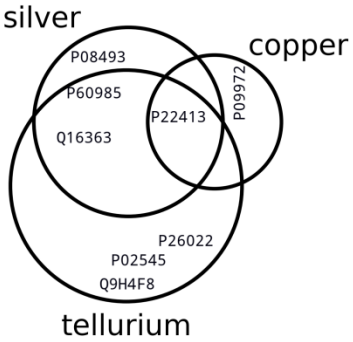

Fig. 2b. *Extremely changing EV-related proteins in the Plastic versus Doped conditions (a few identified proteins in common between experimental conditions)*

Conclusions

An analysis pipeline to identify extremely changing EV proteins has been evaluated, aiming at characterizing processes underlying the role of silver, copper, and tellurium doping in bioactive glasses. The present results suggest that doped bioactive glasses, compared to non-doped ones, activate a set of peculiar EV-related proteins greatly modified and specific for each metal. On the other hand, the presence of the biomaterials activates a different set of proteins, some of them in common between experimental conditions.

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Lipid and metabolic profile of circulating plasma compared in Parkinson's disease patients undergoing several types of therapy

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Summary: *Integration of multi-omics analysis is the best approach to study neurological disease. In this way, we designed a lipid and metabolic profile of subgroups of Parkinson's disease patients to better understand the pathway involved in disease pathophysiology and drug response.*
Keywords: *Lipidomics, Parkinson's disease, deep brain stimulation*

Introduction

Parkinson's disease (PD) is a complex neurodegenerative disorder characterized by the impairment of several cellular pathways including autophagy, mitochondrial metabolism, oxidative stress, and vesicular trafficking, leading to the formation of alpha-synuclein aggregates [1]. Currently, only symptomatic therapies are available and long-term medical treatments are associated with a range of adverse effects. It is well demonstrated that circulating lipids and metabolites may mirror the alteration of metabolic pathways at cellular level, even at neuronal one [2]. In this study, we explored whether long-term treatment of PD patients induced alteration on metabolism.

Experimental

We performed a multi-omics approach to profile lipidomic in plasma as well as we used a multiplex ELISA assay to measure circulating marker associated to lipids mobilization and catabolism (leptin, ghrelin, FABP3 and FABP7), inflammation (TNF-alpha, Gro-alpha), neurotrophy (BDNF) and neurodegeneration (GFAP). The study cohort included three groups of PD patients, undergoing different therapies, and healthy subjects matched for age, sex, and BMI. Specifically, the PD cohort included patients treated for at least 10 years with L-Dopa standard therapy (n=15), or with deep brain stimulation (DBS) and L-Dopa maintenance therapy (n=16), and patients taking only dopamine agonists (n=15) and controls (n=15).

Results

We found an extensive dysregulation of lipids in PD patients compared to healthy subjects. This effect was more evident when comparing patients treated with DBS *vs* L-Dopa. Moreover, the PD patients not treated with L-Dopa showed a decrease of proteins involved in active fatty acid metabolism compared with control group, PD patients treated with L-Dopa and DBS patients. The DBS group also showed an increase of peripheral inflammation marker.

Conclusions

These preliminary data are encouraging to extent this analysis to a larger cohort of patients to discover novel biomarkers for the monitoring of the therapy and to identify drug targets that will improve long-term treatments for PD.

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Data-Independent Acquisition of EPS-Urine: A Predictive Model for Prostate Cancer

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Summary: *The number of prostate cancer (PCa) cases is increasing every year. Because PSA-based screening shows low sensitivity and specificity, the detection of new biomarkers is very important. This work illustrates a predictive model capable of classifying PCa better than PSA alone.*
Keywords: *Prostate cancer, Data-independent acquisition, Machine Learning*

Introduction

Prostate cancer (PCa) is the most common diagnosed neoplasia in the male population[1]. To date, the diagnosis of PCa is based on the digital rectal exam (DRE) and serum prostate specific antigen (PSA) assay. Considering the low specificity and sensitivity of PSA, the discovery of new biomarkers is needed to improve the diagnosis of PCa.

In this work, expressed prostatic secretion (EPS)-urine were analysed by data-independent acquisition (DIA) thereby detecting even low-abundance proteins. Overall, 2615 proteins were quantified. After combining the panel of quantified proteins with the clinical parameters, the obtained matrix was analysed by Machine Learning algorithms. This approach has allowed to select the components of our predictive model: two clinical variables and two proteins. Altogether, our predictive model correctly classified PCa patients better than PSA alone.

Experimental

Sample processing

EPS-urine samples from patients with PCa (n=73) and from patients with benign prostatic hyperplasia (BPH, n=60) were collected.

EPS-urinary proteins were digested by Filter-Aided Sample Preparation (FASP) on Microcon-10 Centrifugal Filter Units (Millipore) as indicated in our previous work[2]. The obtained peptide mixture was purified by two purification steps: (i) strong cation exchange (SCX) to remove detergent and (ii) C18 StageTip to discard the salts (Figure 1).

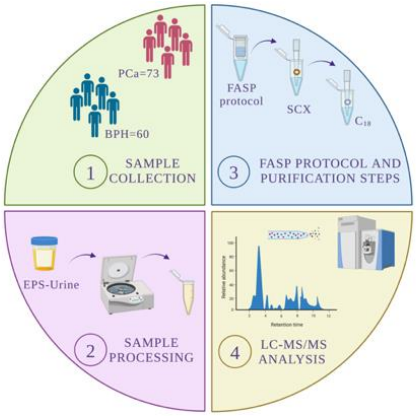

Figure 1. *Steps of our workflow*

Spectral library

Peptides from a pool of protein digests from 22 PCa patients (11µg) were divided in 10 fractions by high- pH reversed phase C18 fractionation and analysed by LC-MS/MS.

LC-MS/MS analysis

Proteomic profile was analyzed by nLC (Easy nL-1000, Thermo Scientific, Bremen, Germany) coupled to a Q-Exactive mass spectrometer (Thermo Scientific, Bremen,Germany). In details, peptides were separated by binary gradient using mobile phase A (0.1% formic acid (FA), 2% ACN) and mobile phase B (0.1% FA and 80% of ACN) with a flow equal to 230 nL/minute. Peptide elution was obtained with the following gradient: from 3% B to 25% B in 90 minutes, from 25% B to 40% B in 30 minutes and from 40% B to 100% B in 8 minutes.

The 10 fractions were analyzed by data dependent acquisition (DDA) using a top-12 method: full scan range 350-1800 m/z, resolution of 70.000,

precursor ion isolation window equal to 1.6 m/z and MS/MS resolution of 35.000.
EPS-urine samples were analysed by mass spectrometer operating in DIA mode using the following settings: full scan range 350-1200 m/z at the resolution of 17.500 and DIA scans with the resolution of 35.000. In details, our DIA method enclosed in total 26 consequent windows of which 20 with isolation width equal to 20 m/z, 5 windows with width of 50 m/z and 1 with width of 200 m/z.

Data processing
DIA analysis was performed by Spectronaut (version 13.0) and the list of quantified proteins was merged with the clinical parameters (total PSA, ratio PSA free/total PSA and prostatic gland size). The matrix enriched with the clinical parameters was processed by features selection (Pearson correlation coefficient, Chi-square test, recursive feature elimination). Following the feature selection Machine Learning algorithms such as random forest, support vector machine, decision tree, K-nearest neighbors were trained.

Results and Discussion

Overall, in our dataset 2615 proteins were identified and quantified thus obtaining a high urinary proteome coverage.
The matrix composed by quantified proteins and clinical parameters was analysed by algorithms of features indicating as best candidates four variables: sempahorin-7A (sema7A), secreted protein acid and rich in cysteine (SPARC), FT ratio, and prostate gland size[3]. To build a predictive model, our dataset was split into two groups: the training set (90% of samples) and validation set (10% of samples). Moreover, the training set was further divided into two sets, one to train the ML models in classifying the samples, and the other to evaluate the prediction power of the predictive model according to a 10-fold cross-validation strategy. On the testing set the model was evaluated across the following parameters: AUC, accuracy, specificity, sensitivity. Finally, the accuracy of the model was evaluated through the validation set (10% of dataset, corresponding to 12 samples).
In the validation set our predictive model classified correctly the 83% of patients (PCa/BPH) demonstrating a discrimination power higher than that of PSA alone (tree out five of the ML) also accompanied by higher specificity.
The two proteins belonging to the model, SEMA7A and SPARC, showed lower levels in the EPS-urine of patients with PCa than those with BPH. In details, SEMA7A is a protein involved in both physiological and neoplastic events such as cell invasion and migration[4]. Since SEMA7A showed higher levels in EPS-urine of patients with BPH, it could be hypothesized that it plays a protective role in the development of cancer. In the PCa context, one work reports that an important increase of SEMA7A levels is associated with overexpression of ERG in mouse prostate organoids[5].
SPARC is a glycoprotein involved in the tissue remodelling, but its role in PCa is controversial and correlated to its localization (stromal or tumour). The up-regulation of SPARC in tumour is correlated with the epithelial-mesenchymal transition (EMT)[6] and metastasis while stromal-derived SPARC hinders PCa cell growth[7].

Conclusions

DIA analysis allowed to identify low abundance proteins thus leading to a deep proteome coverage of EPS-urine sample. In the light of the interesting results, it is necessary to enrol a higher number of patients in the validation cohort to evaluate the discrimination power of our predictive model.

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Neurotoxic Effects of Legacy and Alternative PFAS on Earthworm Neural Systems

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Summary: *This study investigated the neurotoxic effects of new generation Perfluoroalkyl ether substances (PFAS) on earthworm neural systems. They found that PFAS like GenX and PFOA impacted neurotransmitter levels and acetylcholinesterase activity. These findings suggest potential harm to the human nervous system and the need for more research on PFAS neurotoxicity.*
Keywords: *acetylcholine, gaba, neurotransmitters*

Introduction

New generation Per and polyfluoroalkyl substances (PFAS) often contain one or more ether groups in their chemical structure to reduce persistence in the environment and potential bioaccumulation in biota. However, very little is known about their toxicological properties, which is why they are also referred to as "poor data" PFAS.This study investigated the neurotoxic effects of PFAS on earthworm neural systems, with potential implications for the human nervous system (NCS). Utilizing four PFAS congeners (PFOA, GenX, PF4MOBA, PF3MOPrA) across a concentration range of 0.6-229 microM, we assessed the impact on the main inhibitory and excitatory neural pathways in *Eisenia fetida* muscles which are known to express genuine gaba-a and nicotinic acetylcholine receptors. Place formulas along with the text left aligned.

Experimental

Neurotransmitter levels were measured in SN10 tissue extracts by means of High-resolution LC-MS/MS analysis in a Thermo Scientific Vanquish UHPLC system coupled to a Q-Exactive mass spectrometer interfaced with a heated electrospray ionization source and a hybrid quadrupole-orbitrap mass analyzer. The mass spectral data for quantitation were acquired in PRM mode according to an inclusion list. Up to 50 different neurotransmitters could be evaluated.

Results

Our results showed that GenX, as well as PFOA affected the level of gaba-a at both low and high concentration levels (respectively 0.6 and 229 microM) and in same time increased those of acetylcholine. GenX also affected the level of serotonin and PFOA those of its precursor tryptophan. PF4MOBA showed similar results, but they were not always significant at all concentrations. Acetylcholinesterase activity (AChE) was consistent with results found for the neurotransmitter being significantly modulated (inhibited) by the three compounds. The full set of molecules is currently ongoing and will complete the big picture of PFAS neurotoxicity in oligochaetes. Our findings clearly demonstrated a significant impact of PFAS compounds on the earthworm neural systems. Moreover, the study provides valuable insights into the potential harmfulness of these compounds on the human NCS, suggesting the importance of considering PFAS as emerging neurotoxicants.

Conclusions

These results contribute to the understanding of PFAS neurotoxicity and its potential effects on broader ecological and human health perspectives, warranting further research and consideration within the neuroscience community.

Spectronaut, MaxDIA and DIA-NN: benchmarking of software tools for DIA proteomics

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Summary: *DIA data analysis presents a statistical and computational challenge due to the large amount of input material generated by mass spectrometers. In this study, we aimed to compare the performance of three different DIA data interpretation tools, Spectronaut, DIA-NN, and MaxDIA.*
Keywords: *data-independent acquisition; spectral library; software tools.*

Introduction

In mass spectrometry-based proteomics, proteins undergo processes of extraction, solubilization, and digestion[1], producing peptides that can be analyzed by liquid chromatography and tandem MS (LC-MS/MS). In DIA mode, multiple ions are co-isolated and co-fragmented, generating multiplexed MS/MS spectra. This study compares the quantitative performance of three different software tools, MaxDIA[2], DIA-NN[3] and Spectronaut.

Materials and methods

HEK 293 cell lysate, Pisum sativum and Soybean powders were dissolved in 100 mM Tris-HCl and 1% SDS (v/v) solution buffer. Proteins were subjected to reduction and alkylation and the three proteomes (Human, Soybean and Pisum sativum) were mixed in two different blends: mixA contained 250 µg of Human proteins, 20 µg of Pisum sativum proteins and 30 µg of Soybean proteins, mixB enclosed the same quantity of Human proteins and 10 µg and 40 µg of Soybean and Pisum sativum proteins respectively (Table 1). Protein fold-change ratio (mixA/mixB) was 1 for Human proteins, 3 for Soybean proteins and 0,5 for Pisum sativum proteins. Two different amounts of proteins (1 and 10 µg) for mixA and mixB were digested in quadruplicate with Filter aided sample preparation (FASP[4]) and Protein aggregation capture (PAC[5]) approach. The removal of detergents was performed by strong cation exchange (SCX) StageTip, and half of the eluate for both digestion protocols was purified. After purification step, peptides were eluted, dried at 30°C and resuspended in 0.1% formic acid.

LC-MS/MS analysis

Peptides mixtures were analysed in DIA mode by an Easy nL-1000 chromatographic instrument coupled on-line to an Exploris 4800 mass spectrometer (both from Thermo Scientific, Bremen, Germany) through a nanoESI interface.
A peptide library was created by fractioning 20 µg of total peptides (mixA and mixB) in 10 fractions on C18 StageTip and analysing them in DDA mode.

DIA Data processing

Raw files were analysed using three different software: MaxDIA and Spectronaut was performed using our spectral library, while DIA-NN was performed in library-free mode. Spectronaut was also performed in the Direct-DIA mode. Statistical analyses were performed in Perseus (version 2.0.6.0): to detect the differentially expressed proteins between mixA and mixB, Student's test was used by setting the S0 value to 0.2 and filtering by Permutation based-FRD equal to 0.05.

Results

For each tested condition, sensitivity, specificity, and precision were calculated through these ratios:
(i) Specificity: TN/(TN+FP)
(ii) Sensitivity: TP/(TP+FN)
(iii) Precision: TP/(TP+FP)
In terms of specificity, the three data analysis software attained a comparable result, with the highest values (about 0.99) achieved by MaxDIA and DIA-NN (PAC-10 µg with SCX). Of note, identifications yielded with DIA-NN were twice compared to those obtained with MaxDIA.
Concerning the sensitivity (Figure 1), the best result (0.83) was obtained by processing the data with Spectronaut in library-free mode (PAC 1 µg), demonstrating its ability to better quantify low abundance proteins.
In terms of precision (Figure 1), MaxDIA returned the highest average value (0.78) but also the lowest number of identifications. However, DIA-NN showed a good compromise between a deep

proteome coverage and an adequate precision value (average 0.71).

Conclusions

In summary, excellent quantitative performance in bottom-up proteomics can be achieved with Spectronaut, DIA-NN, and Max-DIA. However, each software tool encloses its own strengths and weaknesses, and there is still much work to be done to establish unambiguous guidelines for the optimal interpretation of different DIA datasets.

Table 1. Human, Soybean and Pisum sativum protein amount in mixtures A and B

mix	Human	Soybean	Pisum sativum
A	250 µg	30 µg	20 µg
B	250 µg	10 µg	40 µg

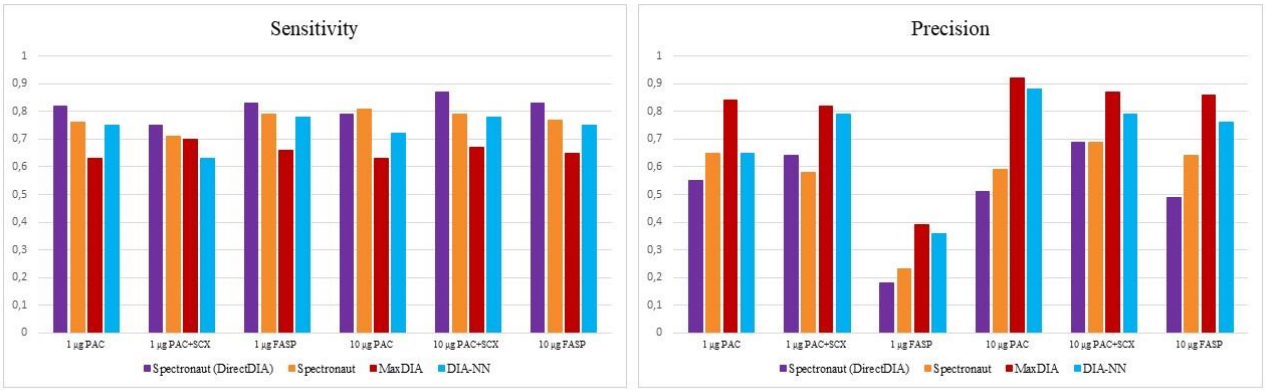

Fig. 1. Average of sensitivity and precision obtained with FASP and PAC digestion approaches (1µg and 10 µg in quadruplicate) by using Spectronaut, MaxDIA and DIA-NN for data analysis.

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