EUPA 2018

XII EUPA CONGRESS
Translating genomes into biological functions

16–20 June 2018
Santiago de Compostela

JOINT MEETING WITH VII SEPROT CONGRESS
Dear colleagues,

It is for us a pleasure to welcome you to Santiago de Compostela for the celebration of the XII EuPA Congress, a joint meeting with the Spanish Proteomics Society (SEProt) and the Portuguese Proteomics Association (ProCura), both celebrating their 7th Congress. Santiago de Compostela is a historical UNESCO-protected city, famous for its pilgrims' way and its 520 year old university. It is the capital of Galicia and is very close to North Portugal, which makes it an ideal location for this joint meeting.

We have prepared an interesting program for you that combines a variety of events, several of which are specially devoted to young researchers. Overall, we had close to 200 abstracts submitted, from which 69 have been selected for oral presentations distributed between the different topic sessions. In addition, we have 11 prestigious invited speakers who will be giving plenary lectures. Please check out our website (www.eupa2018.com) to download the complete program. We are especially pleased to have Prof. Ruedi Aebersold to give the Opening Lecture of the congress. Prof. Aebersold will be also named Honorary Member of SEProt for his outstanding contribution to the Proteomics field.

Besides the main scientific sessions, we will have various parallel activities. For instance, the EuPA Bioinformatics Community (EuBIC) initiative organizes a bioinformatics bazaar that will be available during the congress. In addition, the Young Proteomics Investigator Club (YPIC) is running a specific session on Sunday 17th early afternoon, before the Opening Session. Moreover, YPIC will also organize meet-the-expert breakfast sessions during the congress. We would like to encourage young researchers coming to the congress to participate actively in all these activities devoted to them. In line with the latter, we also have an exciting educational program that is taking place on Saturday 16th at the Faculty of Medicine, next to the congress venue. We have two workshops run in parallel: one organized by EuBIC, focused on bioinformatics, and a second one, organized by EuPA, focused on Cytoscape. We hope many of you decided to register for these events.

One of our priorities, as congress organizers, has been to facilitate young investigators to come to the meeting so we kept and even decreased the congress fees compared to previous years. Moreover, EuPA and SEProt awarded 20 travel grants (10 each) for young researchers. In addition, we have a Young Investigator Prize (YIP), selected among those presenting their application following the acceptance of an abstract. Prizes to the best posters will be also given during the closing ceremony. Talking about prizes, we have two important ones to be announced during the closing ceremony: the Juan Pablo Albar Proteome Pioneer Award 2018, sponsored by EuPA, and the SEProt-Juan Pablo Albar Prize, sponsored by Bruker. The former intends to honour a person involved in EuPA with a leading and long-standing involvement in the promotion of the principles of sharing and integrating resources for the development of excellent research in the Proteomics field. The SEProt prize acknowledges outstanding proteomic-related scientific activities by Spanish scientists carrying out their main work in Spain.
Finally, we cannot conclude without thanking the support of all our sponsors; without them, this congress would not be possible. Lunch seminars supported by these companies will take place during the congress so please check out the congress website for further information. As recognition, EuPA will award the company that best contributed to the scientific/technological advances in proteomics in the last year with the EuPA Industry Award, a ceremony taking place at the closing session of the congress.

We hope you will enjoy our congress and your time in Santiago de Compostela, a place to discover; do not miss the opportunity to walk the streets of the old city and enjoy the gastronomy and amiability of the composteláns. You will have time for science and much more.

Benvidos and have a great congress!!

Dr. Ángel García
President of the Spanish Proteomics Society
Email: angel.garcia@usc.es

Dr. Deborah Penque
President of the Portuguese Proteomics Society
Email: deborah.penque@insa.min-saude.pt
# PLENARY SESSIONS

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POSTER
The Proteotype: Integrator of Biological Information and Determinator of Phenotypes

Ruedi Aebersold,
Department of Biology, Institute of Molecular Systems Biology, ETH Zurich and Faculty of Science, University of Zurich

Biological or clinical phenotypes arise from the biochemical state of a cell or tissue which, in turn, is the result of the composition of biomolecules and their organization in the cell. At present, there is neither a comprehensive theory nor computational models that generally predict phenotypes from the genomic measurements. Nevertheless such predictions are frequently attempted, particularly in clinical research, exemplified by personalized/precision medicine. It is therefore an important question which type(s) of molecular information either by themselves or integrated will increase the ability to predict phenotypes from molecular measurements, compared to the present state.

In this presentation we will argue that the proteotype, defined as the composition and modular organization of the proteome in a particular cellular instance, is particularly informative. We will discuss experimental, mass spectrometry based methods to measure the proteotype and inference algorithms to quantify the modular organization of the proteome. We will then examine to what extent the proteotype correlates with quantitative phenotypes and how proteotype data are effectively integrated with other types of molecular patterns or biochemical knowledge to increase the predictability of phenotypes. Selected examples will be used to illustrate the general points.
PROTEOMICS AND DATA INTEGRATION FOR UNRAVELING
THE MYSTERIES OF SPERMATOGENESIS

Charles Pineau, Emmanuelle Com, Nathalie Melaine, Régis Lavigne, Mélanie Lagarrigue, Blandine Guével and Laetitia Guillot

PROTIM, Inserm UMR_S1085 – Irset, Campus de Beaulieu, 35042 Rennes, France

Background: The strikingly complex structural organization of the mammalian testis in vivo creates particular difficulties for studies of its organization, function and regulation. These difficulties are particularly pronounced for investigations of the molecular communication networks that govern spermatogenesis within the seminiferous tubules. The use of classical molecular and cell biology approaches to unravel this complexity has proved problematic, due to difficulties in maintaining differentiated germ cells in vitro, in particular. This lack of a suitable testing ground has led to a greater reliance on high-quality proteomic and genomic analyses as a prelude to the in vitro and in vivo testing of hypotheses.

Methods: In this presentation, we will highlight the options currently available for research, as used in our laboratory, in which proteomics and integrative genomics strategies are applied to the study of spermatogenesis in mammals. We will comment on results providing insight into the molecular mechanisms underlying normal and pathological spermatogenesis and on new perspectives for the treatment of male infertility in humans. Finally, we will discuss the relevance of our strategies and the unexpected potential they offer in the study of male reproduction, within the framework of the Human Proteome Project.

Results: The data obtained contribute to partly understand: i) some of the cellular cross-talks established between the somatic Sertoli cells and the germ cell lineage; ii) the formation of the male gamete; and iii) its post-testicular maturation.

Conclusions: Integrative genomics is becoming a powerful strategy for discovering the biological significance hidden in proteomic datasets. Using the described integrative strategies, we succeeded in gaining new insight into spermatogenesis and to better understand the pathophysiology of testicular function, with promising clinical applications in the context of male infertilities.

Keywords: Testis, Spermatogenesis, Male infertilities, Integrative proteomics
OMICS tools to characterize platelet function

Florian Beck,1 Jörg Geiger,2 Stepan Gambaryan,3 Fiorella Solari,1 Stefan Loroch,1 Nadine J. Mattheij,4 Kerstin Jurk,3 Julia Burkhart,1 Christian Fufezan,1 Johan Heemskerk,4 Ulrich Walter,5 Rene Zahedi,7 and Albert Sickmann1

1 Leibniz-Institut für Analytische Wissenschaften–ISAS–e.V., Dortmund, Germany; 2 Interdisciplinary Bank of Biomaterials and Data, Würzburg, Germany; 3 Sechenov Institute, Russian Academy of Sciences, Saint Petersburg, Russia; 4 Cardiovascular Research Institute Maastricht, Maastricht, The Netherlands; 5 Center for Thrombosis and Hemostasis, Mainz, Germany; 6 Institute for Biology and Biotechnology of Plants, Münster, Germany; 7 Lady Davis Institute, Jewish General Hospital, Montreal, Canada

Background:

More than 130 years ago, it was recognized that platelets are key mediators of thrombosis and hemostasis. Nowadays, it is established that platelets participate in additional physiological processes and contribute to the genesis and progression of cardiovascular diseases. Anti-platelet treatment is of fundamental importance in combatting functions/dysfunction of platelets in the pathogenesis of cardiovascular and inflammatory diseases. Owing to their anucleate nature (dys)function of platelets is likely to be completely attributable to alterations in protein expression patterns and post-translational modifications. Consequently, platelet proteomics will represent an invaluable tool for characterizing the fundamental processes that affect platelet homeostasis and thus determine the roles of platelets in health and disease.

Methods:

Fresh blood was obtained from healthy volunteers who had not received any medication for two weeks. Whole-blood collection and platelet isolation were performed as described previously (Beck at al., Blood, 2014) Platelet preparations were > 95 % pure, contamination with erythrocytes and leukocytes were 1/104 platelets and 1/106 platelets. For temporal ADP profiles, aliquots were stimulated with ADP and/or Iloprost, or remained unstimulated.

Results:

In the blood flow, the activation of platelets not contributing to thrombus formation can be reversed by prostacyclin exposed by the endothelium. We wondered whether we could use temporal phospho-proteomics to study this reversal activation and thus quantified changes between (1) unstimulated platelets and platelets stimulated (2) for with ADP, (3) ADP followed by Iloprost. We further merged all our temporal platelet phosphorylation data sets to identify phosphorylation sites showing an inverse response. We hypothesize that these might be central players governing platelet homeostasis and representing suitable candidates.

Conclusions:

Platelet phosphorylation profiles induced by the two fundamental counterparts ADP and prostacyclin produce a complex but consistent picture. A remarkable number of phosphorylation sites and changes have been observed in platelets for the first time; however, the function of many of those is still unknown. It will be important to understand which of the observed initial and later phosphorylation events are mediated by triggering the P2Y1 and P2Y12 receptors, and subsequent integrin αIIbβ3 outside-in signaling. The identification of reciprocal protein phosphorylation may allow the identification of potential marker proteins representing a specific functional state of platelets. By integrating biochemical and functional data/models and quantitative proteomic data, novel pharmacologic targets can be identified that might pave the way toward precision medicine.

Keywords:

Mass spectrometry, quantitative proteomics, platelets, clinical proteomics
Dissecting EGF receptor signaling in-vivo by

Quantitative Interaction Proteomics and Phosphoproteomics

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**Background:** Quantitative phosphoproteomics is emerging as a powerful technology for global analysis of cellular signaling networks. In particular, tyrosine phosphorylation (pTyr) is of great importance in eukaryotic cells due to its crucial role in regulating intracellular signaling networks controlling cell fate decisions such as proliferation, migration, differentiation, cell cycle progression and apoptosis. EGF receptor signaling is often dysregulated in lung cancer, which makes phosphoproteins involved in this pathway attractive therapeutic targets.

**Methods:** EGF stimulated lung tissues and lung cancer cells were harvested, proteins extracted and digested with trypsin. Phosphopeptides were enriched by TiO2 and analysed by nanoLC-MS/MS on Q Exactive MS/MS. Phosphopeptides were identified and quantified using MaxQuant.

**Results:** I will present how we employed quantitative phosphoproteomics to delineate EGF receptor signaling in-vivo in lung tissue and delineated the signaling complexes recruited to all regulated phosphotyrosine sites. We quantified thousands of phosphorylation sites as a function of ligand and stimulation time, and thereby revealed EGF receptor-specific phospho-regulation of key adaptor and signaling molecules, which fine-tune cell migration and proliferation. A large-scale phosphopeptide interaction screen allowed us to identify readers of the regulated phosphotyrosine sites and thereby assign function to them. Using this strategy we worked out the molecular mechanisms underlying the oncogenic properties of an EGF receptor lung cancer mutation.

**Conclusions:** Based on a multidisciplinary approach, which combines quantitative phosphoproteomics, interaction proteomics and functional assays, we can identify EGF-dependent mechanisms for the control of wildtype and mutated EGF receptor signalling, which specify long-term cellular outcomes.

**Keywords:** Phosphoproteomics, EGF receptor, lung cancer, quantitative interaction proteomics, cell signalling.
USING A MASS SPECTROMETER AS A MICROSCOPE:
A PHYSICAL MAP OF A HUMAN CELL

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Background: Compartmentalization is essential for all complex forms of life. In eukaryotic cells, membrane-bound organelles, as well as a multitude of protein- and nucleic acid-rich subcellular structures, maintain boundaries and serve as enrichment zones to promote and regulate protein function, including signaling events. Consistent with the critical importance of these boundaries, alterations in the machinery that mediate protein transport between these compartments has been implicated in a number of diverse diseases. Understanding the composition of each cellular “compartment” (be it a classical organelle or a large protein complex) remains a challenging task. For soluble protein complexes, approaches such as affinity purification other biochemical fractionation coupled to mass spectrometry provides important insight, but this is not the case for detergent-insoluble components. Classically, both microscopy and organellar purifications have been employed for identifying the composition of these structures, but these approaches have limitations, notably in resolution for standard high-throughput fluorescence microscopy and in the difficulty in purifying some of the structures (e.g. p-bodies) for approaches based on biochemical isolations.

Methods: We employed proximity-dependent biotinylation (BioID) coupled to mass spectrometry to profile in HEK293 cells: 1) classical subcellular compartment markers and 2) components of RNA-associated P-bodies and stress granules. All experiments are performed in stable inducible isogenic cells, and purifications are in at least biological duplicates. In all cases, confidence scoring was performed against sets of negative controls using the SAINT software tools, and data analysis and visualization was performed through ProHits-LIMS and ProHits-viz. Websites for the interrogation of the datasets are built in-house.

Results: Our RNA granule and body dataset (139 baits) enabled us to define the composition and organization of stress granules and P-bodies in human cells, based on analysis of correlated prey profiles (Youn et al., Mol Cell, 2018). The same strategy is now employed on the compartment marker dataset (192 baits), resulting in the assignment of ~4,000 proteins to one or more distinct subcellular localizations. We will describe the creation of a "cell-map" website to help the community score and analyze their own BioID datasets.

Conclusions: BioID coupled to mass spectrometry is a powerful and scalable method to define organellar composition at different levels of resolution (bait coverage largely drives the granularity in the resulting networks). While the work presented was performed in the HEK293 cell model, by moving the probes to a regulatable lentiviral vector system, other cell types, including primary cell and patient-derived cell lines, can now be profiled.

Keywords: Proximity-dependent biotinylation, BioID, subcellular organization, cell biology, lentiviral delivery
TAILS Positional Proteomics Identification of LUBAC Regulation by MALT1 and An Allosteric Pharmacologic Molecular Corrector Rescue of NFkB Activation

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Background: Uncontrolled and upregulated proteases are attractive drug targets in a variety of malignancies, inflammatory and autoimmune diseases. However, increasing protease activity to correct delayed or insufficient protease expression or activity in disease is chemically and therapeutically challenging. The paracaspase MALT1 is central for transducing lymphocyte antigen receptor activation of NF-kB.

Methods: We developed nanomolar, selective allosteric inhibitors of MALT1 protease activity that bind by replacing the side chain of Trp580 and locking the protease in an inactive conformation. Interestingly, we had previously identified a patient homozygous for a hypomorphic MALT1 mutation at Trp580 (tryptophan to serine) who suffered from combined immunodeficiency. N-terminal positional proteomics was performed on the homozygous MALT1 mutant B lymphocytes using Terminal Amino Isotopic Labeling of Substrates (TAILS).

Results: The mutation weakened the interaction between the paracaspase and C-terminal immunoglobulin domains of MALT1 resulting in protein instability (Tm 45.9°C versus 52.5°C) and consequently reduced MALT1 function and protein levels. Due to this specific mode of action and the coincident location of the MALT1-Trp580Ser mutation in the MALT1 immunodeficient patient, we reasoned that such an allosteric compound may stabilize the MALT1-Trp580Ser mutant protein by replacing the absent tryptophan residue. We found that the new allosteric inhibitors also bind MALT1-Trp580Ser, stabilizing the mutant protein and increasing the Tm to that of wild-type MALT1. In so doing the small molecule allosteric nanomolar inhibitors restored MALT1 protein levels in MALT1(mut/mut) lymphocytes, rescuing canonical NF-kB and c-Jun N-terminal kinase (JNK) signaling31, and improving the proteolytic activity of MALT1-Trp580Ser to restore substrate cleavage.

Conclusions: Thus, a low molecular weight pharmacological corrector compound rescued protease deficiency by substituting for the mutated residue to restore function and increase cleavage activity, inspiring potential precision therapies to increase mutant enzyme activity.

Keywords: N Terminomics, TAILS, precision medicine, pharmacological corrector, linear ubiquitin, LUBAC, HOIL1, HOIP, SHARPIN, MALT1, protease, lymphocytes.
(Phospho)Proteomics for marker and target discovery in cancer

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The general goal of our clinical cancer proteomics efforts is to disclose tumor biology and drive improved diagnostics, management and treatment of cancer (Jimenez et al., Clin. Prot. 2018). Label-free mass spectrometry-based proteomics (both DDA and DIA) is optimally suited for large-scale clinical sample profiling.

I will highlight our work in cancer addressing: 1. protein markers for improved colorectal cancer screening (n=313, De Wit et al., Ann. Int. Med., 2017), 2. colorectal cancer subtyping/ prognostic markers (n= 164) and 3. phosphoproteomics for response prediction (preprint in Biorxiv describing our integrative inferred kinase activity scoring pipeline for single samples).

Ultimately cancer proteomics powered by precise measurements and dedicated analysis will realize the full potential of multi-parameter diagnostics and personalized medicine.
The RNA binding proteome in time and space

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In most cells, mRNA species are spatially located and translated in a highly controlled manner involving the interaction of cis- and trans-acting factors with RNA-binding proteins (RBPs). Aberrant translation of proteins in the wrong subcellular location can give rise to multiple disease states. In order to determine where and how intracellular RNA species are located in steady-state, it is necessary to efficiently extract RNA species and their coordinating protein binding partners. Interrogation of RBPs has centred round UV crosslinking RNA to protein coupled with oligo(dT)-based capture, an approach which favours mRNAs over other RNA species. To overcome this bias, recent methods have employed the use of specialised bases incorporated into RNA during in vivo transcription that can be biotinylated.

Here we present the orthogonal organic phase separation (OOPS) method, a simple method to extract UV crosslinked RBPs that does not necessitate the incorporation of specialised bases or rely on the presence of 3’ poly A tails. This highly efficient method enables reproducible recovery of RBPs or protein-bound RNA (PBR), free protein and free RNA, from a single sample, in an unbiased manner compatible with downstream proteomics and RNA sequencing. Using this method we demonstrate that all long RNAs in the cell are bound by proteins and identify protein-binding sites across the transcriptome in a high throughput manner. Furthermore, we reveal previously undetected RBPs that are located across all membrane-bound organelles. We also identify a core set of RBPs found in all human cell lines tested to date. As the OOPS method does require the presence of 3’ poly A tails, we show its versatility by cataloguing bacterial RBPs. Given that OOPS dramatically reduces the amount of sample needed, we also demonstrate that we can access changes in the RNA-binding proteome during the cell cycle, shedding light on cellular mechanisms that are influenced by the dynamic interactions of RNA and protein.

Finally, we show that subcellular fractionation methods such are LOPIT (localisation of organelles proteins using isotope tagging), are compatible with OOPS, obtaining, for the first time, spatial information for RBPs in UV-cross-linked cells.

**Keywords**: RNA binding proteins, subcellular location, spatial proteomics
A proteomic map of the target landscape of clinical kinase inhibitors

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Background: Kinase inhibitors have developed into important cancer drugs because de-regulated protein kinases are often driving the disease. Close to 40 such molecules have been approved for use in humans and several hundred are undergoing clinical trials. As most compounds target the ATP binding pocket, drug selectivity among the 500 human kinases is a recurring question. Clinically speaking, polypharmacology can be beneficial as well as detrimental. Therefore, knowing the full target spectrum of a drug is important but rarely available.

Methods: We have used a quantitative chemical proteomics approach to profile 240 clinical kinase inhibitors in a dose dependent fashion in cancer cell lysates to identify thousands of drug-protein interactions.

Results: The data revealed previously unknown targets for established drugs, offered a perspective on the druggable kinome, highlighted (non)kinase off-targets, and suggested potential therapeutic applications. Integration of phosphoproteomic data refined drug-affected pathways, identified response markers, and strengthened rationale for combination treatments. We exemplify translational value by discovering SIK2 (salt-inducible kinase 2) inhibitors that modulate cytokine production in primary cells, by identifying drugs against the lung cancer survival marker MELK (maternal embryonic leucine zipper kinase), and by repurposing cabozantinib to treat FLT3-ITD – positive acute myeloid leukemia.

Conclusions: The translational value of this project is apparent from the fact that the drug:target information generated in this project is now used in the molecular tumor board of the Comprehensive Cancer Center Munich in order to develop patient-specific recommendations for cancer patients.

Keywords: clinical kinase inhibitors; cancer therapy; chemical proteomics; quantitative mass spectrometry
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THE HISTONE CODE STUDIED BY BOTTOM-UP LABEL-FREE MASS SPECTROMETRY: HOW COMPREHENSIVE IS THE CURRENT PICTURE?

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Background: Histone posttranslational modifications (hPTM) regulate a plethora of fundamental biological processes by mediating transcription of genes. As the image of the epigenetic landscape is gradually sharpening, the position of hPTM herein becomes increasingly defined. With this improved view also comes the growing sense that it is the interplay between different biochemical changes that dictates the final biological outcome. However, studying the proverbial grammar emerging from the combination of hPTMs, the so-called "histone code", still is no sinecure.

Methods: Mass spectrometry is currently the only technique holding the promise of simultaneously annotating and quantifying enough different hPTM combinations to mine the histone code to a considerable depth. Label-free bottom-up quantification is an important MS approach used to study the dynamics in this histone code.

Results: State-of-the-art label-free DDA MS can now annotate 200-300 different histone peptides. Still, this field is relatively young and it requires ongoing development of dedicated workflows in terms of sample preparation, acquisition strategies and especially data analysis. Here we show that the intrinsically stochastic nature of DDA sampling and the limitations of MS1 quantification that comes with it, will probably never allow drilling to the bottom of the histone code. The use of DIA strategies (e.g. SWATH and HDMSE) therefore seems to be a logical next step.

Conclusions: MS on histones is unique in that it studies the dynamics of PTM combinations on top of a known, relatively short protein backbone. It thus requires a different way of thinking both in term of identification and quantification. No doubt, this field will develop into a mature branch in proteomics because its potential is practically limitless: all studies involving Eukaryotes, both fundamental and applicable, can benefit from a better understanding of the dynamics of the "histone code", be it on Animalia, Plantae, Fungi or Protista.

Keywords: histone code, combinatorial PTMs, DIA
Dissecting the Molecular Portrait of Ground State Pluripotency with Quantitative Proteomics

Martinez-Val, A., Lynch, C., Serrano, M., Muñoz, J.

**Background:** Typically, mouse embryonic stem cells (mESCs) cultured in serum fluctuate between a “primed” and a “naïve” state of pluripotency. Whilst the primed state is prone to differentiation, the naïve state shows higher maintenance of its self-renewal capacity. In fact, naïve mESCs are considered as the ground state of pluripotency and an *in vitro* surrogate of pre-implantation embryos. Inhibition of GSK3 and ERK (known as 2i) can capture this ground state indefinitely in culture. Interestingly, inhibition of CDK8 (a regulator of RNApolII) seems to stabilize mESCs towards the ground state. Whether these two mechanisms, *i.e.* 2i and CDK8i, converge in a similar ground state it is not clear.

**Methods:** Here, we used isobaric labelling quantitative proteomics to characterize the molecular mechanisms regulated by 2i and CDK8i in mESCs during their transition towards the groundstate pluripotency. To this end, we analysed, at unprecedented depth levels, (i) the early signalling events that are triggered by inhibition of such kinases, (ii) how the global proteome is remodelled and (iii) the changes in their metabolites profiles as result from all these molecular changes.

**Results:** Our data show that, after long-term culture, CDK8i induces a set of protein changes that significantly overlap with those observed by 2i. Most importantly, this protein signature appear during embryogenesis at the inner cell mass of the blastocyst. Moreover, a high-resolution temporal analysis of 2i and CDK8i demonstrated that similar mechanisms are activated/inhibited during this transition, albeit significant differences were found between them in metabolic routes. To further investigate these changes, an unbiased metabolomic analysis allowed us to link changes in expression levels of enzymes to their metabolite products.

**Conclusions:** Together, these results demonstrate that direct inhibition of RNApol II activity induces acquisition of the ground state by similar mechanisms as 2i, suggesting a potential convergence between these routes.

**Key words:** pluripotency, quantitative proteomics, phosphoproteomics, metabolomics
Quantitative proteomics and whole transcriptomics sequencing of Progeria-derived cells point to a key role of nucleotide metabolism in premature aging

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Background:

Hutchinson Guilford Progeria Syndrome (HGPS) or Progeria is a fatal disease with a very low incidence characterized by a typical clinical picture of elderly pathologies. HPGS-affected patients begin to show symptoms of accelerated aging at age of two, and typically die in the second decade of life due to cardiovascular deficiencies. The causal agent is a point mutation in Lamin A gene, generating a truncated isoform called Progerin -PG- or Δ50 Lamin A. Our aim for this study was to combine both shotgun proteomics and NGS to unravel new molecular pathways modulated in HGPS.

Methods:

Human HGPS-derived and healthy parental control cell lines were analyzed by iTRAQ (LTQ-Orbitrap Velos platform) and RNAseq (Illumina HiSeq 1500 platform). Processing of the raw data was done using Proteome Discoverer 2.1 and Bowtie2 and TopHat, respectively. Real-Time PCR was done using LightCycler® 480 SYBR Green I Master and immuno-histochemistry in ZMPSTE 24 −/− mouse liver sections was done with anti-PRPS1 antibody (Abcam). All the experiments were done at least in triplicate and results were considered significant when p-value ≤ 0.05.

Results:

After selection of modulated proteins and transcripts and crosschecking of the results a small set of proteins were selected for validation. One of those proteins, Ribose-phosphate pyrophosphokinase 1 (PRPS1), is essential for nucleotide synthesis. PRPS1 loss-of-function mutants present lower levels of purine. PRPS1 protein and transcript levels are detected as significantly decreased in HGPS cell lines vs. healthy parental controls. This modulation was orthogonally confirmed by targeted techniques in the cell lines and also in an animal model of Progeria, the ZMPSTE24 knock-out mouse.

Conclusions: All together, our results strongly suggest that nucleotide and, specifically, purine-metabolism, are altered in premature aging, opening a new window for the therapeutic treatment of the disease.

Keywords: Aging, nucleus, metabolism
Searching for Ghost Proteins Interactome

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In the commonly accepted dogma, eukaryotes mRNA codes for a single protein following Kozac consensus sequence; but it has been shown that proteins could be translated from alternative Open Reading Frame (ORF) such as translation from 5'UTR, 3'UTR or with a shift on the CDS domain. These non-predicted proteins have been named alternative proteins (AltProt) or Ghost proteins in opposition to reference proteins and constitute a hidden proteome. Recently, re-interrogation of data in-silico using libraries predicting these Alternative Proteins has led to the identification of more than 13,000 new proteins. Despite the function of these proteins has not been yet elucidated, preliminary data show that they would have important role in the regulation of reference proteins.

Mass Spectrometry based methods were shown to be central for the direct observation of these ghost proteins which were observed both using Bottom-Up or Top-Down strategies. Depicting the function of these ghost proteins is now the key issue. The get insight into the function of these ghost proteins we are searching for their interactome at a large scale looking at Protein-Protein Interaction (PPI). Over the past years several methods have been developed to study PPI. One of these is Cross-Linking Mass Spectrometry (X-Link MS) using different chemical reagents such as DSS, BS3, DSSO and DSBU coupled to MS analysis. Using appropriated cross-linkers the strategy can be applied in-vivo enabling to get of the interaction between the different proteins and build-back the network and pathways in which they are involved. This workflow was applied in-vivo using cell lines of glioblastoma (NCH82) to get deeper information on the implication of ghost proteins in the pathology. This will be further extended to cancer cells in presence of absence of anti-tumor drugs in order to dig into ghost proteins functions in normal and physio-pathological conditions.

Keywords:
Alternative Protein, Ghost proteins, Mass spectrometry, Protein-Protein interactions, Crosslink-MS

2Delcourt, V. et al. Small Proteins Encoded by Unannotated ORFs are Rising Stars of the Proteome, Confirming Shortcomings in Genome Annotations and Current Vision of an mRNA. Proteomics, Ahead from publication 2017.
Macrophages reactivation inside glioma microenvironments through PC1/3 inhibition associated with TLR3 activation

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**Background:** Inside immunosuppressive tumor environment, infiltrated macrophages present anti-inflammatory phenotype promoting tumor growth and T cytotoxic cells function suppression. In this way, therapeutic strategies need to be developed against this immunosuppression. We have previously showed that proprotein convertase 1/3 (PC1/3) inhibition led to an abundant secretion of immune factors and orient macrophages towards a pro-inflammatory (1, 2) and anti-tumoral phenotype (3). Macrophages activation is enhanced when PC1/3 inhibition is combined with Toll Like Receptors (TLRs) stimulation (3, 4). Previous studies have also suggested that after TLR3 stimulation, macrophages secrete factors that exerted a toxic effect on glioma cells (5).

**Methods:** Thus, the combination of TLR3 receptor agonist, PolyIC, with PCs inhibitor, which targets several proproteins convertases (furin, PC1/3, PACE4), on the activation and anti-tumor factor secretion by macrophages was studied. The effect of different PolyIC or PCs inhibitor concentration were investigated on C6 glioma cell line and NR8383 macrophages secretion by mass spectrometry and also on C6 viability through MTS test. Then, the efficacy of the combination between PolyIC and PCs inhibitor on the glioma cells viability and invasion was analysed either by direct co-culture between macrophages and glioma cells or indirect culture through macrophages conditioned media. Finally, the anti tumoral activity of macrophages extracellular vesicles was investigated on spheroid C6/NR8383 after PolyIC stimulation.

**Results:** PCs inhibition and PolyIC stimulation lead to an overexpression of pro inflammatory and anti tumoral factors by macrophages. Moreover, the association between PCs inhibitors and TLR3 stimulation lead to a decrease both of viability C6 and spheroid invasion.

**Conclusions:** The association between TLR3 activation and PCs inhibition shows good anti tumoral activity on rat glioma cell line. The results of this study could lead to a new therapeutic strategy for glioma treatment.

**Keywords:** Glioma, macrophages, extracellular vesicles

**References**
ROLE OF EXOSOMAL CX43 IN MELANOMA PROGRESSION.

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Background: Loss of gap junction intercellular communication (GJIC) and/or downregulation of connexins (Cxs) have been reported in different cancer cell lines as well as in tissues of many tumor types including melanoma. Cxs have been described as tumor suppressors in earlier stages of melanoma. However during tumor cell invasion and metastasis their role is a matter of some controversy. Extracellular vesicles (EVs) and exosomes released by cells participate in cell communication and can be involved in tumor progression. The transmembrane protein connexin43 (Cx43) was found in exosomes and participate in the transfer of information to the target cell though gap junctions (GJs).

Methods: Ectopic expresión of Cx43 was performed using vectors and electroporation. Protein levels and cellular sublocalization were studied by western-blot and immunofluorescence. Exchange of lucifer yellow (LY) was used to check gap junction intercellular communication (GJIC). Exosomes were isolated by ultracentrifugation and analysed using the NanoSight instrument and electron microscopy. The protein content was analysed by LC-MS/MS using a 6600 triple Tof (Sciex).

Results: Exosomes were efficiently isolated from human melanoma cells lines, however Cx43 was only present in exosomes derived from the melanoma cells that overexpressed Cx43 (A375Ma2-Cx43). When different melanoma cell lines were exposed to exosomal Cx43, these vesicles decreased cell proliferation and blocked colonies grown. The analysis of the protein content revealed 464 proteins exclusively present in exosomes positive for Cx43 compared to exosomes without Cx43, isolated from melanoma cell lines. Several of identified proteins are related with regulation of apoptosis such as APAF-1. We also identified proteins that regulate p53 expression, the CDKN2A anti-proliferative activity and the EGFR signaling pathway.

Conclusions: Our results indicate that exosomal Cx43 through its scaffolding function could be involved in the recruitment of proteins and other compounds to the exosomes switching the role of these EVs in melanoma. Further understanding of the role of Cx43 in the exosomes will have implications for the development of new therapeutic strategies as drug carries and delivery vehicles to combat metastasis in melanoma.

Keywords: Connexin43, melanoma, exosome.
**Conclusions:** Our results indicate that exosomal Cx43 through its scaffolding function could be involved in the recruitment of proteins and other compounds to the exosomes switching the role of these EVs in melanoma. Further understanding of the role of Cx43 in the exosomes will have implications for the development of new therapeutic strategies as drug carries and delivery vehicles to combat metastasis in melanoma.
Mechanisms of IL-33 activation through proteolytic maturation by environmental allergens analyzed by mass spectrometry

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Background Interleukin-33 (IL-33) is an epithelial cytokine critically involved in type 2 immunity, allergic inflammation and asthma, which activates specific target immune cells such as group 2 innate lymphoid cells (ILC2s). IL-33 contains a C-terminal bioactive IL1-like domain, and can be regulated through cleavage in its central domain by activator proteases. We explored here the mechanisms of IL33 activation by allergens, and its action on ILC2s.

Methods: Recombinant human IL-33 full-length protein, either untreated or incubated with allergens extracts, was digested using specific proteolytic enzymes and analyzed by nanoLC-MS/MS on a highly sensitive Orbitrap Fusion mass spectrometer, to map the central domain of the protein and identify neo N-terminal peptides resulting from allergen cleavage in this region. To characterize the proteome of ILC2s and its modulation following IL33-stimulation, mouse ILC2s were isolated from lungs and analyzed by single-shot nanoLC-MS/MS followed by label-free quantification.

Results: We found that all environmental allergens analyzed (including fungi, house dust mite, cockroach and pollens) possess proteolytic activity and cleave IL-33 in the central “sensor” domain, generating mature C-terminal forms which were shown to be potent inducers of type-2 innate immune responses in vivo. Stimulation of ILC2 target cells using these activated IL-33 forms induced the significant modulation of about 200 proteins, including IL-5 and IL-13 but also several cell surface proteins and transcription factors. Interestingly, our unbiased proteomic approach revealed that stimulation of ILC2s with a combination of IL-33 and other cytokines specifically induced a major up-regulation of IL-9, a key driver of chronic and allergic inflammation at mucosal surfaces.

Conclusions: Our data reveal a novel mechanism for the induction of allergic type 2 inflammation through the direct cleavage of IL-33 by many allergen proteases (1). Moreover we provide the first proteomic characterization of ILC2s and their response to IL-33.

Keywords: IL-33, allergy, proteolytic processing

(1) Cayrol et al, Nat. Immunol, in press
A novel Data-independent proteomics approach (DiS) reveals the mechanism of super-assembly of respiratory complexes III and IV

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Respiratory chain complexes can super-assemble into quaternary structures called supercomplexes that optimize cellular metabolism. The interaction between complexes III (CIII) and IV (CIV) is modulated by supercomplex assembly factor 1 (SCAF1, also known as COX7A2L). The discovery of SCAF1 represented strong genetic evidence that supercomplexes exist in vivo. SCAF1 is present as a long isoform (113 amino acids) or a short isoform (111 amino acids) in different mouse strains. Only the long isoform can induce the super-assembly of CIII and CIV, but it is not clear whether SCAF1 is required for the formation of the respirasome (a supercomplex of CI, CIII2 and CIV)1,2,4–6. Here we show, by combining deep data-independent-based proteomics (DiS) and immunodetection analyses, that SCAF1 is always required for the interaction between CIII and CIV and that the respirasome is absent from most tissues of animals containing the short isoform of SCAF1, with the exception of heart and skeletal muscle. We also used directed mutagenesis to characterize SCAF1 regions that interact with CIII and CIV. Furthermore, we find that the CIV subunit COX7A2 is replaced by SCAF1 in supercomplexes containing CIII and CIV and by COX7A1 in free CIV dimers, and that dimers seem to be more stable when they include COX6A2 rather than the COX6A1 isoform. These results reflect the improved skills of the new DiS proteomics approach to characterize multiprotein structures and provide a molecular model that explains how the expression of CIV protein isoforms regulate OXPHOS supercomplex assembly.


Proteomic analysis of regulatory T-cells reveals the importance of Themis1 in controlling their suppressive function

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Background:

Regulatory T-cells (Treg) represent a minor sub-population of T-lymphocytes that are of paramount importance for establishing self-tolerance, controlling inflammatory responses and maintaining immune homeostasis. Here, we present a large-scale quantitative proteomic study that defined a specific “signature” of Treg and identified some candidate proteins that may contribute to their suppressive function, such as Themis1.

Methods:

Treg and conventional T-lymphocytes (Tconv) sub-populations were carefully sorted by flow cytometry, and we performed global label-free quantitative mass spectrometry to compare their proteomes. This comprehensive data set of seven biological repeats was compared with transcriptomic data, as well as with other recently published proteomics data sets of CD4⁺CD25⁺ T-cells in order to define a robust set of protein markers characterising the Treg subset of T-lymphocytes. Themis1 was consistently down-regulated in Treg cells, and its role in regulating Treg function was characterised through in vitro co-culture tests and animal models of inflammatory bowel disease.

Results:

Besides “historical” proteins that characterise Treg cells, our study identified numerous new proteins that are significantly up- or down-regulated in Treg versus Tconv. We thus refined and increased the list of protein markers that discriminate these two T-cell populations of distinct functions at the protein level. We then focused on Themis1, a protein consistently under-expressed in Treg, and previously proposed as a new target gene that could participate in the pathogenesis of immune diseases. We showed both in vitro and in vivo that overexpression of Themis1 in Treg leads to an increase of their suppressive functions suggesting that it represents a checkpoint control of the suppressive function of Treg.

Conclusions:

Our label free quantitative strategy allowed a better characterisation of the Treg cell lineage, and we characterised the molecular mechanisms underlying the potential role of Themis1 in these cells. It was published in Molecular & Cellular Proteomics in 2017.

Keywords:

Regulatory T cells, Conventional T cells, Bottom-up proteomics, Label-free quantification
In-Depth Proteomic Characterisation of Rare Haematopoietic Progenitors in the Foetus and Adult

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Background: Blood stem and progenitor cells in the foetus and adult possess distinct molecular landscapes that regulate cell fate and change their susceptibility to initiation and progression of haematological malignancies. However, up until recently, the molecular mechanisms that govern the differences between foetal and adult blood cell development have been poorly explored on the proteomic level. In our latest work, we characterised and compared the proteomic makeup of foetal and adult haematopoietic stem and progenitor cells (HSPCs) (Jassinskaja et al., 2017, Cell Reports). Using mass spectrometry-based quantitative proteomics, we identified major differences in several important cellular processes, as well as ontogeny-specific expression of protein families that had not previously been described to play a role in early blood cell development, highlighting the need for a shift towards proteomic-centric approaches in the field. In order to further increase our understanding of normal and malignant blood cell development during ontogeny, we are now directing our efforts towards optimisation of our proteomic workflow to enable investigation of the proteomic ontogenic changes that occur in rare, stringently defined lineage-biased haematopoietic progenitors that are susceptible to leukaemic transformation.

Methods: We have FACS-sorted murine primary haematopoietic progenitor cells and applied StageTip-based methods and TMT-labelling for sample preparation and pre-fractionation in combination with multi-notch synchronous precursor selection MS3 scanning on Orbitrap Fusion.

Results: We have confidently identified and quantified the larger part of the cellular proteome in less than 100,000 primary FACS-sorted haematopoietic cells.

Conclusions: We believe that our approach will grant novel insight into normal and leukaemic haematopoiesis during foetal and adult life, as well as make global mass spectrometry-based proteomic studies a viable option in areas of research where availability of starting material is limited.

Keywords: quantitative proteomics, haematopoiesis, developmental biology
Background: Platelets within Platelet Components (PCs) for transfusion release inflammatory molecules that have accumulated during processing; some are occasionally associated with transfusion Adverse Reactions (ARs), despite the prestorage leukoreduction implementation. The rationale of this study is to decipher the proteome platelets in two major types of PCs, buffy-coat-derived pooled PCs (PPCs) and single donor apheresis PCs (SDA-PCs) when found in association with ARs.

Methods: Extensive proteome analysis was performed in 3 PPCs and 3 SDA-PCs, involved in ARs, compared to matched, clinically well-tolerated PCs. Label-Free LC-MS/MS method was used for quantitative proteomic analysis of washed platelet pellets. Bioinformatics analysis allowed to characterize differentially expressed (DE) proteins (Fold Change > |1.5|) and dysregulated pathways between ARs and control PCs.

Results: From PPCs and SDA-PCs proteome studies, 1723 and 4116 proteins, respectively, were identified from which 473 and 146, respectively, were DE within the two studied groups. The bioinformatics interpretation of the DE proteins revealed an important enrichment in the platelet activation and degranulation as the most significant biological processes associated with ARs. Moreover, inflammatory disorders and mitochondrial dysfunctions were among the most relevant disease mechanisms involved in ARs after platelet transfusion. Three common proteins were significantly dysregulated in both types of investigated PCs: The Mitochondrial carnitine/acylcarnitine carrier protein (SLC25A20) was found decreased while Multimerin-1 (MMRN1) and Calumenin (CALU) were consistently increased PCs involved in ARs compared to matched controls.

Conclusions: Platelets in PCs having led to clinical ARs in the transfused patients were found to exhibit profound proteome change; some changes indicate alteration in the platelet signalosome. This study indicates that novel safety steps can be thought of at least to secure PC transfusion in likely patient categories, when patients are considered either more fragile or at higher risk of manifesting transfusion ARs.

Keywords: Label-Free, Quantitative proteomics, Platelet components, Bioinformatics, Biological Process
Background: Platelet Components (PCs) for transfusion release some inflammatory molecules that have accumulated during processing; some are occasionally associated with transfusion Adverse Reactions (ARs), despite the prestorage leukoreduction implementation. The rationale of this study is to characterize platelet proteome changes in single donor apheresis PCs (SDA-PCs) when found in association with ARs.

Methods: Proteomics analysis was performed in 6 SDA-PCs involved in ARs, compared to 6 matched PCs controls. Label-Free method (LC-ESI Orbitrap Q-Exactive mass spectrometer) was performed for the proteins quantification. Bioinformatics and biostatistics analyzes allowed to identify differentially expressed (DE) proteins (Fold Change > |1.5|). Their impact on biological processes and pathways in case of ARs was studied via a protein-protein interaction analysis (String db) and a functional analysis (Genecodis3, KEGG Pathway).

Results: 332 significant proteins (p<0.05) were found for the 12 SDA-PCs from which 90 were DE (58 hyper-expressed, 32 hypo-expressed) between PCs involved in ARs vs controls. The functional analysis of the DE proteins revealed an important enrichment for the oxidative phosphorylation including mostly hyper-expression, a notable cytoskeletal modification towards a strong cell remodeling, and an increase of the inflammation process in case of ARs. These dysregulated proteins are mostly implicated in signaling pathways including oxidative phosphorylation, actin cytoskeleton regulation, MAPK signaling pathway and AMPK signaling pathway.

Conclusions: Platelets in PCs having led to clinical ARs in transfused patients were found to exhibit profound proteome changes impacting some interesting signaling pathways. These impacts strongly suggest a platelet cytoskeleton remodeling in response of oxidative stress increasing inflammatory process. This study helps to better understand the physio-pathological aspect of the ARs and could contribute to a better transfusion safety for transfused patients.

Keywords: Platelet proteomics, Bioinformatics, Biological Process, Signaling pathway
DIA+: A NOVEL DATA-INDEPENDENT ACQUISITION METHOD COMBINES MULTIPLE PRECURSOR CHARGES TO BOOST PEPTIDE SIGNAL.

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Background: Data-independent acquisition (DIA) methods aim to expand the benefits of low-throughput targeted proteomics to proteome-wide analyses. These methods rely on the use of several broadband isolation windows that select and fragment all peptide ions within a cycle. Isolation windows differ in that (I) they exhibit different widths, (ii) they are acquired either sequentially or in a non-consecutive order, and (iii) they are either juxtaposed or overlapped. Here we present DIA+, a novel DIA multiplexing scheme with isolation windows that combine signals from identical peptides with different charges.

Methods: Peptide mixtures derived from digested HeLa cell extracts were injected in triplicates in an Orbitrap Fusion Lumos mass spectrometer equipped with a chromatographic nano-UPLC system (Proxeon-1000) and a 50-cm C18 column (EASY-Spray; 75µm, PepMap RSLC C18, 2µm particles, 45ºC) using 120-min chromatographic gradients (ACN:H2O, 1% FA). All DIA methods were acquired with a first MS1 scan (Orbitrap detection at 60K) followed by different quadrupole isolation schemes, HCD fragmentation (28% CE) and MS2 detection in the Orbitrap at 30K. Data was analyzed using DIA Umpire v2.1 with the search engine Comet. Peptides and proteins were filtered at 1% FDR and their quantification was based on the sum of the six most intense fragment ions and the six most intense peptides respectively.

Results: DIA+ is based on the co-isolation of charge +2 and +3 precursor ions from identical peptide sequences to combine their signal and therefore, increase the number of MS2 peptide fragment ions detected. DIA+ combines three non-consecutive m/z ranges, based on the mass difference between charge +2 and +3 peptides, into a composite 24 Da isolation window. Forty of these combined 24 Da windows cover a range of 400-1350 m/z. The performance of DIA+ was compared with other reference methods in the field, and with a DIA+ control method in which each 24 Da isolation window results from the combination of three consecutive m/z ranges. The combination of improved signal-to-noise and sequence coverage increases the confidence on peptide identification and results in a significant increase in the number of identified peptides and proteins. It is worth noting that increased signal-to-noise has direct implications in the quality of peptide quantification, and that an increase in peptide sequence coverage benefits applications such as site-localization of post-translational modifications and the identification of single-point mutations or polymorphisms.

Conclusions: Novel data independent acquisition method with improved sensitivity and peptide sequence coverage.

Keywords: DIA, Data-independent acquisition, charge, protein quantification
The C/EBPα interactome unravelled by a novel mass spectrometric screening method

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Background:

C/EBPα is a pioneering transcription factor involved in cell fate decisions and differentiation in several cell types. Several lines of evidence suggest that C/EBPα acts as a hub for many components of the epigenetic and gene regulatory machinery. C/EBPα has a modular structure, is intrinsically disordered and extensively post-translationally modified, all contributing to the functional plasticity of the protein. The goal of the project is to dissect and map the PTM dependent interactome of C/EBPα.

Methods:

We probed 271 C/EBPα derived tiling peptides, including peptides with PTMs, for protein interactions. Protein extracts were prepared from differentiating myeloid cells for interaction with C/EBPα peptides using a method termed “protein interaction screen on a peptide matrix” (PRISMA). The resulting interactome was cross-validated by BioID experiments.

Results:

The screen produced a high quality interactome of C/EBPα in differentiating myeloid cells and revealed footprints of interacting protein complexes mapped to the C/EBPα sequence in a PTM dependent fashion. Hotspots of protein interactions correlate with molecular recognition features (MoRF) within disordered regions and PTM sites.

Conclusions:

PRISMA is a valuable tool for interactome mapping in a PTM dependant fashion. Identified binding partners help to understand the functionality of C/EBPα and will facilitate the rational design of C/EBPα mutants that will be tested in a trans-differentiation system.

Keywords: protein interaction screen, short linear motifs, PTMs, transcription factor
DETERMINATION OF THE SIGNALING PATHWAYS INVOLVED ON HEME-OXYGENASE 1 OVER-EXPRESSION IN EPCs IN ATHEROSCLEROSIS.

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Abstract EuPA 2018: Disease Proteomics

**Background:** Cardiovascular diseases (CVD) remain the first cause of death in the world, being atherosclerosis, a pathological process that affects vascular vessels; the main factor involved in all CVDs. The use of endothelial progenitor cells (EPCs) in CVD cell therapy constitutes a promising approach due to their vascular regenerative properties. However, the mechanisms by which they exert their beneficial effects remain unknown. In our group we have recently described how EPCs become activated in response to their stimulation with atherosclerotic factors ex vivo\(^1\). Among the over-expressed proteins, we found heme-oxygenase 1 (HO-1), a protein known to promote angiogenic, anti-inflammatory and antioxidant effects. The identification of the signaling pathways involved in HO-1 over-expression would help to enhance the protective effect of EPC on atherosclerosis.

**Methods and results:** EPCs were incubated ex-vivo in presence/absence of previously collected atheroma plaque secretomes, as described (1). Proteomic analysis was carried out and protein identification reported the over-expression of HO-1. Some proteins such as NRF2, HSF1, KEAP1 or NQO1, known to participate in the over-expression of HO-1, were not initially identified in the proteomic approach. Thus, western-blot for these proteins as well as q-PCRs analysis have been performed to determine which of these protein and gene changes appeared in EPCs in response to the atherosclerotic factors. The results obtained will be discussed.

Global proteome landscape during antigen-dependent B-cell differentiation reveals dynamic cell signaling profiles across distinct B-cell subpopulations

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Human B-cell differentiation has been extensively investigated on genomic and transcriptomic grounds; however, no studies have accomplished so far detailed analysis of antigen-dependent maturation-associated human B cell populations from a proteomic perspective. Here, we investigate for the first time the quantitative proteomic profiles of B-cells undergoing antigen-dependent maturation using a label-free LC-MS/MS approach applied on 5 purified B-cell subpopulations (naive, centroblasts, centrocytes, memory and plasma B cells) from five reactive tonsils. Our results revealed that the actual differences among these B-cell subpopulations are a combination of expression of a few maturation stage-specific proteins within each B-cell subset and maturation-associated changes in relative protein expression levels. The considerable overlap of the proteome of the 5 studied B-cell subsets strengthens the key role of the regulation of the stoichiometry of molecules associated with antigen recognition and presentation, cell survival and other signaling cascades crucial for the transition between each B-cell maturation stage.

Keywords: B-cell differentiation, naive B cell, centroblast, centrocyte, memory B cell, plasma cell, quantitative proteomics, gene expression microarrays, immune system.
N-TERMINOMICS AS A TOOL FOR IDENTIFYING PROTEOLYTIC CLEAVAGE OF *Toxoplasma gondii*

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**Background:** Apicomplexan parasites such as *Toxoplasma gondii* cause significant mortality and morbidity in humans and livestock. These parasites employ specialized secretory organelles (micronemes, rhoptries and dense granules (GRA) to invade and survive within host cells. We recently identified the small GTPase Rab11A as an essential regulatory factor of dense granule exocytosis. GRA proteins represent major virulent factors that are exported from the parasite into the host cells and modulate key immune and metabolic responses. A recent study showed that certain GRA proteins possess an export element, which is the site of a proteolytic cleavage that is subsequently acetylated and acts as a sorting motif for export [1]. However, the mechanisms regulating parasitic protein export into the host cell remain mostly unexplored.

**Methods:** To address this question, we have undertaken an N-terminome analysis on the lysate and secreted fraction of control parasites compared to parasites over-expressing a mutated version of Rab11A blocked in secretion, using the doublet N-terminal oriented proteomics (dN-TOP) approach [2] based on a double light/heavy trimethoxyphenylphosphonium (TMPP) labeling of alpha-amines. Protein extracts were submitted to TMPP labeling followed by 1D SDS-PAGE separation. Gel bands were digested with trypsin prior to nanoLC-MS/MS analysis (nanoUPLC-Q-Exactive plus Orbitrap).

**Results:** The N-terminome analysis of the secreted fractions allowed us to identify more than 300 secreted parasite proteins and 55 N-terminal peptides among which about 85% and 55% were found to be acetylated in control and mutated parasites respectively.

**Conclusions:** After comparison with the mutant strain, we were able to identify novel uncharacterized Rab11A-dependent secreted proteins defined by the presence of a cleaved signal peptide and N-terminal acetylation, which represent novel putative virulent factors essential for parasite survival.

**Keywords:** N-terminomics, *Toxoplasma gondii*, dN-TOP strategy, TMPP labeling

Shotgun proteomics of human pancreatic islets: effect of cytokines exposure

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Background: The effect of cytokines on β-cell function and survival was studied in β-cells as INS1-E and a different role was suggested for INF-γ and IL-1 which act on activation of cellular defense mechanism and cell functions, respectively. In addition, pro-inflammatory cytokines also showed to induce β-cell apoptosis and endoplasmic reticulum stress. In order to investigate the effect of cytokines on human islets, the complete proteome before and after exposure to cytokines was analyzed using label free shot-gun proteomics.

Methods: Aliquots of 40 µg of human islets protein extracts (n=3), with and without cytokines treatment, were loaded onto 12% acrylamide resolving gel. After separation, gel pieces (13 pieces for lane) were excised from the gel and the proteins were identified by Shotgun methodology after an in-gel trypsin digestion. Mass spectrometry data were acquired according to the novel label free quantitation workflow developed by Bruker Daltonik.

Results: Around 3000 proteins were identified and out of 307 differentially expressed proteins, 184 resulted increased (among these chemokines, oxidative stress related proteins and immunoproteasome proteins) and 123 reduced (i.e cathepsines, antioxidant proteins, Krebs enzymes) after treatment with cytokines. Ingenuity pathways analysis highlighted the activation of upstream regulators such as STAT1 and 2, NFKb, JAK1, and inhibition of MAPK1, atypical chemokine receptor 2, transcription intermediary factor 1-alpha and small ubiquitin-related modifier 3. Finally, the treatment with cytokines induced a significant decrease (-28±10%) of insulin secretion with respect to control, together with an increase of apoptotic beta cells and a reduction of volume density of grain of insulin.

Conclusions: Overall our results show how the detrimental effects of cytokines treatment in human pancreatic islets could be associated to proteome changes. Further studies are necessary to clarify the correlation between these deregulation and type 1 diabetes features.

Keywords: cytokines, human pancreatic islets, type-1 diabetes.
Extensive proteomic analysis of lipid rafts composition on resting and activated platelets.

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Background: Lipid rafts, also named glycolipid-enriched membrane domains (GEMs), are known to contain key receptors and signalling proteins that are essential for platelet activation. In the present study, we have performed an extensive proteomic analysis of these domains in resting and activated platelets.

Methods: Lipid rafts were isolated by a procedure based on their insolubility in the non-ionic detergent Brij-58 and their ability to float in sucrose density gradient after ultracentrifugation. After an initial approach to select the best protein digestion method, we performed a comparative proteomic analysis of platelets in resting state and activated with two major platelet receptors agonists: the Glycoprotein VI (GPVI)-specific agonist collagen-related peptide (CRP) and the C-type lectin-like receptor 2 (CLEC-2)-specific agonist Rhodocytin. Following FASP (Filter aided sample preparation) digestion, tryptic peptide samples were analysed by LC-MS/MS in a LTQ Orbitrap XL. Database search was performed with the Proteome Discoverer v1.4 software at FDR = 1%.

Results: Only identifications detected in at least 3 of the 4 biological replicates analysed were taken into account for comparison. Thereby, 48 proteins were selected from basal samples, 48 from CRP samples and 42 proteins from Rhodocytin samples. Remarkably, over 90% of the proteins identified were previously found associated to lipid rafts. The 20 most abundant proteins in each condition, based on the number of peptide-spectrum matches (PSM), remain mostly unaltered under the different stimulation conditions and are related to cell signalling and cell response processes (e.g. tyrosine-protein kinases Lyn and Fyn).

Conclusions: This novel study of platelet lipid rafts protein composition illustrates that these domains are constitutively enriched in signalling proteins in resting platelets and its composition remains markedly stable upon activation of Immuneceptor tyrosine-based activation motif (ITAM)-signalling pathways through the GPVI and CLEC-2 receptors.

Keywords: Platelets, Lipid rafts, CLEC-2, GPVI
Proteomic Signature of PDAC Cells with Mutant p53

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Background: The p53 tumor suppressor plays a crucial role in the prevention of oncogenic transformation through the removal or enduring growth arrest of potentially neoplastic cells. Upon cellular insults, activated p53 serves as a sequence-specific transcription factor to control the expression of specific genes, thereby stimulating various biological processes. In contrast to the tumor suppressive roles of wild-type p53, mutant p53 proteins have been shown to support cancer progression by enhancing the ability of cancer cells to invade and metastasize and to confer chemo-resistance. Pancreatic ductal adenocarcinoma (PDAC) is a very aggressive disease with a five-year survival of less than 5%. The tumor suppressor TP53 gene is mutated in about 50-75% of PDAC patients.

Methods: The secreted proteins of PDAC cell lines with mutant and wild type p53 were identified and quantified through a shotgun proteomic strategy: the proteins were extracted from the conditioned media and subjected to proteolytic digestion and then analysed by LC-MS/MS. To state the direct involvement of mutant p53, transfection of siRNAp53 to specifically knock-down (KD) the endogenous mutant p53 was performed in both wt and mutant p53 PDAC cell lines. To confirm the results the overexpression of mutant p53 was also performed.

Results: Using the shotgun proteomics approach and the sequential window acquisition of all theoretical fragment-ion spectra (SWATH) we were able to quantify hundreds of secreted proteins and to identify potential biomarkers of mutant p53. Furthermore the specific mRNA expression of selected proteins was investigated by qRT-PCR assay. We discovered a number of common proteins having opposite regulation after overexpression and knock-down of mutant p53. These proteins might constitute a sort of secreted signature driven by the hot-spot mutant p53 in PDAC and will be validated in serum samples of PDAC patients having WT or mutant TP53 gene.

Conclusions: These data might suggest the identification of targeted therapies specifically addressed to inhibit growth of PDACs carrying oncogenic mutant p53, which are strongly resistant to traditional chemotherapies.

Keywords: mutant p53; secretome analysis; pancreatic cancer
Unveiling the Human Ancient Proteome

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Background: Mummies are very important not only for the study of archaeology and paleopathology but also to study the conservation of ancient human proteins and their post translation modifications. In fact, since they are well preserved, the extraction and analysis of proteins is still possible. The ‘bog body’ are human remains found in wetland peat deposits.

Methods: The human remains of two bog bodies, the ‘Zweeloo Woman’ and the ‘Weerdinge Couple’, were analysed using shotgun proteomics. Proteins from bones, intestines, and several types of tissues were extracted and trypically digested before LC-MS/MS analysis.

Results: The proteomics revealed the presence of hundreds human proteins but also several post-translational modifications due to post-mortem hydrolytic and oxidative damage.

Conclusions: These data showed the most complete ancient human proteome and that mass spectrometry offers new perspectives for the study of ancient material, which often is not suitable for DNA analysis.

Keywords: Ancient proteome; Proteomics; Mummies; Post-mortem modifications.
Study of Mitochondrial Proteome Changes Due to eIF6 Depletion with SWATH-MS Acquisition

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Background: Eukaryotic Initiation Factor 6 (eIF6) is an initiation factor that binds 60S ribosomal subunits and has an anti-association property, by hampering 60S premature joining to 40S. In general, eIF6 is rate limiting for tumour onset and progression. eIF6 haploinsufficient cells are normal, but not efficiently transformed in vitro. Mitochondria are the main compartments of energy production, and some lines of evidence have shown that mitochondrial alterations contribute to the development of metabolic syndrome.

Methods: We analysed, by uLC-QTOF-MS/MS exploiting the SWATH-MS (Sequential Window Acquisition of all Theoretical fragment ion spectra) acquisition, the expression of mitochondrial proteome of AML-12 (non-tumourigenic murine liver hepatocytes) cell line, where eIF6 was down-regulated by shRNA, and of three different tissues from wild type and +/− mice for eIF6 (liver, muscle and brain). The SWATH-MS acquisition method is a high throughput label-free method for protein quantitation that combines the traditional shotgun proteomics with the quantitative accuracy and reproducibility of selected reaction monitoring (SRM).

Results: We found that depletion of eIF6 by shRNA induces profound and varied impact on mitochondrial proteome, impairing the energy production, steering the metabolism toward the up-regulation of aerobic glycolysis and the inhibition of oxidative phosphorylation.

Conclusions: SWATH-MS proteomics is a valid tool to study the in vitro effect of gene depletion. In particular, our results demonstrated the presence of several pathways and biological processes, involved at the mitochondria level, that are affected by the eIF6 depletion.

Keywords: Mitochondria proteome; eIF6 depletion; SWATH-MS; cell proteomics
COMPREHENSIVE QUANTIFICATION OF THE MODIFIED PROTEOME REVEALS OXIDATIVE HEART DAMAGE IN MITOCHONDRIAL HETEROPLASMY

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Background: Posttranslational modifications hugely increase the functional diversity of proteomes. Recent algorithms based on ultratolerant database searching are forging a path to unbiased analysis of peptide modifications by shotgun mass spectrometry. However, these approaches identify only half of the modified forms potentially detectable and do not map the modified residue. Moreover, tools for the quantitative analysis of peptide modifications are currently lacking.

Methods: Here, we present a suite of algorithms that allow comprehensive identification of detectable modifications, pinpoint the modified residues, and enable their quantitative analysis through an integrated statistical model. These developments were used to analyze the impact of mitochondrial heteroplasmy on the proteome and on the modified peptidome in several tissues from 12-week old mice.

Results: Our results represent the first quantitative characterization of the complete landscape of posttranslational modifications in a biological model, and reveal that heteroplasmy mainly affects cardiac tissue, inducing oxidative damage to proteins of the oxidative phosphorylation system.

Conclusions: Our data provide a molecular mechanism that explains the structural and functional alterations produced by heteroplasmy in heart mitochondria.

Keywords: heteroplasmy; posttranslational modifications; mitochondria; oxidative phosphorylation
Quantitative proteomics reveals neuronal ubiquitination of Rngo/Ddi1 and several proteasomal subunits by Ube3a, accounting for the complexity of Angelman syndrome

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Background: Angelman syndrome is a complex neurodevelopmental disorder caused by the lack of function in the brain of a single gene, UBE3A. The E3 ligase coded by this gene confers its substrates with K48-linked ubiquitin chains, a signal historically considered to signal for degradation by the proteasome. However, a change in protein abundance is not proof that a candidate UBE3A substrate is indeed ubiquitinated by UBE3A.

Methods and results: We have here used an unbiased ubiquitin proteomics approach, the bioUb strategy, to identify 79 proteins that appear more ubiquitinated in the Drosophila photoreceptor cells when Ube3a is over-expressed. We found a significantly high number of those proteins to be proteasomal subunits or proteasome-interacting proteins, suggesting a wide proteasomal perturbation in the brain of Angelman patients. We focused on validating the ubiquitination by Ube3a of Rngo, a proteasomal component conserved from yeast (Ddi1) to humans (DDI1 and DDI2). Ube3a-mediated Rngo ubiquitination in the fly neurons was confirmed by immunoblotting. Using human neuroblastoma SH-SY5Y cells in culture, we also observed that human DDI1 is ubiquitinated by UBE3A, without being targeted for degradation. We have also observed that DDI1 is expressed in the developing mice brain, with a significant peak at E16.5.

Conclusions: Our data suggest that DDI1 has biological functions not yet described that could be of relevance for Angelman syndrome clinical research. Additionally, regulation of the proteasome by UBE3A explains how a single E3 ligase is responsible for such a complex syndrome.

Keywords: Ubiquitin, Angelman, neuronal, proteostasis, PTMs, quantitative proteomics
A missing value-compatible algorithm to calculate absolute phosphorylation stoichiometry from LFQ-DIA, SILAC and TMT-based data

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Background: Comprehensive mass spectrometry (MS)-based proteomics is now feasible but reproducible quantification of post-translational modifications (PTM) such as phosphorylation remains challenging. Recently, our group compared LFQ, SILAC and MS^2/MS^3-based TMT quantification methods for cell signaling studies, and showed that reliable quantification of phosphopeptides is dependent on accuracy, precision and peptide coverage of the chosen method (Hogrebe et al., Nat Com 2018). Based on our findings, we introduced a linear modeling-based algorithm which exploits the multiplexed aspect of MS^3-measured TMT data to calculate absolute phosphorylation stoichiometry for all conditions at the same time. While promising, this approach is limited to currently eleven TMT channels, and impaired by so-called ratio compression of extreme phosphopeptide changes. The depth and high accuracy of LFQ with data-independent acquisition (DIA) could overcome these limitations (Kelstrup et al., J Proteome Res 2017). On peptide-level however, even LFQ-DIA suffers from a high number of missing intensity values, which is incompatible with the linear modeling approach.

Methods: Here, we expanded our stoichiometry algorithm for use with any type of missing value-containing data, including LFQ-DIA. We achieve this by introducing a peptide consolidation step, which combines different charge states and PTM-modified peptide variants into a single peptide form, thereby eliminating missing values between them.

Results: We tested our new approach with a LFQ-DIA-measured dataset of RPE1 cells stimulated with EGF and different kinase inhibitors in a total of 17 conditions, which resulted in more than 7000 stoichiometry values. Hierarchical clustering of stoichiometry data distinguished different concentrations of the same inhibitor precisely, and stoichiometry changes provided information complementary to traditional peptide ratio-based analysis.

Conclusions: We are working on releasing our algorithm as an R-plugin in Perseus, which will allow the scientific community to extract absolute phosphorylation stoichiometry information from any type of quantitative phosphoproteomics data.

Keywords: phosphoproteomics, stoichiometry, DIA, LFQ, TMT
Quantitative phosphoproteomics reveals novel phosphosites downstream Kappa-opioid receptor in human spermatozoa


*Equal contribution

**Background:** Human spermatozoa express G protein coupled receptors (GPCRs) which actively participate in the regulation of their fertilizing capacity. Due to the incapacity of human spermatozoa to express new proteins, they may possess different signalling pathways underlying GPCRs compared to those present in somatic cells. Therefore, the aim of this study is to reveal which are those specific molecular mechanisms activated downstream GPCR in human spermatozoa.

**Methods:** We carried out phosphoproteomic approaches in order to study the signalling pathways underlying GPCRs, specifically the kappa-opioid receptor (KOR). Human spermatozoa were stimulated at different time points with U50488H, the specific agonist. After sample processing, we performed phosphopeptide enrichment with TiO2 beads followed by the chemical labeling with TMT to further analyze them by a Q Exactive Mass spectrometer (Odense, DK). MS raw files were processed with MaxQuant software v1.3.0.7 and Perseus bioinformatic program. Functional assays were conducted by flow cytometry using the anti-CD46 antibody.

**Results:** U50488H regulates the phosphorylation of novel phosphosites belonging to human sperm specific proteins. These phosphosites could be involved in the regulation of a novel calcium signaling pathway which differs from those found in somatic cells supporting the idea that GPCRs present unique features in their molecular mechanisms in human spermatozoa. This finding could describe the first steps of the signalling pathways activated via KOR and which lasts in an inhibition of the acrosome reaction, as the functional studies confirm.

**Conclusions:** The human sperm GPCRs regulate different signalling pathways comparing to the ones described in somatic cells via the phosphorylation of sperm specific proteins. As the spermatozoa are transcriptionally and translationally silent cells, the post-translational modifications such as phosphorylations play a key role in the fulfillment of the different physiological functions.

**Keywords:** Opioid system, Kappa-opioid receptor, acrosome reaction, signaling pathways.
Histidine phosphorylation as another dimension of the phosphoproteome
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Background
Histidine phosphorylation is known to have crucial biological functions in prokaryotes. In recent years compelling evidences that histidine phosphorylation also plays important biological roles in eukaryotes gradually accumulated. Nevertheless, the lack of adequate analytical methods to study the unstable histidine phosphorylation constitutes to this day the biggest hurdle to the large-scale study of histidine phosphorylation and ipso facto to the unraveling of the full extent of its biological roles.

Method
Here we present - to this day - the sole method capable of enriching histidine phosphorylation and providing site-specific localization and quantification1. We demonstrated for the first time that in the right conditions, Fe³⁺-IMAC is suitable for the enrichment of histidine phosphorylation at the peptide level. Along the way, we identified nucleic acid-containing biomolecules as the main contaminants in phospho-enriched samples after affinity chromatography. We tackled this problem by developing a robust and efficient sample preparation protocol, incorporating nucleic acid enzymatic digestion and protein precipitation2.

Results
As a result, we were able to improve the number of STY phosphorylation sites identified by more than 50% in human cell lines, and by more than 10 times in bacteria, which are notoriously difficult to analyze. After further optimizations, we applied this workflow to study phosphorylation in E. coli, which resulted in the broadest reported bacterial phosphoproteome coverage. Remarkably, around 10% of the identified phosphosites were on histidine residues. We show that our method is sensitive and reproducible in term of both identification and quantification. In addition, we report for the first time that the phosphohistidine immonium ion can be used as a diagnostic tool to unambiguously localize histidine phosphorylation.

Conclusion
This work paves the way for the study of the elusive histidine phosphorylation and more generally of long-neglected phosphorylation dynamics in microorganisms.

Keywords
Phosphoproteomics, histidine phosphorylation, IMAC, phosphopeptides enrichment

Application of thiol redox proteomics to clinical samples – valve heart disease and ischemic stroke

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Background:

Aortic valve disease is the most common valve disease in Western countries, and the main indication for surgical valve replacement. Two presentations with different pathogeny predominate: degenerative aortic stenosis is the most frequent while aortic insufficiency has lower prevalence. On the other hand ischemic stroke is caused by a blood clot that blocks a blood vessel in the brain. Differentiating stroke etiology is essential for treat patients after post-stroke rehabilitation in order to avoid a stroke recurrence. We aim to study changes in protein levels and posttranslational modifications that explain differences among aortic and insufficiency diseases and may provide candidate biomarkers for differentiating among clinical entities.

Methods:

We have analysed aortic valves obtained from patients with aortic stenosis, patients with aortic insufficiency or valves from hearts replaced during organ transplantation in which the normal function of the valve was previously assessed. Samples from ischemic stroke were collected by thrombectomy and classified attempting to their etiology. Gelsilox methodology and isobaric labelling have been used in order to estimate the relative quantitation of protein level and the relative cysteine-containing peptides in their reduced and oxidized forms.

Results:

Statistical analysis using WSSP method for both samples allowed us to establish differences in peptide, protein and category level when clinical groups are compared. While protein and category analyses discriminated stenosis and healthy valve clinical groups, cysteine modifications discriminated insufficiency and healthy groups. Regarding stroke thrombi samples, protein quantitation allowed to distinguish both clinical groups.

Conclusions:
Combination of quantitative proteomics and thiol redox proteomics, and the use of complementary statistical analysis methods allows enhancing the classification of samples from different clinical groups, providing further clues into the pathological mechanisms and biomarkers candidates.

**Keywords**: aortic valve disease, ischemic stroke, quantitative proteomics, thiol redox proteomics, clinical proteomics.
Background: We evaluate the performance level of the first serial trapped ion mobility spectrometry quadrupole time of flight (TIMS-QTOF) instrument enabling a “Parallel Accumulation - Serial Fragmentation” method (PASEF, Meier et al., JPR 2015, PMID: 26538118) on phosphopeptide samples.

Methods: IMAC phosphopeptide enrichments from 200 µg proteolytic digests of Hela protein digests and from 330 µg proteolytic digests of HEK cells were HPLC separated (nanoElute, Bruker Daltonics) on 250 mm pulled emitter columns (IonOpticks, Australia) and analyzed on a high-resolution timsTOF Pro instrument (Bruker Daltonics) on different LC gradients from 30 - 90 min. Post-processing analysis was performed in Mascot 2.5.1 (MatrixScience). Peptides and phosphopeptides were filtered to <1% PSM FDR.

Results: High-throughput DDA measurements acquired from phosphopeptides enriched from 200 µg of Hela protein digest starting material resulted in 14,000 unique peptides identified after a 90 min LC-TIMS-PASEF-MS experiment. Injection of 8% of the HEK preparation resulted in more than 9600 unique phosphopeptides identified after a similar LC-TIMS-PASEF-MS experiment. The TIMS separation also allowed to separate and fragment coeluted isobaric phosphopeptides that were only differing by the phosphorylation site that was occupied.

Conclusions: The sensitivity of TIMS-PASEF experiments is preserved @ MS/MS acquisition speeds exceeding 100 Hz. Furthermore, the ion mobility dimension separation facilitates the detection and fragmentation of phosphopeptides. These combined features will enables to make a significant step towards comprehensive proteome analysis.

Keywords: TIMS-PASEF, PTMs, Phosphoproteomics.
Innovative glycoproteomic approaches for cancer biomarker discovery

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Background:

The high mortality and poor prognosis observed in cancer reflects the late diagnosis due to the appearance of clinical symptoms only when the cancer has progressed. Early detection, as well as a patient-oriented therapy, are some of the most promising approaches to improve the long-term survival of cancer patients. Therefore, there is a pressing need for the development of new biomarkers, to improve diagnosis, prognosis and treatment of cancer patients 1.

Glycosylation is one of the most abundant and complex forms of post-translational protein modifications (PTM) and the most important to consider for the cell surface and secreted proteomes. Cancer cells exhibit a marked disorganization of the secretory pathway organelles, ER and Golgi. This change, combined with altered expression of glycogenes, means that glycosylation pathways are dramatically affected in cancer cells leading to the expression of cancer-associated glycoforms of glycoproteins in cancer cells 1.

The challenge to identify protein alterations in cancer has started to be faced more than 60 years ago. In the last decade, advances in protein analytical techniques with the advent of "omics" and mass spectrometry approaches, namely cancer (glyco)proteomics, combined with focused and well-designed experimental approaches are leading to significant findings in biomarker research 2,3,4,5.

Methods:

Development of glycoproteomics approaches for cancer biomarker characterization, including:

- glycoprotein enrichment by lectin affinity chromatography and immunoprecipitation from cancer cell lines protein extracts and serum
- western blots and lectin blots against cancer associated glycan antigens
- characterization of glycans and glycoproteins by mass spectrometry
- glycan in cell and in tissue detection by immunoflourescent labeling

Results:

We highlight results from 3 studies:

i) Serum glycoproteomic analysis using 2D-gel electrophoresis, Western blot, and MALDI-TOF/TOF mass spectrometry led to the identification of circulating proteins carrying cancer-associated altered glycans 4.

ii) Cellular- and receptor-specific glycan profiling of ErbB2-overexpressing NCI-N87 cells unveiled a heterogeneous glycosylation pattern harboring the tumor-associated sialyl Lewis a (SLea) antigen 4.
iii) Glycosylation characterization of exosomes subpopulations from different cancer cell lines.

Conclusions:

The study of cancer-associated glycosylation by glycoproteomics approaches, including detailed glycosylation characterization by mass spectrometry, is essential for the development of novel cancer-associated glycoprotein biomarkers.

Keywords: Glycosylation, cancer, biomarkers, glycoproteomics

References:


Acknowledgments

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Rapid Phosphoproteomics by HIFU-TiO$_2$-SCX-LC-MS/MS

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**Background:** To date, substantial efforts have been directed toward identification and characterization of the maximum number of phosphorylation sites per experiment. Thus, a new strategy for the Rapid Phosphoproteomics Analysis is presented in this work.

**Methods:** The performance of the method was established for the global phosphoproteome analysis of un-stimulated human Jurkat leukemia T cells (E6.1). The proposed method is based on six main steps: (a) cell lysis and protein extraction (time: 45 min), (b) in-solution trypsin digestion accelerated under an ultrasonic field provided by high-intensity focused ultrasound (HIFU) (time: 10 min), (c) a single step of phosphopeptide enrichment using TiO$_2$ (time: 90 min), (d) fractionation by strong cation exchange chromatography (SCX) (time: 60 min), (e) analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a high-resolution LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) (time: 60 min/run), and (f) data analysis using BYONIC and SEQUEST-HT (Proteome Discoverer 2.1, Thermo Fisher Scientific) (time: 60 min).

**Results:** Using this accelerated workflow, 15,367 phosphorylation sites from 13,029 different phosphopeptides belonging to 3,163 different phosphoproteins were efficiently identified with high-throughput and reproducibility in less than 15h.

**Conclusions:** Results demonstrates that the present strategy, HIFU-TiO$_2$-SCX-LC-MS/MS, is the fastest analytical method reported to date for generating reproducible large-scale phosphoproteomics datasets in a limited time (<15h).

**Keywords:** Phosphoproteomics, high-intensity focused ultrasound (HIFU), TiO$_2$, strong cation exchange chromatography (SCX), mass spectrometry, human Jurkat T cells.
Differential phosphorylation in human FETUIN-A may provide a switch to prevent soft tissue calcification in patients in peritoneal dialysis

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Background: Peritoneal dialysis (PD) is a form of renal replacement in chronic kidney disease [1]. During long-term PD, morphological changes occur in the peritoneum leading to changes in solute and water conductance. These include expansion of extracellular matrix to form fibrous tissue leading to thickening of the membrane and neovascularization, resulting in ectopic calcification [1]. Vitamin D is a crucial regulator of bone metabolism and calcium homeostasis; nevertheless, recent research suggests that its biological action goes beyond this and also include regulation of inflammation and angiogenesis, as well as cell growth, differentiation, and apoptosis of many cell types. One of the earliest pathologic features of CKD patients is active vitamin D deficiency as conversion of vitamin D3, 25-hydroxyvitamin D3(25D), into the bioactive form, 1,25-dihydroxyvitamin D3 (1,25D), occurs mainly in the kidney [1, 2]. Increasing data show that vitamin D receptor agonists therapy decreases proteinuria, may reduce renal damage progression, and improves cardiovascular outcomes in CKD patients [2].

Methods: In the present work, we carried a time-resolved proteomics study (T0, T1 and T3) on peritoneal dialysate effluent from of patient treated with a vitamin D receptor agonist to evaluate effect of this treatment in CKD patient. The PDE samples were treated as described previously [1] and analysed by 2D-GE and nanoLC-ESI-qTOF-MS/MS to determine altered protein expression levels in response to treatment.

Results: In our work, we have identified key changes in phosphorylated Fetuin-A in response to vitamin D receptor agonists therapy, which might be correlated to decrease in calcification risk.

Conclusions: Vitamin D receptor agonists therapy in CKD hold a great potential to manage soft tissue calcification in patients in peritoneal disease, therefore contributing to decrease morbidity and mortality in these patients.

Keywords: Protein phosphorylation, Human FETUIN-A, peritoneal dialysis

B-cell chronic lymphocytic leukemia (B-CLL) is a blood cancer with highly heterogeneous genomic alterations and altered signaling pathways. To date, no studies have investigated the B-CLL proteome. Here, we report the overall (quantitative) proteome of B-CLL and CLL-like monoclonal B-cell lymphocytosis (MBL) primary cells and the phosphoproteins potentially involved in the baseline-pathological B-cell behavior using a high-resolution mass spectrometry-based approach. Overall, 2,970 proteins and 327 phosphoproteins were quantified across the five studied tumor samples (ProteomeXchange-PXD005997) including 329 phosphopeptides reported here for the first time. Although the five tumor proteomes shared a significant overlap (73%), the phosphoproteomes varied significantly highlighting the importance of B-CLL phosphosignature investigations. Despite such heterogeneity, tumor B-cells from different CLL and MBL patients also displayed common phosphoproteins such those involved in BCR-signaling, cell-cell interactions, and NF-kB/STAT3 pathway. Notably, these phosphoprotein profiles were independent of the cytogenetic alterations and/or IGHV mutational status of tumor cells. Despite the great similarity observed between CLL and MBL cells, they showed different levels of phosphorylated ARIH2 and PTPN11 suggesting a role in disease progression. Therefore, our results provide new insights into the global proteome and phosphoproteome of B-CLL and MBL cells and the immune signaling pathways involved in its development and progression.
Depicting the platelet CLEC-2 signalling cascade by a phosphotyrosine proteomic approach: impact of secondary mediators.

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Background: CLEC-2 (C-type lectin-like receptor-2) is a membrane receptor highly expressed on platelets that is known to play a relevant role in thrombosis and cancer metastasis. The CLEC-2 signalling cascade involves the participation of several tyrosine-kinases such as Src family kinases (SFK), and Syk, among others. CLEC-2 signalling is reinforced by the release of secondary mediators (ADP and TXA2) upon primary receptor activation. In the present study we performed a comparative phosphotyrosine proteomic analysis of basal and CLEC-2-activated platelets.

Methods: Platelets were activated with the CLEC-2 specific agonist Rhodocytin in presence and absence of the secondary mediators inhibitors Apyrase and Indomethacin. After FASP (Filter aided sample preparation) digestion of platelet lysates, tryptic peptides were immunoprecipitated with a phosphotyrosine specific antibody and then analysed by LC-MS/MS in a LTQ Orbitrap XL. Database search was performed with the Proteome Discoverer v1.4 software at FDR=1%. Phosphosites probabilities were predicted by the ptmRS tool.

Results: Only phosphopeptides identified in at least 3 of the 4 biological replicates analysed were taken into account for comparison, that is, 57 phosphopeptides for basal samples, 43 for rhodocytin-stimulated samples in presence of secondary mediators inhibitors, and 102 for rhodocytin-stimulated samples in absence of inhibitors. Our results suggest that phosphorylation of the kinases PKC, Tec, BTK, Fer and MAP is highly dependent on secondary mediators, whereas the adapter LAT, and VASP are properly phosphorylated upon CLEC-2 receptor activation on its own. Interestingly, numerous signalling proteins such as Src, Lyn, G6f, G6b and integrin β3 were found constitutively tyrosine-phosphorylated on resting platelets.

Conclusions: This novel study allowed the identification of the main players in the CLEC-2 cascade mapping relevant tyrosine phosphorylation sites. The relevance of the identified phosphorylations for protein interactions in the CLEC-2 signalosome is currently under study.

Keywords: Platelets, phosphoproteomics, phosphotyrosine, CLEC-2
Proteome-wide redox profiling in mesothelial cells during experimental peritoneal dialysis by strategic thiol reactive labeling

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Peritoneal dialysis (PD) is a renal replacement therapy for patients with end-stage renal failure. PD uses the peritoneum as semi-permeable membrane to remove uremic toxins and water from the patient by a glucose-based PD fluid (PDF). PDF strongly contribute to generation of reactive oxygen species (ROS) in the peritoneal cavity, which induce alterations to proteins, thus impairing cell viability and progression of PD-related local and systemic side effects. We aim to analyze the impact and specific targets of ROS during PD and potentially cytoprotective and anti-oxidative mechanism of glutamine-supplementation of PDF.

To establish an analytical redox-proteomics workflow for studying oxidative stress in peritoneal mesothelial cells we used a gold-standard model of redox-stress ($H_2O_2$) and PDF-induced stress. Levels of oxidative stress were first analyzed by intracellular fluorescent ROS staining, superoxide dismutase (SOD) activity and formation of advanced oxidation protein products (AOPP). Alterations of the redox proteome were analyzed in a comprehensive redox proteomics approach by either directly or indirectly labeling cysteine residues with fluorescent dyes (redox-2D-DIGE) or isobaric tags (redox-TMT).

SOD, AOPP and intracellular ROS showed induction of significant levels of oxidative stress by PDF and $H_2O_2$ treatment and a reduction of these parameters by the addition of glutamine. The proteomics approach, which labels the reduced or oxidized cysteines revealed a decrease in reduced and increase in oxidized cysteines in all treatments compared to control. 2D-gels allow global visualization of the reduced and oxidized cysteines of a complex protein sample and generation of redox profiles for individual protein spots (540 spots by direct labeling and 362 by indirect). LC/MS redox proteomics confirmed the quantitative levels seen on cysteine oxidation and additionally allowed identification of individually oxidized proteins.

Redox proteomics of peritoneal cells could represent a novel approach for the identification of biomarkers for improving PD and to evaluate antioxidant therapeutic interventions.
AN INTACT WORKFLOW FOR HUMAN CANCER CELL LINE PHOSPHOPEPTIDE ANALYSIS (10/15) (POSTER ONLY)

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Background: Immobilized metal affinity chromatography (IMAC) using a nitrilotriacetic acid (NTA) chelating ligand functionalized with Fe(III) is one of the most widely adopted phosphopeptide enrichment techniques for LC/MS applications. Agilent AssayMAP Bravo platform is able to provide a fully automated phosphopeptide enrichment workflow using high capacity Fe(III)-NTA cartridges. In this study, we evaluated one of the main factors affecting the performance of the enrichment, the ratio of total peptide sample amount to affinity resin used in our protocol.

Methods: The Agilent AssayMAP Phosphopeptide Enrichment V2.0 App was used for automated phosphopeptide enrichment with Fe(III)-NTA cartridge (5 µL bed volume). Human MCF7 breast cancer cell line tryptic digest was loaded onto the cartridges with four different sample to cartridge ratios. Nanodapter was used to convert 1290 UHPLC to nanoflow UHPLC which was coupled to a mass spectrometer through the Agilent nanoESI source. With a 90-min nanoLC gradient, 6550 iFunnel Q-TOF was used for phosphopeptide discovery. MRM analysis of 20 light and heavy pre-spiked phosphopeptide standards was performed on 6495B QQQ to measure their overall recovery from the enrichment procedure.

Results: In the pre-enriched sample, about 1.5% of peptides were identified as phosphopeptides. After enrichment, phosphopeptide selectivity was routinely above 90% across all 12 enriched samples. The total number of phosphopeptides increased with the increase of the sample amount to cartridge ratio. The recovery of the standard phophopeptides are generally consistent across different ratio.

Conclusions: AssayMAP Bravo provides fully automated, highly selective and reproducible enrichment for phosphopeptides. Nanodapter effectively converts UHPLC to nanoflow UHPLC which allows users to have both standard flow and nanoflow LC in one system. Together with nanoESI source, 6550 iFunnel Q-TOF offers the ultimate sensitivity for phosphopeptide identification. 6495B Triple Quadrupole offers fast, accurate and robust MRM based peptide quantitation.

Keywords: AssayMAP, Fe(III)-NTA, phosphopeptide enrichment, peptide quantitation

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Quantitative analysis of protein redox posttranslational modifications in haematopoietic stem and progenitor cells

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Background: The impact of redox homeostasis on the initiation and the progression of haematopoietic malignancies has been highlighted. To date, the precise mechanism underlying redox regulation of blood cell development in foetal and adult haematopoiesis remains unclear. However, a critical role for oxidative modifications of protein thiols has been pointed out. Our recent findings showed significantly decreased levels of proteins involved in redox homeostasis and antioxidant defence in foetal liver haematopoietic stem and progenitor cells (HSPCs) compared to adult bone marrow HSPCs. We suppose that insufficient protection of protein thiols in foetal HSPCs lead to extensive oxidative damage upon leukaemia initiation in these cells. The goal of our work is to characterize proteins susceptible to thiol oxidative modifications in foetal haematopoiesis.

Methods: To achieve our goal, we applied sequential iodoTMT labelling strategy and nanoLC-MS3 method to FACS sorted primary cells. Foetal and adult Lineage- Sca-1+ c-Kit+ (LSK) HSPCs were acquired by FACS sorting of cells extracted from mouse foetal liver (FL) at day 14.5 of gestation and adult bone marrow, respectively. Free and oxidized protein thiols in cell lysate were sequentially labelled using an isobaric set of thiol-reactive iodoTMT-sixplex reagents. After digestion, iodoTMT-labelled peptides were enriched using anti-TMT resin and analysed by nanoLC-MS3 using Thermo Scientific Orbitrap Fusion.

Results: In total, we identified 4184 Cys peptides corresponding to 2216 protein groups with the average 87% efficiency of the enrichment in three replicates of mouse FL and ABM HSPCs. We found in total 98 proteins with significant change of oxidation between FL and ABM HSPCs. 96 proteins were significantly oxidized in FL compared to ABM. These proteins were enriched for biological processes related to protein translation.

Conclusions: We identified candidate proteins that are promising targets for further investigations of their role in haematopoiesis in relation to oxidative changes.

Keywords: Redox proteomics, PTMs, ROS, haematopoiesis, haematopoietic stem cells, iodoTMT label
Applied phosphoproteomics: Selective view on cGMP/PKG stimulation by Riociguat versus cAMP/PKA stimulation in human platelets

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Background: Human platelets are tightly regulated by multiple vasoactive substances or drugs including endothelial-/drug-derived NO and prostacyclin (Iloprost). NO activates the soluble guanylyl cyclase (sGC)/cGMP pathway and inhibits platelets, often in synergy with prostacyclin. cGMP effects in platelets are poorly understood and sometimes controversial. Previously, we already characterized the human platelet proteome (Burkhart et al. Blood 2012), and dynamic changes of the phosphoproteome upon Iloprost and ADP treatment (Beck et al. Blood 2014; Beck et al. Blood 2017).

Methods: Human platelets were either stimulated with Iloprost (cAMP/PKA) or Riociguat, a robust, cAMP-independent sGC-stimulator. Changes in protein phosphorylation were studied by tailored, large-scale quantitative LC-MS/MS phosphoproteomics workflows employing two different proteases. A targeted LC-MS/MS workflow was created to validate preliminary results.

Results: In total we quantified 8181 phosphorylation sites from 2249 proteins across three biological replicates. Riociguat increased (>1.5-fold up) and decreased (>1.5-fold down) phosphorylation levels in 345 and 94 proteins, respectively. These covered many platelet functions, including 24 protein kinases (e.g. MYLK, CAMKK1/2, CDK16/17/18, BRAF) with increased and 4 protein kinases (KALRN, KSR2, PAK2, WNK1) with reduced levels. Comparison of the Riociguat- and Iloprost phosphoproteomes showed a significant overlap but also specific Riocigat/cGMP effects. The top-list of Riociguat affected phosphoproteins includes known PKG substrates (e.g. VASP, ITPR1, MRVI1/IRAG) but also novel candidates including the PP2A inhibitor ENSA. A draft method for result validation by targeted LC-MS/MS
showed to be able to reproducibly monitor 47 putatively regulated and biologically meaningful phosphopeptides.

**Conclusions:** This first human platelet phosphoproteome of the Riociguat/sGC/cGMP pathway demonstrates an unexpected magnitude, diversity and involved protein kinase network. The Riociguat/sGC/cGMP and Iloprost/cAMP substrates show a remarkable overlap with only few specific events. Our data also provide a number of novel PKG substrates including the PP2A inhibitor ENSA which require further study.

**Keywords:** Bioanalytics, nano LC-MS/MS, phosphoproteomics, platelets, signaling
Quantitative Persulfide Site Identification highlights a direct involvement of H$_2$S in Amyotrophic Lateral Sclerosis

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**Background:** Hydrogen sulfide (H$_2$S) is one of the gasotransmitters that participates in multiple biological signalling pathways. Moreover, H$_2$S mediates the formation of cysteine persulfides (R-S-SH), which affect the activity of target proteins. The biological reactivity of the sulphur atom in cysteine is a significant factor in determining susceptibility to oxidative damage and neurodegeneration.

In our previous study, we demonstrated toxic levels of H$_2$S in Amyotrophic lateral sclerosis (ALS)$^1$. To better clarify the H$_2$S-ALS interaction, this study aims to detect and quantify persulfide-containing peptides in the ALS proteome.

**Methods:** Proteomics analysis was performed on spinal cord of transgenic SOD1$^{G93A}$ mice at symptomatic disease-stage. MS analysis included both complementary approaches: DDA analysis on a UHPLC-Q Exactive Plus (Thermo Scientific) and ion mobility–enhanced DIA analysis by Expression configuration mode (HDMS$^e$) on Sinapt G2-Si MS equipped with an ACQUITY MClass System (Waters). For peptide identification, Xcalibur and Waters raw files were analyzed by Peaks v6.0 and PLGS v3.0.2 (Waters) softwares, respectively. Quantitative analysis was performed using MaxQuant software.

**Results:** We developed a MS-based workflow for the enrichment and site specific identification of cysteine persulfides in tissues, modifying the already published protocol for cells$^2$. First, thiols and persulfides were labelled using iodoacetyl-PEG2-Biotin; single peptides containing either labelled persulfides or cysteines were enriched using streptavidin agarose beads and then eluted. All the fractions were analyzed by both MS approaches. DDA and DIA analysis showed an overlap of 54 proteins with persulfide containing peptides differently expressed in SOD1$^{G93A}$ than SOD$^{WT}$. Among them, e.g. superoxide dismutase, peroxiredoxin 6 that are known to be redox-regulated.

**Conclusions:** by this innovative MS-based approach on tissues, it is assumed that H2S-induced persulfide formation on several proteins (e.g. SOD1) may play a role in the pathogenesis of ALS.


**Keywords:** ALS, H2S, mass spectrometry, proteomics, sulphhydration
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Abstract

**Background** Data clustering is indispensable for identifying biologically relevant molecular features in large-scale experiments with thousands of measurements at multiple conditions. Optimal clustering results yield groups of functionally related features that may include genes, proteins and metabolites in biological processes and molecular networks. Omics experiments typically include replicated measurements of each feature within a given condition to statistically assess feature-specific variation. Current clustering approaches ignore this variation by averaging, which often leads to incorrect cluster assignments.

**Methods** We present VSCell that accounts for feature-specific variance. Based on an algorithm derived from fuzzy clustering, VSCell unifies statistical testing with pattern recognition to cluster the data into feature groups that more accurately reflect the underlying molecular and functional behavior. We apply VSCell to artificial and experimental data sets comprising hundreds to more than 80,000 features across 6-20 different conditions including genomics, transcriptomics, proteomics and metabolomics experiments.

**Results** VSCell avoids arbitrary averaging methods, outperforms standard fuzzy c-means clustering and simplifies the data analysis workflow in large-scale omics studies. In particular, we show i) high-resolution recognition of underlying patterns with more efficient removal of incorrectly assigned cluster members; ii) improvement of clustering results for unfiltered data; iii) more precise enrichment of features with common biological functions; iv) summarization of peptides to proteins can be avoided by direct application of the clustering.

**Conclusions** VSCell is publicly available and includes statistical tests, estimation of cluster number, variance-sensitive clustering, and gene set enrichment by DAVID.

Download VSCell at [https://bitbucket.org/veitveit/vsclust](https://bitbucket.org/veitveit/vsclust) or access it through [computproteomics.bmb.sdu.dk/Apps/VSClust](http://computproteomics.bmb.sdu.dk/Apps/VSClust)

**Keywords** Cluster analysis, Statistics, Omics data
PROTEOGENOMICS OF ADENOSINE-TO-INOSINE RNA EDITING IN MODEL ORGANISMS

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Background: Proteogenomics combines multi-omics data to provide comprehensive knowledge on molecular repertoire of living organisms. RNA editing catalysed by RNA-dependent adenosine deaminase (ADAR) enzymes may recode mRNA to provide new proteoforms. Consequences of RNA editing at the proteome level were still not studied in model animals. Using publicly available and own shotgun proteome data, we have identified A-to-I editing events in brain proteomes of fruit flies and mice.

Methods: Next-generation sequencing open data on RNA editomes for both model animals were converted for proteomic search of edited proteins. Publicly available and own high-resolution shotgun proteome data were used to identify edited peptides by X!Tandem search engine with calculation of the separate FDR. Some findings from fruit fly study were validated at the mRNA level and by targeted mass-spectrometry with isotope-labelled standards.

Results: Using three independent datasets of Drosophila melanogaster head or brain proteome, about 70 sites of coding RNA editing were identified, of them 7 were found in all 3 datasets, and about 30 in all datasets. The function of synaptic vesicle exocytosis was substantially enriched between edited proteins. Some findings of more common editing events were validated by MRM targeted proteome analysis, with measurement of the ratio between edited and intact counterparts in fruit fly brains. As for mice data, up to 15 editing events was identified in mouse brain datasets. It was shown that RNA editing was more common in invertebrates than in mammals. Still, findings in mouse brain were in good correspondence with prior art. In particular, canonical editing events in glutamate receptors, such as Gria2-4 products, were detected.

Conclusions: Proteogenomics provides successful identification and quantitation of protein changed as a result of mRNA editing. The targeted approach may be used to elucidate function of edited proteins in various disease and environmental models.

Keywords: proteogenomics, RNA editing, fruit fly, mouse, central neural system
CREATING A COMPREHENSIVE FUNCTIONAL MAP OF THE HUMAN PHOSPHO-PROTEOME

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Background: In recent years we have developed an infrastructure to enable robust data sharing of mass spectrometry proteomics data in the public domain, including the world-leading PRIDE database and related tools, open data standards and the establishment of the worldwide ProteomeXchange Consortium of proteomics repositories. Thanks, among other efforts, to the success of PRIDE and ProteomeXchange, the proteomics community is now widely embracing open data policies. This plethora of data is being increasingly reused by the research community, e.g. in proteogenomics approaches, creation of spectral libraries and innovative meta-analysis studies. In this context, I will explain an in-house project aiming to build a functional and comprehensive version of the human phospho-proteome, using public datasets as the base.

Methods: First, we manually selected and further curated 118 phospho-enriched PRIDE public datasets coming from human cell lines and tissues (in non-diseased conditions). We re-analysed all the datasets together in a conservative way using MaxQuant, obtaining 120,000 phospho-peptides. However, it is well-known that phosphorylation sites are not equally relevant from a functional point of view. We then developed a machine-learning approach to develop a phosphorylation functional ranking score for each phosphosite.

Results: The features used in the approach were selected based on different groups of properties: regulatory information, the amino acid sequence, the three-dimensional structure, the available MS evidence from the analysis, and taxonomic data. Different approaches were developed for Ser/Thr and for Tyr phosphorylation. The resulting functional score has also been validated in vivo.

Conclusions: This study highlights just one of the many possible directions in reuse of public proteomics data.

Keywords: proteomics repositories, public data reuse, human phospho-proteome, machine learning.
Benefits of rapid profile of modifications with SpecOMS

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Background: A number of open modification search (OMS) methods have been developed in recent years because up to 75\% of the spectra may remain unidentified after a conventional analysis. The presence of modifications is considered the most likely explanation for this low rate of identification. However, until recently, OMS methods were not used routinely because they were confronted to both excessive calculation times and loss of sensitivity.

Methods: The SpecOMS software is able to identify tens of thousands of MS/MS spectra within minutes without applying a mass filter of any kind on a standard workstation. Then, SpecOMS can reveal the full profile of modifications displayed by complex peptide mixtures very quickly. SpecOMS v1.2 is now available at https://github.com/matthieu-david/SpecOMS.

Results: We will illustrate on several spectra datasets downloaded from PRIDE how to take better advantage of these modification profiles whether it is to master the artifacts of an experiment, to maximize the sensitivity of a search or to identify rarer PTMs. In particular, we will show how to configure SpecOMS and how to evaluate the FDR to obtain a better sensitivity than cascaded approaches applying successive conventional searches with variable modifications.

Conclusions: Even with a large search space, OMS methods can identify a greater number of spectra than conventional methods under certain conditions. In addition, OMS methods open the way to the discovery of a wide variety of modifications. The bottlenecks that limited the use of OMS methods are being resolved.

Keywords: Bioinformatics; MS/MS spectra interpretation; open modification search; pattern discovery; post translational modifications
Scop3P: the bridge between human phosphosites, protein structure and PRIDE data

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Presentation type: ‘Oral or Poster’

Background

Protein phosphorylation is one of the most important and best-studied post-translational modifications because of its involvement in the regulation of many biological processes. This is reflected in its association to human diseases such as cancer and metabolic disorders. Phosphorylation can alter the physico-chemical properties, conformation, stability and flexibility of proteins. The accuracy of identification, annotation and functional analysis of these phosphosites is therefore crucial for understanding the regulatory roles of protein phosphorylation. Available resources on phosphorylation usually contain sequence and phosphosite information, but only rarely structural information.

Methods

All available human phosphosites were obtained from the latest release of UniprotKB, and of all experimentally determined phosphosites from PRIDE. Moreover, these sites are mapped on to three dimensional protein structures where available, in PDB. Other secondary structural informations were retrieved from PDBe.

Results

Scop3P: a database of human phosphosites, provides residue and structural level annotations of all known human phosphosites. Every phosphorylation is also annotated with valuable structural information, including structural propensity, solvent accessibility, probability of being a disordered region, and the frequency of observation of this phosphosite in PRIDE experiments.

Conclusions

Scop3P will thus be a valuable resource for the visualization of phosphosites onto protein structures, and for understanding protein phosphorylation structure-function relationships.

Keywords: Phosphorylation, PTM, Protein structure, Public repositories
Systems proteomics of gene expression

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Background: Genes are not randomly distributed in the genome. In humans, 10% of protein-coding genes are transcribed from bidirectional promoters and many more are organised in larger clusters. Intriguingly, neighbouring genes are frequently coexpressed but rarely functionally related. We show that coexpression of bidirectional gene pairs, and closely genes in general, is buffered at the protein level (Kustatscher et al, Mol Syst Biol, 2017). We find that co-regulation of spatially close, functionally unrelated genes is pervasive at the transcriptome level, but does not extend to the proteome. We then demonstrate the implications of this for functional genomics (Kustatscher et al, under revision).

Methods: We integrate genomic, epigenomic, Hi-C, transcriptomic and proteomic datasets to study gene expression, and to understand how mRNA expression differs from protein expression. In a second step, we construct ProteomeHD, a large proteomics dataset documenting the response of 10,323 proteins to 294 biological perturbations. We then use machine-learning to create a proteome covariation map that reveals detailed functional associations between human proteins.

Results: We present evidence that non-functional mRNA coexpression in human cells arises from stochastic chromatin fluctuations and direct regulatory interference between spatially close genes. Grouping human genes together along the genome sequence or 3D structure is associated with reduced expression noise, supporting the hypothesis that the selection for noise reduction is a major driver of the evolution of genome organisation. Building up on these insights, we demonstrate the power of functional proteomics by identifying a new organelle connection between peroxisomes and mitochondria.

Conclusions: mRNA expression reflects gene function and genomic position, protein levels reflect specifically gene function. Functional proteomics finds new organelle interaction.

Keywords: Proteomics, Transcriptomics, HiC, Epigenomics, Functional proteomics
ProteoRE, a Galaxy-based infrastructure for annotating and interpreting proteomics data.

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**Background:** with the increased simplicity associated with producing MS-based proteomics data, the bottleneck has now shifted to the annotation of large lists of proteins for biological interpretation purpose. The ProteoRE (Proteomics Research Environment) primary aims to fulfil this need by centrally providing an online research service to biologists/clinicians without programming expertise to analyze their proteomics data in a collaborative and reproducible manner. ProteoRE is built upon the Galaxy web-based technology, a scientific workflow system allowing for data integration, data and analysis persistence and providing interfaces for users to interact with tools dedicated to the functional and the visual analysis of proteomics datasets.

**Methods:** using output files from quantitative proteomics software (e.g. MaxQuant, Proline…) as a starting point, tools and interfaces have been designed in close collaboration with biologists and clinicians on the basis of real case studies. ProteoRE’s tools have been implemented either by reusing tools (from the Galaxy Tool Shed) or by wrapping Bioconductor packages and external code, then beta-tested by “naïve” end-users.

**Results:** Two case studies have been considered: the first one consists in interpreting a proteins identification list from a human sample [Lacombe et al., 2018] while the second entails selection of biomarkers candidates. Tools implementing the corresponding workflows include i. data manipulation (filtering, identifiers conversion, cross-comparison), ii. protein list annotation (using public data resources), iii. functional and pathways analyses (GO terms frequencies, enrichment analysis) along with graphical representations. A first version of ProteoRE integrating these tools and datasets with online support is in free access: [http://www.proteore.org](http://www.proteore.org)

**Conclusions:** While Galaxy-based tools offers services for proteomics identification (e.g. MS data conversion, protein database tools, search algorithms), tools focusing on proteomics functional analysis are still lacking. ProteoRE is an attempt to fill this gap with the hope of promoting proteomics data in the Life Science community.

**Keywords:** Proteomics data annotation, functional analysis, web server, computer application, Galaxy
Facilitating MALDI-TOF mass spectrometry data analysis with S2P and Mass-Up

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Background: Mass spectrometry-based technologies applied to proteomics such as MALDI-TOF-MS (mass spectrometry using matrix assisted laser desorption ionization coupled to time of flight analyzers) are able to analyze large batches of samples quickly, producing large amounts of high-throughput data. Key to successful analysis of such datasets are bioinformatics tools allowing researchers to automatically preprocess, store and analyze them in a robust and effective way. The purpose of this work is to present two open-source, free-to-use desktop applications for the analysis of mass spectrometry data: S2P (http://www.sing-group.org/s2p), and Mass-Up (http://www.sing-group.org/mass-up).

Results: S2P is a desktop multipurpose application specifically created to perform fast processing of 2D-gel and MALDI-MS protein identification-based data. S2P allows researchers (i) to collect Progenesis SameSpots reports into a full table where all samples can be compared and analyzed, (ii) to link spots to protein identifications obtained with Mascot, and (iii) to view and export data in different ways, among other tasks. Mass-Up is a desktop multiplatform application aimed to allow researchers to perform complete analyses of MALDI-MS data. Mass-Up allows data preprocessing as well as subsequent analysis including (i) quality control, (ii) biomarker discovery, (iii) clustering, (iv) biclustering, (v) three-dimensional PCA visualization and (vi) classification.

Conclusions: S2P and Mass-Up are two open-source, free-to-use applications that allow wet lab researchers and life scientists without advanced bioinformatics skills to analyze their datasets in an easy and straightforward manner. So far, different research groups have successfully used them for conducting multiple proteomics-based research projects.

Keywords: bioinformatics, proteomics, data analysis, Mass-Up, S2P.
Action plan to decipher functions of Chr18 encoded proteins in the frame of neXt-CP50 Challenge

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Background: Within the framework of the Human Proteome Project new initiative for creating functional annotations is launched – neXt-CP50 Challenge. The Russian consortium participates in the project, focusing on the human chromosome 18. According to NextProt (v2.12.0 2017-08-01), for 16 of 275 protein-coding genes there is no information on the functions of the corresponding proteins.

Methods: Two proteins were selected as priority (Q96DM3, Q9P260). For selecting these proteins the functionality of the knowledgebase on the human chromosome 18 was used (www.kb18.ru). A comparative meta-analysis of the extent of research, experimental results, and the variety of proteoforms was carried out for all 16 protein-coding genes. In the context of this work, protein function was understood as its interrelation with other proteins with already known functions (guilt by association), chemical compounds, metabolic pathways and diseases. The main research method was text-mining analysis of unstructured data available in the form of abstracts of scientific publications in the PubMed library.

Results: The algorithm proposed for creating functional annotations was tested on the group of chromosome 18 proteins with experimentally confirmed functions. The results are compared with the data of STRING, GeneMANIA resources and other platforms designed for creating functional annotations. With the use of the algorithm, semantic maps were constructed reflecting the functional specificity of the selected proteins.

Conclusions: For the selected proteins of the human chromosome 18, bioinformatic hypotheses were obtained that describe the functional specificity of these proteins. Experimental validation of the predictions obtained involves the analysis of AP-MS data (affinity purification–mass spectrometry) and the results of assessment of protein-protein interactions performed with an optical biosensor.

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Keywords: HPP, text-mining, interactomics, Chromosome 18, proteome
Comparison of different approaches for the creation of spectral libraries for data independent acquisition analyses

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Type of presentation: Oral or poster

Background: Data-independent acquisition (DIA) is gaining more and more interest as a method for reliable and comprehensive label-free quantification of proteomics data and was already successfully used in several clinical and biomarker discovery studies. DIA though is dependent on good spectral libraries for the peptide respectively protein identification, and there are many methods available for the creation of these.

Methods: As a ground-truth dataset, we created samples with eight spike-in proteins in five different states together with a complex matrix of full C2C12 cell lysates. On this dataset we tested several different approaches for the creation of spectral libraries: different amounts of loaded protein mass per sample, different numbers of DDA runs without fractionation, as well as with peptide and protein fraction before MS analysis. Furthermore, we evaluated the directDIA approach of Spectronaut Pulsar (Biognosys) as stand-alone method, as well as a merge together with the before mentioned DDA runs. Furthermore, we compared the analyses with a stand-alone DDA analysis of the same sample.

Results: Depending on the used method, the spectral libraries vary in their ability to identify and thus quantify the peptides in the analysed samples. As we generated a ground-truth dataset, we were able to not only evaluate how many proteins could be identified, but also give estimates on how well the quantification worked. This was analysed as well on a qualitative level (were the proteins found to be differential) as well as quantitative (how well was the actual fold change mirrored by the analyses).

Conclusions: The method of choice for the creation of spectral libraries depends on the availability of samples, which can be used for spectral library generation, as well as the effort taken to pre-fractionate samples and the available MS machine runtime. We will give some considerations of which method to take, depending on these limiting factors.

Keywords: data independent acquisition, label-free quantification, spectral library
Efficient and user friendly label-free quantification with the Proline software suite

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Background: Label-free quantification based on precursor ion intensity is a widely used method for quantifying differentially expressed proteins across different conditions or samples. An ideal software solution should allow the production of reliable and comprehensive results, and be flexible enough to allow the integration of existing tools without compromising ease-of-use. Importantly, it should also offer a graphical user interface (GUI) to manually check and fix erroneous quantitative results (bad signal extraction or wrong mapping between identification and quantification data).

Methods: To meet these objectives we developed the Proline software, a next-generation tool based on a modular data processing toolbox. Imported and generated data are persisted in a relational database (PostgreSQL), providing thus a very high sustainability and traceability of the produced results. An original signal detection algorithm was developed taking advantage of the recent mzDB file format. To assess the performance of the label-free module, we used a proteomic standard dataset composed of an equimolar mixture of 48 human proteins (UPS1, Sigma) spiked at different concentrations into a yeast cell lysate background.

Results: Compared to MaxQuant, Proline shows a lower rate of missing values and a better similarity between observed and expected ratios, on the used standard dataset. Moreover it is faster than existing solutions because it leverages the optimized mzDB file format, either for signal processing or raw data display. Visual inspection of the results can be performed through two user-friendly GUIs, Proline Studio (desktop application) and Proline Web (for remote access). Thanks to its architecture Proline may interoperate with any programming language and could be easily plugged into another workflow system.

Conclusions: The benchmarking study shows that Proline provides high quality label-free quantitative results and constitutes a very interesting alternative to competing solutions, combining robustness, performance, modularity and user-friendliness.

Keywords: quantification, label-free, tools, algorithm, software, visualization
Artifactual methionine-to-isothreonine conversion during sample preparation in proteogenomics

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Background

Met-to-isoThr conversion in proteogenomic data was noted by us [Chernobrovkin et al., 2015] and nearly led to a false discovery of a mutation. As Met is known to turn into isoThr under the action of iodoacetic acid [Gundlach et al., 1959] it was hypothesized that this event may occur during the alkylation step of sample preparation.

Methods

Deep proteomic data was taken from ProteomeXchange and searched against a corresponding database using MaxQuant with the introduction of the Met->Thr modification as a variable one along with other default parameters. Then, the datasets having the average of modified Met more than 1% were selected and the modification rate and the influence of the neighboring amino acids were analyzed. Since [Muller et al., 2017] has the data acquired after different sample preparation protocols applied, the Met-to-isoThr modification frequency has been compared according to the reagents used.

Results

According to our observations, the use of iodoacetic acid increases the level of Met-to-isoThr modification. Also, the modification happens more often, if the samples are prepared in-gel. The analysis of the neighboring amino acids shows that Pro following Met in a peptide sequence increases the level of Met-to-isoThr modification if calculated respectfully to the number of MetPro combinations in all identified peptides.

Conclusions

- As iodoacetic acid amplifies Met-to-isoThr modification, it should be avoided during proteogenomic, sample preparation.
- Despite the fact that today iodoacetamide is the most popular alkylating agent, it seems to lose its position to chloroacetamide that causes less Met-to-isoThr modifications.
Especial cases of proteomic studies: which splice-form should be treated as canonical?

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Background: Thousands of genes expressed in a particular cell determine the functionality of the cell. Each step in the flow from DNA via RNA and finally to protein supplies the cell with a potential control point for self-regulation of its functions. This control is implemented by alternation of the amount and type of proteoforms the cell generates by “fine adjustment” of different aberrations. One of the fundamental resources of such aberrations is alternative splicing. The difference between splice-forms is defined by a combination of exons. While the majority of proteomic studies are devoted to examination of canonical sequences, we focused on annotation of prevailing splice-forms in different biomaterials and the opportunity of their detection and annotation.

Methods: RNAseq data of normal liver tissue, lung, bladder, kidney, stomach and esophagus, publicly available in SRA database, were analyzed to identify the prevailing form (canonical or aberrant) by level of expression for each gene. The transcriptomic data were processed by PPLine software. The frequency of detection of proteotypic peptides in proteomic experiments was calculated according to the GPMdb. Data of protein-protein interaction was obtained from IntACT database and using method of virtual co-precipitation for interpretation results of AP-MS experiments.

Results: According to the results of the transcriptomic analysis of six tissues, splice-forms prevail over canonical forms in more than 2.7 thousand cases. Notably, that in 1.6 thousand cases gene products were presented only by splice-forms. According to the UniProt database, there are about 550 thousand of proteotypic peptides in human proteome: 50% of them correspond both to canonical and splice-forms, 40% (~223 thousand) are specific only for canonical form, and 4% are specific exclusively for splice-forms. On example of Chromosome 18 for 10% genes, we determined the functional differences between splice forms based on the interactomic data.

Conclusions: Knowledge about prevailing form is crucial on every step of a proteomic study – from experiment design to data interpretation.

Acknowledgements: El.P., Ol.K., Ek.P. and Ek.I. acknowledge the Leading Scientific School of Prof. Andrey Lisitsa (№NSh6313.2018.4).

Keywords: Human Proteome Project, proteoforms, alternative splicing, splice-forms, RNA-seq, mass-spectrometry
• The in-solution trypsin digestion is more preferable than the in-gel digestion in cases when Met-to-isoThr modification can cause artifacts.
• In most cases the Met-to-isoThr modification rate does not exceed 1% and is not crucial for shotgun proteomic analysis. However it should be taken into account in proteogenomic workflow as it might lead to false discoveries.

Keywords

methionine, isothreonine, homoserine, sample preparation, proteogenomics
Chess in proteomics: winning combination of 2DE and MS/MS

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Background: Proteome heterogeneity is an unavoidable factor that complicates proteomic studies. Necessity for investigation of such events as alternative splicing, single amino acid polymorphisms and posttranslational modifications at protein level is determined by significant influence of these events on expression and functional properties of proteins. Careful analysis of proteome, which takes into account aberrant proteoforms, provides a basis for understanding the machinery of complex biochemical systems.

Methods: Basing on the results of RNASeq analysis of HepG2 cell line, we created a transcriptome-specific library, containing 52 thousand protein sequences, encoded by 12 thousand genes. Such library allows to focus on individual variations and helps to avoid uncontrolled extension of search area by populational data, thus reducing FDR. One of the most popular methods of proteomic analysis – shotgun mass spectrometry – is characterized by low (ca. 20%) coverage of protein sequence. Short peptides, detected with shotgun-MS, often do not allow to distinguish highly homologous proteoforms. To empower shotgun approach, we added two dimensional electrophoresis (2DE). After 2DE fractioning we cut the gel in 96 cells and analyzed every cell by MS. Fractioning of protein mixture before MS allows to enrich the results with coordinates of proteoform on the gel (pI and MW).

Results: 2DE profiling with further MS analysis allowed to discover over 2358 proteoforms (1658 of which were canonical, 224 – splice-forms, and 115 – with amino acid polymorphisms) encoded by 1904 genes. Without 2DE we identified only 925 proteoforms because of sample heterogeneity and lack of knowledge of physical-chemical parameters of proteoforms, used for specification of certain proteoform.

Conclusions: Effective tandem of 2DE/MS allows us to forecast modifications, which can change physical-chemical parameters (and the location of protein spot on the gel, consequently). Obtained results consist not only of evaluation of proteoforms implemented at the protein level, but also of improvement of experimental approaches to cell proteotyping.

O.K. and E.P. acknowledge the Leading Scientific School of Prof. Andrey Lisitsa (grant the Russian Federation of President NSh- 6313.2018.4)

Keywords: proteomics, proteoforms, proteome heterogeneity, shotgun MS, 2DE
THE FUNCTIONAL MITOCHONDRIAL HUMAN PROTEOME NETWORK

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Background:
Mitochondria are complex organelles of eukaryotic cells. The majority of mitochondrial proteins are present in all cells. However, some proteins are present only in certain tissues or under specific conditions. For this reason, mitochondria contain a great number of proteins that have yet to be identified and characterized. By combining proteomics and computational biology, it would be possible to characterize the human mitochondrial proteome, considering both mitochondrial proteins and their first interactors that influence mitochondrial functionality, maintenance, dynamics and metabolism.

Methods:
We used the neXtProt database (Release 2018-01-17) to generate a list of human mitochondrial proteins (Mitochondrial list). The Mitochondrial list was analysed with the neXtProt SnorQL to retrieve molecular interactors. These data were visualized with Cytoscape 3.6.0 to generate the functional mitochondrial proteome network. Using as reference the sub-cellular location section of the neXtProt database, we analysed the network to highlight mitochondrial proteins that may exhibit dual subcellular localization (e.g., cytoplasm, nucleus).

Results:
The Mitochondrial list was generated by including all mitochondrial proteins (GOLD and SILVER data) annotated in neXtProt (2093 IDs). Then, the neXtProt interaction section was used to obtain the functional mitochondrial proteome network (9053 nodes). 1796 proteins (Mitochondrial nodes) over the 2093 IDs were present in the network, since at least one interaction was retrieved from the database. Moreover, 461 proteins of Mitochondrial nodes interacted each other with GOLD protein-protein interactions. Seventeen mitochondrial proteins of this network were localized also in the cytoplasm, nucleus, Golgi apparatus or endoplasmic reticulum, nine proteins were classified as mitochondrial only, while 435 proteins exhibited dual/triple subcellular localizations.

Conclusions:
This functional mitochondrial proteome network may be a starting point to characterize mitochondrial proteins. Moreover, using a cluster algorithm and performing an over-representation analysis, it will be possible to unravel the contribution of proteins to biological processes and molecular mechanisms controlled by mitochondria.

Keywords: mitochondria, neXtProt, protein-protein interaction, functional network
In-depth proteomic characterization of complex cellular proteomes using the Q-Exactive HF-X and Proline software

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Background: In-depth characterization of cellular proteomes is a major challenge in large-scale discovery studies. Identification and relative quantification of as many proteins as possible, including those expressed at very low copy numbers, is necessary to finely dissect molecular mechanisms and provide detailed cellular phenotyping. To this aim, mass spectrometers with very high sequencing speed and better sensitivity have been recently introduced. Sample fractionation is also often performed in order to improve protein identification. However, systematic fractionation is time-consuming and difficult to implement in studies involving label-free quantitative analysis of large number of samples. We explore here strategies based on data-dependent acquisition methods and MS data processing to increase proteomic depth.

Methods: Whole cellular lysates of mouse primary T cells were analysed on the recent Q-Exactive HF-X using nanoLC-MS/MS in data-dependent acquisition mode. The MS-Angel and Proline software tools were used to process raw files, validate protein identification, build peptide libraries and perform label-free quantification.

Results: Parameters such as sample amount, gradient time, MSMS scan resolution, injection time, and criteria for selection of parent ions were optimized to increase analytical depth in data-dependent acquisition mode on the Q-Exactive HF-X. Moreover, by matching MS peptide signals from a library of identified peptide ions constructed in Proline after sample fractionation, we could extend significantly the number of detected peptides in single-shot acquisitions.

Conclusions: We present an analytical workflow and data processing methods for an in-depth proteomic characterization in single-shot LC-MS/MS measurements, allowing the detection of very low abundant proteins playing important roles in T lymphocytes.

Keywords: Q-Exactive HF-X, data-dependent acquisition, bioinformatics
**Proteogenomics: Combining Next Generation Sequencing and Mass Spectrometry in Candida albicans**

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**POSTER**

**Background:** Proteogenomics is a multi-omics research field that integrates genomics, transcriptomics and proteomics. This approach allows the identification of novel peptides in MS/MS searches by including transcriptomics and genomics information not present in the reference protein sequence databases used in MS/MS searches. There are different strategies and data sources used to generate such databases, even though using transcriptomic data from specific experiments is nowadays the most common one. Nevertheless, it is not trivial to create a database, especially if it is not a model organism.

**Results:** In this work, we develop a program to generate databases with SNP (Single Nucleotide Variants), INDELS (Insertions/Deletions) and novel junctions for any organism taking into account transcriptomic information publicly available. The program requires as input a FASTA file with the reference genome, an annotation file and a VCF file with the variations, and returns the database with the reference protein sequences and the variations. As a proof of concept and to create a more complete database for *Candida albicans* we analyzed 11 experiments with 52 RNA-seq datasets of *C. albicans* from GEO and the resulted database was used to do MS/MS searches with all the experiments from Peptide Atlas repository identifying with a 0,01 FDR, 12 new peptides corresponding to 10 different proteins that are in process of validation by polymerase chain reaction (PCR) and re-sequencing.

**Keywords:** Proteogenomics, bioinformatics, transcriptomics, proteomics
Software for quantitative analysis of protein complexes in proteomics data

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Background:

Proteomics provides quantitative information about more than 4000 proteins in a single experiment. While computational methods for protein inference from the mass spectrometry data and statistical analysis of quantitative features are well established, comprehensive biological interpretation of the results remains challenging. Therein, the characterization of the functional role of protein complexes, being main players of cellular regulation, would require versatile software to integrate the available information. Based on our previously published work\(^1\), we present a new software for proteomics data analysis that provides insight into protein behaviour with respect to their role in maintaining and regulating complex composition.

Methods:

We implemented a Shiny web application to quantify and validate the quantitative profiles of protein complexes. The program relies on the protein complex information contained within the Complex Portal\(^2\) and the CORUM Database\(^3\). To test the software we analysed a proteomics dataset from the differentiation of human muscle satellite cells over 6 days period.

Results:

The software tool is capable of 1) identification of protein complexes within a complex set of proteomics results; 2) assignment of complex coverage and quantification scores 3) evaluation of co-expression of the complex subunits and overall expression changes of the complexes throughout the different conditions of the experiment. This is achieved by interactive standardized expression profile graphs heatmaps for fold changes and correlations and linear modelling of expression profiles.

Analysis of the proteome changes occurring during the differentiation of human muscle satellite cells allowed us to show that during myoblasts formation expression of mitochondrial calcium uniporter and F1F0-ATP synthase complexes increase, while ribosome, proteasome, MCM, Arp 2/3, DNA synthesome and RNA polymerase complexes decrease in their abundance.

Conclusions:

This application is, to our knowledge, the first tool to carry out automated and interactive analysis of quantitative information of protein complexes identified and quantified in proteomics experiments.

Keywords: Proteomics, Protein Complexes, Bioinformatics, Quality Control, Data visualisation

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Comprehensive hypothesis-free identification and quantification of the modified proteome.

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**Background:** Post-translational modifications hugely increase the functional diversity of proteomes. Recent algorithms, based on ultra-tolerant database searching are forging a path to unbiased analysis of peptide modifications. However, these approaches identify only half of the modified forms potentially detectable and do not map the modified residue. Moreover, tools for the quantitative analysis of peptide modifications are currently lacking.

**Methods and Results:** Here we present a suite of bioinformatics tools designed to overcome these limitations. We developed Comet-PTM, an improved OS engine that takes into account of the mass-shift produced by the modification in the fragmentation series, producing the same score as a close search using the same mass as a variable modification. In addition, SHIFTS, an algorithm that detects the peaks in the ΔMass distribution and controls the FDR through a conservative, 3-layered approach (Global, Local and Peak FDR). Our tools double the coverage attained by existing algorithms, enabling the generation of FDR controlled comprehensive peptide maps that include practically all the modifications potentially detectable by MS and conventional searches. Our approach also allows detection of accurate location of the modified residue.

For quantifying PTMs, we developed a novel algorithm based on a previously proposed systematic workflow (Navarro et al., 2014). The algorithm includes a peptide-to-protein integration step that quantifies protein values from the unmodified-peptide and then computes the standardized log2-ratio of the modified peptides with respect to these protein values, allowing detection of PTMs whose behaviours deviate significantly from those of the unmodified-peptides from the same protein.

**Conclusions:** These developments were used to characterize the impact of mitochondrial heteroplasmy on the modified peptidome, revealing that heteroplasmy mainly affects cardiac tissue, inducing oxidative damage to proteins of the oxidative phosphorylation system, and provide a molecular mechanism explaining the structural and functional alterations produced in heart mitochondria. (Accepted manuscript: Cell report)

**Keywords:** PTM, Open search, comet-PTM, PTM identification, comprehensive ptm identification, quantification, novel algorithm
The online Tabloid Proteome: an annotated database of protein associations

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Background:

Mass-spectrometry based proteomics produces large amounts of data. While typically acquired to answer specific biological questions, these data can also be reused in orthogonal ways to reveal biological knowledge. Here we present a novel method for such data re-use of public mass-spectrometry based proteomics data.

Methods:

Mass-spectrometry based proteomics experiments were obtained from the PRIDE database and further reprocessed. For the identified proteins, we calculated the co-occurrence score, using Jaccard similarity. Protein pairs with score of at least 0.4 were mapped to five knowledgebases; Reactome, Ensembl, IntAct, BioGRID, and CORUM, to assign potential biological relevance.

Results:

Of the 2325 protein pairs that pass the Jaccard similarity threshold, we have successfully been able to map 81% of pairs (68% with five knowledgebases and 13% with Gene Ontology terms). When randomly selected pairs were mapped to the five knowledgebases, we only found 40 annotated protein pairs (on average). As a result, the difference between real and random data is extremely significant. To extend the usability and accessibility of the detected protein pairs for the research community, we have developed an online Tabloid Proteome database.

Conclusions:

We have successfully been able to map the vast majority of strongly co-occurring protein pairs with existing biological knowledge. Our approach shows that by re-using publically available data in a fully orthogonal way, effectively treating these data as a proteome-wide association study, we can extract various biologically meaningful patterns. Moreover, the online Tabloid Proteome is an easily searchable website that presents protein associations, along with the possible biological annotation of these associations derived from exiting knowledgebases.

Keywords: Protein-association, data re-use, data analysis, online database
SIMPLIFYING THE USE OF ION LIBRARIES DURING DATA PROCESSING OF DATA INDEPENDENT ACQUISITION PROTEOMICS DATA

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Background: As the use of data independent acquisition grows in proteomics research, the need for improved data processing workflows increases. The most common data processing workflow is to use spectral ion libraries to drive targeted extraction of peptide / fragment areas. Increasing the size and quality of the ion library has been shown to increase the proteins reliably quantified. RT correlation between ion library and the dataset is another key factor that determines quality of data extraction. Simplifying how ion libraries are built and used during data extraction was explored in this work.

Methods: Two algorithms were explored to simplify the data extraction workflow for SWATH® data within the OneOmics™ Project cloud processing pipeline, the automatic merging of related ion libraries followed by auto RT calibration. During library merging, ProteinPilot™ Software group files are combined in using a non-linear calibration strategy. New peptides are added to existing proteins and new proteins are added if not present in seed library. During SWATH processing, endogenous peptides are automatically selected across time bins and the best peptide are chosen. Best scoring peak groups are used for RT calibration.

Results: Previous data has shown that increasing the size of the ion libraries used provides improved protein quantitation. Therefore the ability to easily combine and align ion libraries for greater depth of sample interrogation would provide a significant workflow benefit. To evaluate the quality of the RT alignment during SWATH processing using the new AutoRT calibration function, it was compared to using the typical RT calibration process using a set of spiked standards peptides. In 33/48 tests, AutoRT approach found similar or more proteins quantified than the standard approach and similar linear fit equations, indicating the new, easier to implement process was as good or better than the currently used standard peptide workflow. The quality of library merging on SWATH results was explored using non-linear retention time alignment. By merging libraries from replicates of a 1D dataset, a 20% gain in peptides was observed. Full pipeline was then used on PBMC and Mouse cell lines to measure performance, where a series of libraries were available to merge.

Conclusions: Non-linear retention time alignment was shown to be effective for merging of libraries with identical or differing gradient lengths resulting in better results.

Keywords: DIA, SWATH, proteomics, libraries, cloud computing
Absolute Quantification of Pseudomonas aeruginosa TonB-dependent Transporters Using PRM and SWATH-MS

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Background: *Pseudomonas aeruginosa* is an opportunistic bacterial pathogen with high intrinsic and emerging acquired resistance to all available antibiotics. A main challenge for developing novel antibiotics is poor compound penetration into bacteria. The most advanced strategy to overcome this uses bacterial active uptake of metal-siderophore complexes by coupling substrate analogues to drugs (so-called “Trojan horse” antimicrobials). In total 36 TonB-dependent transporters (TBDTs) are responsible for active uptake of metallophores across the outer membrane of *P. aeruginosa* and some are currently explored as potential entry points for siderophore-antibiotic conjugates.

Methods: We utilized highly sensitive targeted proteomics technologies (PRM and SWATH-MS) to quantify TBDTs in cultures, animal infection models, and infected human tissues (in which bacteria are trace components). In PRM, ratios between endogenous and isotopically-labelled reference peptides targeting 41 *P. aeruginosa* proteins were used to derive absolute protein abundances. Whole proteome quantification was performed with SWATH-MS. The raw data was acquired with a Q-Exactive HF MS (Thermo Fisher Scientific), and processed with SpectroDive or Spectronaut (Biognosys AG).

Results: The LOQs of our PRM-assays ranged between 10-100 attomol/µg peptide (1-10 copies/cell). The TBDT copies/cell varied strongly between *in vitro* conditions, *in vivo* models, and human tissues. *P. aeruginosa* had consistently high expression of TBDTs for its own metallophores. TBDT abundances for many other substrates were lower and dispersed. *P. aeruginosa* had particularly different properties in *in vitro* culture conditions compared to infection models and patients, but zinc limitation mimicked *in vivo* patterns very well.

Conclusions: Our data reveals challenges for current siderophore-coupled antibiotics, but also opportunities for zinc-metallophore conjugates. Based on our results, we suggest a reverse paradigm for antimicrobial development, which begins with ultrasensitive proteomic analysis of pathogen properties directly in human patient samples, thereafter identifying animal models and *in vitro* screening conditions that reproduce these key pathogen properties.
An Unbiased metaproteomic approach to describe the mucosal microbiome of HIV-exposed African Infant cohort

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Background: Infants born to HIV-infected mothers have an altered cellular immunity despite being HIV-infected themselves. It is known that the gut microbiome is crucial to immune development. Currently, 16S rRNA sequencing, the gold standard for characterising the microbiome, yields limited information on the function of the microbial community, predicted open reading frames might not be expressed under in vivo conditions.

Methods: For this study, infant stool samples, both HIV-exposed and unexposed infants collected at birth and at 4-7 days were analysed to characterise the metaproteome using mass spectrometry-based proteomics. Briefly, cold stored samples were snap-frozen and ground samples were suspended in organic buffer and precipitated proteinaceous material were subjected to in-solution trypsin digestion. Desalted tryptic peptides were analysed in triplicate on the nLC-MS/MS. An in-house developed pipeline was used to construct a metaproteomic database using the Universal Reference Database and generated mass spectrometry data. The smaller database was used for protein and organism identification in MaxQuant-suite.

Results: Using our unbiased approach we identified 3943 proteins for all 22 samples described. The microbial diversity changed dynamically from birth to 4-7 days after birth and the proportion of human proteins identified decreased as the microbial diversity evolved. Using this approach, we identified virus, parasite, bacteria and human proteins in a single sample. The number of proteins belonging to the Bifidobacteriaceae family were significantly different between HEU and HU infants at birth, 1 to 180 respectively. Furthermore, this bacterial family increased dramatically to 1800 proteins within the first week of birth. Members of the Bifidobacteriaceae family are important microbes that colonize the gut of humans and other animals during the early stages of life.

Conclusions: Here we demonstrated that using mass spectrometry-based proteomics approach we were able to construct a metaproteomic profile of the microbiota composition of infant stool samples.

Keywords: metaproteomics; microbiome; HIV
**Listeria monocytogenes** planktonic and sessile cells adaptation to different temperatures seen through Shotgun proteomics

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**Background:** *Listeria monocytogenes* is a foodborne pathogen that can cause invasive severe human illness (listeriosis) in susceptible patients. Consumption of refrigerated ready-to-eat foods causes most human listeriosis cases. Although initial contamination levels in foods are usually low, the ability of these bacteria to survive and multiply at low temperatures allows it to reach levels high enough to cause disease. It can grow in a broad spectrum of temperatures, and as a biofilm-forming bacterium, it can modify their cell envelope composition and their metabolism to maintain cell homeostasis. This study explores the changes in protein expression of *L. monocytogenes* surfaceome according to the temperature (10°C, 25°C and 37°C) and the mode of growth (planktonic vs biofilm).

**Methods:** Protein extractions were performed from planktonic or sessile cells in the exponential and stationary phase of growth at each of the three temperatures. Cell envelope proteins are challenging to characterize due to their low abundance and poor solubility. Here, *L. monocytogenes* surface proteins were targeted using three protein extraction methods: trypsin-enzymatic shaving, biotin-labelling of exposed proteins on the surface of intact cells and cell fractionation. The different subproteomes obtained were separated and identified by shotgun proteomics using high-performance liquid chromatography combined with tandem mass spectrometry (LC-OrbiTrap LTQ Velos, ThermoFisher Scientific).

**Results:** A total of 191 (biotinylation), 137 (shaving) and 1005 (fractionation) proteins were identified, the biotinylation method having the lowest percentage of contamination by cytoplasmic proteins (43%). Many of the identified proteins are connected with thermoregulation, some are mainly overexpressed at 37°C, the temperature at which *L. monocytogenes* is virulent, and others at the low-temperature condition, as those prevailing in food workshops.

**Conclusions:** These comparisons of protein expression throughout several conditions will enrich databases and help to model the regulatory circuitry that drives the adaptation to environments.

**Keywords:** *Listeria monocytogenes*, Temperature adaptation, Biofilm, Surfaceome, Shotgun proteomics
QUANTITATIVE PROTEOMICS TO STUDY NITROGEN AND CARBON METABOLISMS IN THE MARINE CYANOBACTERIUM PROCHLOROCOCCUS

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Prochlorococcus requires the capability to accommodate to environmental changes in order to proliferate in oligotrophic oceans, particularly regarding to nitrogen and carbon availability. A precise knowledge of the composition and changes of the proteome is necessary to gain fundamental insights into such responses. Here we present the most recent advances in the proteome analysis of the important model cyanobacterium Prochlorococcus. Quantitative proteomics was applied to investigate proteome changes in Prochlorococcus as a response to variations in carbon and nitrogen availability. On the one hand, we report the effect of glucose addition on the metabolism of Prochlorococcus. Our studies indicate that glucose is actively taken up by Prochlorococcus, but its uptake does not significantly alter the trophic ways of this cyanobacterium, which continues performing photosynthesis. Therefore Prochlorococcus seems to remain acting as a fundamentally phototrophic organism, capable of using glucose as an extra resource of carbon and energy when available in the environment. On the other hand, we present the effect on the proteome of nitrogen limitation promoted by azaserine, an inhibitor of the glutamate synthase, a key enzyme in nitrogen assimilation in cyanobacteria. Statistically significant changes were observed for 408 proteins, the majority (92.4 %) being down-regulated after 8 h of treatment. We observed a strong decrease in ribosomal proteins upon azaserine addition, while many transporters were up-regulated. The regulatory proteins PII and PipX were down-regulated and the global nitrogen regulator NtcA was up-regulated. Furthermore, our data indicate that NtcA participates also in the regulation of photosynthesis in Prochlorococcus. Our results suggest that Prochlorococcus responds to the lack of nitrogen by slowing down translation, while inducing the photosynthetic cyclic electron flow and the biosynthesis of proteins involved in nitrogen uptake and assimilation. Funded by Gobierno de España (BFU2016-76227-P), Junta de Andalucía (P12-BIO-2141) and Plan Propio Universidad de Córdoba.

Keywords: Prochlorococcus, marine cyanobacteria, carbon metabolism, nitrogen metabolism
Candida albicans INDUCES CHANGES IN HUMAN MACROPHAGE PROTEINS INVOLVED IN RNA-SPLICING, PROTEIN SYNTHESIS AND APOPTOSIS

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Background: Macrophages are involved in the primary human host response to Candida albicans. After pathogen recognition, a cascade of signaling pathways is activated and ATP binding proteins are crucial for its regulation. Here, we performed a quantitative proteomic approach to study human macrophage proteins after interaction with C. albicans cells using an ATP-binding protein enrichment approach.

Methods: THP1 cell-line was used and cells were labelled by SILAC. Monocytes were differentiated into macrophages by adding PMA. After incubation of the macrophages with C. albicans (MOI of 1) for 3 hours, protein lysates were enriched in ATP-binding proteins using the ActivX kit. Proteins were in-gel digested using trypsin. Mass spectra were acquired on a LTQ-Orbitrap Velos mass spectrometer.

Results: Overall, 547 non-redundant proteins, including 137 ATP-binding proteins, were quantified. Among them, 59 proteins were differentially abundant during macrophage interaction with C. albicans. GO term enrichment analysis showed that the more abundant proteins during interaction were involved in protein synthesis and RNA splicing whereas the less abundant proteins were related to proteolysis events and apoptosis. Western blotting and SRM were used to validate the abundance of MAP2K2, PRDX5 and NDKA. Forty-five protein-protein associations were found using the STRING software. Furthermore, several upstream regulators were predicted using the Ingenuity® Pathway Analysis program, including miR21 and miR124. We checked the abundance of these two miRNA finding no statistical differences. We checked the activation of miR155 and miR146 in response to C. albicans and LPS. No upregulation of these mi-RNAs with live C. albicans cells was observed, suggesting a decrease in the negative feedback of the pro-inflammatory response of macrophages in response to live C. albicans.

Conclusions: This work was useful for the discovery of new human macrophage proteins that are involved in host-pathogen interaction.

This work was supported by FP7-PEOPLE-2013-ITN, IMRESFUN Project and MINECO Bio2015-65147-R.

Keywords: macrophages, SILAC, Candida albicans, ATP-binding proteins
DATA-INDEPENDENT ACQUISITION:

HIGH-THROUGHPUT PROTEOMIC TOOL TO MONITOR THE RESPONSE OF Candida albicans TO HUMAN MACROPHAGES

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Background: The opportunistic human fungal Candida albicans is the main pathogen responsible for severe infections in immunocompromised patients. The innate immune system constitutes the first defensive line against this pathogen. This comprises phagocytes such as macrophages that recognize the fungus and engulf it. However, C. albicans also presents some mechanisms to survive to this attack. In order to go deep in the knowledge of the C. albicans response to macrophages, we carried a data independent acquisition mass spectrometry analysis (DIA).

Methods: Four replicates of C. albicans cytoplasmic extracts were collected after 3, 6 and 9 hours of interaction with human derived macrophages (THP1). Samples were analysed in a QExactive mass spectrometer firstly by data-dependent acquisition mode to obtain an ion library, and then by using independent acquisition mass spectrometry to quantify all the proteins from the library generated.

Results: We constructed an extensive spectral library including above 2300 proteins (40% of C. albicans proteome). There were significant increase and decrease in the abundance of more than one hundred proteins after interaction with macrophages. Proteins related with nucleosome organization and chromatin remodeling such as Hta1, Pob3, Nhp6A and orf19.6090 decreased consistently at the three time points. Nevertheless, one histone related with DNA damage (Htz1) was remarkably more abundant after interaction at all time points tested. Additionally, other processes were specifically modified at each time point i.e. at 6 hours a high number of proteins from the mitochondrial respiratory chain decreased which are being validated. At the last time point (9h) few changes in protein abundance were found which support a recovery phase of the fungus.

Conclusions: Overall, this analysis provides a comprehensive understanding of the proteome dynamics of C. albicans in order to avoid macrophage phagocytosis rendering new clues to fight against this fungi.

This work was supported by MINECO (BIO2015-65147-R)

Keywords: C. albicans, macrophage, proteomic, data-independent acquisition
SIGNATURES OF COMMENSAL AND PATHOGENIC
STAPHYLOCOCCUS EPIDERMIDIS: A GENOMIC AND
PROTEOMIC APPROACH

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Background: Staphylococcus epidermidis include the skin microbiota and contribute to homeostasis and protection against pathogens. However, they are the most frequent cause of medical device-associated infections. Skin isolates belonging to clonal complex 2 (CC2) lineage are the major colonizers sharing their ecological niche with other minor genetic backgrounds (non-CC2). CC2 strains are the more frequent in infection, however the reasons are poorly understood.

Methods: Genomic DNA from a CC2 and a non-CC2 S. epidermidis strain were sequenced using HiSeq. Genomes were annotated using RAST and Blast2go. Tryptic peptides resulting from the total proteome were analysed by SWATH MS. Growth rates at different pH, biofilm formation, metal and antibiotic resistance and proteolytic assays were conducted.

Results: The comparison between the genomes of both S. epidermidis strains showed the presence of 81 and 57 specific genes, respectively. Comparative proteomics of both strains has extended knowledge on these metabolic differences. A total of 668 proteins were quantified, of which 194 and 163 were accumulated in CC2 and non-CC2 strains, respectively. The most active processes in the CC2 strain were those related to antibiotic resistance, virulence, genetic transposition, membrane trafficking, L-arginine degradation, and pentose-phosphates, peptidoglycan and D-alanyl-lipotheichoic acid biosynthesis. On the other hand, the non-CC2 strain showed an increased expression of proteins involved in metal ion resistance, detoxification and transport associated to osmoregulation, DNA repair, urea degradation, citrate cycle and proteolytic activity. Results of phenotypic assays corroborate the omics conclusions.

Conclusions: Although both CC2 and non-CC2 strains are colonizers of human skin, they display totally different metabolic and phenotypic profiles in the same environmental conditions, suggesting each type of strain plays a specific role in skin ecology. Overall, having the opportunity, CC2 strain appears to be better equipped to cause infection than non-CC2 strain.

Keywords: Staphylococcus epidermidis, pathogen, proteomics, genomics

* these authors contributed equally to this work
Rapid determination of host-pathogen quaternary protein structures in complex biological samples

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A central challenge in medical microbiology is to develop and apply new experimental approaches to characterize the highly dynamic protein-protein interaction networks forming at the host-pathogen interface. Here we demonstrate a combined ‘protein-centred’ and ‘bacterial-surface centred’ affinity-purification DIA-MS experimental set up to determine that the interactome formed between the bacterium Streptococcus pyogenes and its human host is highly interconnected and composed of several large inter-species protein complexes.

To further determine the quaternary structure of these protein complexes we applied a targeted chemical cross-linking mass spectrometry strategy (TX-MS) that relies on a novel combination of chemical-cross linking, high-resolution mass spectrometry and high-accuracy protein structure modelling. Applying TX-MS to the M protein, the most important virulence factor on the S. pyogenes surface, enabled the construction of a high-resolution model of a strikingly intricate 1.8 MDa protein complex formed between the M protein and human plasma proteins. The model was based on a dense network of XL distance constraints composed of over 200 distinct inter-protein cross-links and reveals a highly organized quaternary structure with no steric competition between the interacting proteins.

The established quaternary structure explains how S pyogenes efficiently use the repeat regions in the M protein to line up several plasma proteins along the full-length protein to prevent phagocytosis, inhibiting complement activation and securing nutrients and at the same time masking conserved surface epitopes in the binding interfaces with human proteins. The model contributes to the understanding of the relationship between the molecular organization of the M protein and the interaction will human host proteins, which may have implications for the design of new vaccines for S pyogenes.
Background: Malaria, caused by the Plasmodium parasite, represents a major health and economic problem globally, yet despite extensive research, over a third of Plasmodium genes are still largely uncharacterised. Physical protein associations are central to most cellular processes and their study can assist in assigning protein function and mechanism of action. Previous protein interaction studies at scale in malaria have relied on in silico predictions of functional association and low coverage yeast two-hybrid. In recent years, chromatographic fractionation coupled with quantitative mass spectrometry has been useful in describing protein interactions at proteome level in mammalian cells and other simpler organisms.

Methods: To investigate protein interactions in Plasmodium at systems level we combined blue native polyacrylamide electrophoresis with quantitative mass spectrometry and machine learning.

Results: We generated eighteen high-throughput proteome fractionation datasets from the schizont stage in three solubilisation conditions across three different Plasmodium species. Our integrative approach identified over twenty thousand protein interactions, the majority of them novel, organized into 600 complexes.

Conclusions: These data represent the first high-confidence large-scale Plasmodium protein interaction map. Our network reflects the native organisation of core conserved cellular processes, sheds light on the function of currently uncharacterized Plasmodium proteins, provides mechanistic and structural insight and reveals potential malaria therapeutic targets.

Keywords: malaria, Plasmodium, interactome, protein interactions, blue native PAGE
Potential role of acetylation on bacterial metabolic pathways

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Background: Post-translational modifications (PTMs) of bacterial proteins were almost unknown until the advent of mass spectrometry. Mass spectrometry-based proteomics has revealed that prokaryotes are able to modify their proteins with a high number of PTMs which have influence on bacterial physiology and virulence. Acetylation is the most studied PTM in prokaryotic organisms. Proteins involved in metabolism being the main group of acetylation targets. This modification has been related to the degree of virulence, adaptation to environmental conditions, and persistence in several bacterial species. In this study, acetylomes of Brachyspira hyodysenteriae and B. pilosicoli have been characterized. These species are known gut pathogens; B. hyodysenteriae is the causative agent of swine dysentery and B. pilosicoli causes colonic spirochaetosis in pigs and is responsible for a human form of the disease.

Methods: Bacterial acetylomes were prepared by immunoprecipitation with specific anti-acetyl Lys antibody and analysed in an Orbitrap Fusion Lumos™ Tribrid coupled to a nanoLC system. Raw data were processed with Proteome Discoverer and filtered at 0.1% FDR using Percolator. Localization probability for each site was calculated using ptmRS.

Results: 3142 and 5496 acetylated peptides (3221 and 5579 sites) were identified in B. hyodysenteriae and B. pilosicoli, respectively. The two sets of acetylated proteins were found to be enriched in proteins involved in metabolic pathways and the biosynthesis of secondary metabolites.

Conclusions: We have reported a different degree of proteome acetylation in these species, being B. pilosicoli proteome the most acetylated one. We have shown that the main targets of acetylation were proteins involved in metabolism and that the differences observed are reflected in the different composition of the components involved in the Acetyl-CoA/Acetate metabolic pathway.

Keywords: Brachyspira, PTM, acetylome, metabolism.
Evaluation of protein extraction protocols
for shotgun metaproteome characterization

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Background: Efficient and robust protein extraction protocols are prerequisite however, still a challenging task when samples with higher variability of cell structure and cellular organization have to be analysed. Indeed, optimized protocols maximize total protein extraction yield and prevent selective depletion of species with higher resistance to cell lysis.

Methods: Altogether 8 protein extraction protocols including, ultrasonic, bead-beating and freeze-thawing with different buffer/detergent systems were evaluated independently on a microbial community (MC) comprising of 3 Gram–positive (GPB), 2 Gram–negative (GNB) bacteria and Saccharomyces cerevisiae. Protein extraction efficacy comparison was conducted using (1) total protein quantification assay, (2) SDS-PAGE and (3) nano-LC-MS/MS approach after filter-aided sample preparation. Protein identification was performed using PLGS while for Gene Ontology protein annotation STRAP was employed.

Results: SDS or urea buffer in combination with bead beating protocols resulted with the two highest protein yields. The same protocols did not result with the highest number of protein identifications but, almost equal representation of GPB, GNB and yeast proteins was reached for urea protocol. Interestingly, for other protocols were mechanical cell disruption was implemented, increased GNB protein identifications were noticed among which two sonication protocols resulted with the highest number of protein identification. For the same protocols protein yields remained low. Vast majority of identified proteins were cytoplasmic or ribosomal, involved in cellular processes and with binding, catalytic or structural molecule activity.

Conclusions: Overall, results demonstrate that protein extraction protocols impact metaproteomic results but if GPB, GNB and yeast proteins are of interest we recommend combination of urea buffer and multiple bead beating steps protocol.

Keywords: shotgun proteomics, metaproteomics, sample preparation, protein extraction, microbiota
Study of the secretome of two uropathogenic Escherichia coli strains with different antibiotic susceptibility.

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Background: Escherichia coli is one of the most frequently isolated bacteria in septicaemia, being many of the strains resistant to β-lactamic antibiotics. The current diagnostic methods are based on the culture and isolation of the microorganism from blood, taking several days the diagnosis and the setting of the proper antibiopolitical susceptibility profile. Thus, the availability of a rapid and accurate diagnostic method capable of detecting and identifying the pathogens causing septicaemia and certain mechanisms of resistance to antibiotics would be an important achievement, lowering the septicaemia mortality rates. Therefore, the objective of our work is to put the basis for a diagnostic method capable of detecting proteins secreted by the microorganisms in the serum sample.

Methods: The secretomes of one of the most prevalent strains in septicaemia, E. coli ST131, and a collection strain E. coli ATCC25922 were analyzed by LC-MS/MS in an Orbitrap FusionTM LumosTM TribridTM (Thermo Scientific). Proteins were identified using the Protein Discoverer 2.2 (Thermo Scientific) software.

Results: 831 proteins common to both strains were identified. Of them, only the ones showing less variability and bigger sizes were analyzed, since these proteins would probably be the ones mainly detected in serum. Besides, characteristic virulence factors of uropathogenic strains were also identified for both strains, and some proteins specific for each strain, such as proteins from flagella and fimbriae and the β-lactamases, very important for the establishment of the treatment.

Conclusions: the identification of these proteins put the basis for a future rapid diagnostic method for septicaemia.

Keywords: E. coli, diagnostic, beta-lactamases
THE ANTIBODY-MEDIATED IMMUNE RESPONSE TO INVASIVE CANDIDIASIS DISCRIMINATES BETWEEN Candida albicans YEASTS AND HYPHAE

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Background: An important virulence trait of Candida albicans is its ability to switch between yeast and hyphal growth under specific host environmental stimuli (also known as dimorphic transition). Both morphological forms are pathogenic and show differences in their cell surfaces that affect host-pathogen interactions. However, little is known about the antibody-mediated immune recognition of their cell surface-associated proteomes in invasive candidiasis (IC). Methods: We assessed the antibody responses to the C. albicans cell surface-associated proteomes from yeasts and hyphae in IC patients using computational immunomics. Proteomic data were then validated using follow-up functional assays. Results: A total of 27 cell surface-associated proteins (CSPs) were differentially immunorecognized in yeasts and hyphae during IC. Capture ELISAs on selected CSPs confirmed serological proteome analysis data. Unsupervised clustering analysis revealed two antibody-reactivity signatures that separated IC sera hybridized with yeast and hyphal CSPs into two distinct groups. Coordinated antibody responses to two repertoires of CSPs as a function of the growth form were observed in IC. Pairwise correlation and gene ontology term enrichment analyses uncovered discrete subgroups of functionally related CSPs that displayed opposing antibody-mediated immune recognition patterns upon dimorphic transition in IC. Changes in the antigenicity of yeast and hyphal CSPs in IC induced substantial topological reorganization of their immune co-recognition networks. We found that antigenicity of CSPs was modulated in IC by changes in their relative abundance and potential post-translational modifications in their epitopes upon dimorphic transition. Further functional assays demonstrated that specific post-translational modifications affected the CSP antigenicity upon dimorphic switching in IC. Conclusions: This study sheds new light on the antibody-mediated immune recognition of the C. albicans yeast and hyphal surface-associated proteomes in IC. Furthermore, our results may have clinical implications in the development of future diagnostics and vaccines for IC. Keywords: Candida albicans, invasive candidiasis, dimorphic transition, cell surface, immunoproteomics.

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**Pseudomonas aeruginosa PAO1 PeptideAtlas**

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**Background:** PeptideAtlas databases provide new and expanded proteome documentation of microorganisms, thus, the Pseudomonas aeruginosa PeptideAtlas will be an important tool for research in virulence factors of this microorganism. In previous studies, our group and other groups have the plasticity of P. aeruginosa proteome.

**Methods:** The *P. aeruginosa* PeptideAtlas has been developed using the wild-type strain PAO1 under different environmental conditions or stresses to increase the *P. aeruginosa* proteome coverage: stationary phase, anaerobic atmosphere, tetracycline sub-inhibitory concentration, biofilm, 20°C, simulating the environmental growth, and under T3SS-induction conditions. A subcellular fractionation approach has also been used to enrich in inner and outer membrane proteins. The MS system used was an LTQ XL Orbitrap (ThermoFisher) equipped with a nanoESI ion source. The spectrometric analysis was performed in a data dependent mode, acquiring a full scan followed by 10 MS/MS scans of the 10 most intense signals detected in the MS scan from the global list. The full MS (range 400-1800) was acquired in the Orbitrap with a resolution of 60.000. The MS/MS spectra were done in the linear ion-trap. Besides the experiments from our group, other published works that have been uploaded to the public repository PRIDE³ have been included in order to increase the coverage of the *P. aeruginosa* PeptideAtlas: published datasets from Herbst et al. (PX001373); Penesyan et al. (PX020865); Casabona et al. (PX000107) and Robert-Genthon et al. (PX000189).

**Results:** From a total of 5668 predicted proteins, 4161 have been identified, resulting in a coverage of the 73.4% of the whole predicted proteome of P. aeruginosa PAO1.

**Conclusions:** This is the highest published coverage from a bacterial PeptideAtlas, being the ones from *Leptospira interrogans*, *Halobacterium salinarum* and *Streptococcus pyogenes*, 65.9%, 62.7% and 55.7%, respectively.
IDENTIFICATION OF NEW CANDIDATE ANTIGENS TO SUPPORT DIVA STRATEGIES IN BRUCELLOSIS

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Background: Brucellosis remains a significant public health issue that affects the agricultural economy in Mediterranean basin. The disease is widespread zoonosis that infects humans mostly through the consumption of contaminated raw milk and dairy products or by contact with infected animals. Although several control programs have been implemented in the Mediterranean basin, the disease is still prevalent in small ruminants and further actions are required to improve the surveillance. Animal vaccination is an efficacious countermeasure but the current diagnostic tests do not allow the differentiation of infected from vaccinated animals (DIVA), limiting the intervention strategies to fight the disease. In this study, we performed an immunoproteomic characterization of Brucella melitensis vaccine strain Rev. 1 and the wild type strain 16M in order to identify candidate antigens specific for infection or vaccination.

Methods: The B. melitensis vaccine strain Rev.1 and the wild-type strain 16M have been subjected to optimized 2D western blot against immune sera from naturally infected animals using a combined fluorescence-chemilumiscence detection. Western blot (WB) image analysis has been performed with Image Studio 5 (LiCor) and specific immunoreactive proteins were identified by MALDI-TOF/TOF with LIFT (Bruker Daltonics).

Results: Three immunoreactive proteins that specifically recognize 16M strain have been detected and identified by the optimized 2D-WB coupled to MALDI-MS/MS identification

Conclusions: optimized High resolution 2DE and western blotting identified a panel of candidate antigens that could discriminate Infected from Vaccinated Animals, supporting the development of DIVA tests for an improved disease management.

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Keywords: immunoproteomics, Brucella melitensis, zoonoses
Characterization of bacteriophage peptides for pathogen identification by LC-ESI-MS/MS.


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Classical and culture-based methods used in detection of pathogen are very slow and laborious. Recently, new rapid molecular microbial diagnostic methods based on genomics and proteomics have been developed in order to achieve faster and more effectively methods for species detection and identification and even strain biotyping. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) has been used for the analysis of bacterial pathogen strain-specific diagnostic peptides [1]. As bacteriophages are high specific to their host pathogens, their nucleic acids, antibodies, whole phages, phage-display peptides (PDPs), and most recently phage’s receptor binding proteins (RBPs) have been studied as biosensor for pathogen detection [2,3].

In this study, bacterial pathogen tryptic digestion peptides have been analysed by LC-ESI-MS/MS to search for genus and/or species-specific biomarkers useful for a rapid identification. A total of 100µg protein extraction was digested with trypsin, cleaned on a C18 microSpinTM, following by LC-MS/MS analyse. The data was processed by SEQUEST (Proteome Discoverer 1.4 package, Thermo Scientific) against Bacteria in the UniProt/TrEMBL database. The study of bacteriophage resulting peptides of bacterial pathogen led to the discovery of some specific biomarker peptides.

Keywords: pathogen detection, LC-ESI-MS/MS, mass spectrometry, phage peptide biomarker.

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Characterization of peptides for histamine-producing bacteria identification by LC-ESI-MS/MS.

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Some bacterial strains have the ability to decarboxylate specific amino acids. The detection of these strains having amino acid decarboxylase activity is important to lower the risk of biogenic amine in food products, specially histamine. Molecular methods for the detection of histamine-producing bacteria in foods are based on the specific detection of histamine decarboxylase genes by PCR allowing the detection before the histamine is produced. However, the difficulty is the availability of universal primers. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) can provide a useful tool for the detection of strain-specific diagnostic decarboxylase peptides.

In this study, proteins from histamine producing strains were isolated, after tryptic digestion, the resulting peptides were analysed by LC-ESI-MS/MS. A total of 100µg protein extraction was digested with trypsin, cleaned on a C18 microSpinTM, following by LC-MS/MS analyse. The data was processed by SEQUEST (Proteome Discoverer 1.4 package, Thermo Scientific) against Bacteria in the UniProt/ TrEMBL database. MS data was analysed to identify the detected peptides and then compared to databased using Blast tool (NCBI) to achieve peptide/protein identification. Thus, histamine decarboxylase peptides were detected in 2 of the studied strains.

Keywords: histamine-producing bacteria, LC-ESI-MS/MS, mass spectrometry, decarboxylase peptide biomarker.

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Refined gene annotation and identification of recoding RNA-Editing events in a fungal model organism by proteogenomics

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Background:
Costs of whole-genome sequencing have steadily decreased and thus it is readily employed in many research areas, from clinics to biodiversity research. The growing availability of raw genomic sequences demands accurate gene annotation methods. Proteomics data can add valuable information not only by confirming the presence of predicted genes, but also in refining the protein-coding regions, the discovery of priorly unpredicted gene products as well as recoding RNA-editing events. Here, a combined proteogenomics approach was employed to improve the genome annotation of the filamentous fungus S. macrospora.

Methods:
S. macrospora was grown under various nutritional conditions and harvested at different developmental stages. Samples for LC-MS analysis were prepared using bottom-up proteomics workflow. Samples were digested with complementing proteases and subjected to high-/low-pH RP-RP LC separation prior to MS/MS analysis. Peptide identification was performed by a combined approach: MS/MS spectra that were not identified in a first search against a predicted protein database were subjected to a second search against the S. macrospora genome. Furthermore, a de novo peptide sequencing workflow was employed to identify exon-spanning peptides and potential RNA-Editing events.

Results:
By implementing our peptide identification strategy, more than 60% of all potentially protein coding genes could be confidently identified, refinements were made on more than 500 genes (e.g. frame shift, splice variants etc.) and 92 novel genes could be identified. Finally, amongst more than 130 detected recoding RNA-editing events, we could describe 14 cases of stop-loss events, leading to prolonged protein translation and novel C-terminal sequences. These findings could be further validated by targeted-MS technology in combination with stable isotope labelled peptides.

Conclusions:
By combining proteogenomics as well as de novo peptide sequencing workflows, this work not only provides an in-depth refinement of the *S. macrospora* proteome, but also demonstrates the widespread occurrence of recoding RNA-editing events in the fungal kingdom.

**Keywords:** Proteogenomics, de novo peptide sequencing, PRM, RNA editing
High-resolution quantitative proteomics applied to the discovery of biomarkers of innate immune response in tuberculosis.

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Background: Tuberculosis (TB) is caused by the bacterium Mycobacterium tuberculosis (MTB). Roughly one third of the world’s population carries MTB in a dormant form. TB is responsible for the death of more than 1.8 million people each year. EMI-TB is an EU Horizon2020 funded action focused on selecting and developing a novel vaccine candidate for TB. Our specific aim is to establish a panel of protein biomarkers representative of those individuals that had been in contact with a patient with microbiological confirmed pulmonary TB but did not get infected.

Methods: Samples (serum, saliva and sputum) were collected from volunteer patients (TB: pulmonary TB; LTBI: contacts with latent TB infection; non-LTBI: contacts without evidence of latent TB infection). Quantitative proteomics were done using TMT10plex (Thermo) and a LC-Orbitrap Elite platform. Raw data was processed using Proteome Discoverer 2.1 software. Modulated proteins were selected after exhaustive manual review of the processed data and non-parametric statistical analysis with R software.

Results: We have found a unique proteomic signature in the sputum of non-LTBI contacts, consisting in a set of 32 proteins mainly involved in regulation of endopeptidase activity (Cystatin-C, SPINK-5), defense against pathogens (Mucin-7, Lactoperoxidase) and perception of bitter taste (Carbonic Anhydrase-6, Cystatin-S). In order to test the biological and clinical significance of our findings we are starting a validation in a functional model of MTB infection.

Conclusions: This indicates that nasal and oral mucosa play a critical role in the initial entry of the pathogen in the host, opening a new window for eliciting of the mucosal immunity to enhance the innate immune response as the first barrier to fight infection. In order to test the biological and clinical significance of our findings we are starting a validation in a functional model of MTB infection.

Keywords: Tuberculosis, vaccines, innate immune response
Early proteome dynamics of glioblastoma cells during the oncolytic adenovirus DNX-2401 infection

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Background: Glioblastoma multiforme (GBM) is the most common and aggressive type of glioma. Standard treatments confer the patient an overall survival time of 15 months. For this reason, modified oncolytic adenovirus such as DNX-2401, which specifically infects and acts in cancer cells, are being developed. This adenovirus has shown encouraging results in a first-in-human phase I clinical trial of GBM.

Methods: With the aim to design and engineering more specific and potent adenoviral based-vectors, we conducted a cell-wide study of cytosolic, nuclear, and secreted glioma proteomes throughout the early time course of DNX-2401 infection, combining different proteomic workflows.

Results: In addition to severe proteostasis impairment during the first hours post-infection, our data show that DNX-2401 induces a transient inhibition of Signal transducer and activator of transcription 3 (STAT3), Transcription factor AP-1 (c-Jun) between 3 and 10hpi and increasing Nuclear factor kappa-B (NFkB) activity at 6hpi. Furthermore, Delta-24-RGD specifically modulates the activation dynamics of protein kinase C (PKC), extracellular signal–regulated kinase 1/2 (ERK1/2), and p38 mitogen-activated protein kinase (p38 MAPK) pathways early in infection. Also there is a dynamic production of pleiotropic factors to the extracellular environment, suggesting that these secreted molecules may regulate the glioma growth, and differentiation states together with the inflammatory cell recruitment and activation within the first hours post-infection.

Conclusions: We have partially deciphered the molecular events triggered by DNX-2401 prior to the induction of autophagy in glioma cells. Further, cytokines modulated by DNX-2401 in vitro, are being monitoring in the serum from glioblastoma patients at three different time points during DNX-2401 treatment, in order to evaluate the immune response elicited by the therapeutic vector in the clinic.

Keywords: Adenovirus, Delta-24-RGD infection, Proteomics, Glioma
Title:
Parallel reaction monitoring-based strategy to validate colorectal cancer biomarkers in serum/plasma.

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Background:
Colorectal cancer is one of the diseases with highest lethality rates. The identification of robust biomarkers for differentiating disease and healthy patients in early diagnosis is demanded by the clinical community. Here, we aim to identify and quantify colorectal cancer (CRC) serum biomarkers in CRC patients by targeted Parallel Reaction Monitoring (PRM).

Methods:
The project has followed three phases: Identification, Verification and Validation. Different cohorts of pooled serum samples have been used in Identification and Verification phases. A different cohort composed by individual serum samples was used in Validation phase. In Identification phase shot-gun proteomics were performed in an LTQ-Orbitrap-Velos instrument with the assistance of Maxquant (vs 1.5.5.1) and Perseus (vs 1.6.1.1) softwares. In Verification and Validation phases, PRM was developed in a Q-exactive instrument. Targeted analyses were assisted by Skyline software (vs. 3.5.0).

Results:
From Identification phase, 116 potential biomarkers were selected based on their statistical significance and their relative expression in disease stages respect to healthy stage and based on their functional relation with cancer progression. Verification phase was conducted in 2 phases. In the first phase, 19 proteins were filtered, and as a result of the second phase, a list of 8 peptides belonging to 7 proteins were proposed for being validated. In individual serum samples, 6 peptides belonging to 5 proteins could be consistently quantified and validated. Importantly, LCNPTPOFGGK peptide (THSB1 protein) was statistically significant when comparing control and CRC patients. PCA analyses could distribute 80% of control patients separated from 65% of CRC patients. ROC analyses points LCNPTPOFGGK peptide as the best positioned in predicting the separation between control and CRC patients.

Conclusions:
A set of serum peptides could be validated by PRM and proposed as a cluster for differentiating control from CRC patients.

Keywords: colorectal cancer, parallel reaction monitoring, disease biomarkers, diagnosis.
Background: Changes in protein-protein interactions are key events that occur during cancer development. The study of these interactions is fundamental for the understanding of cancer mechanisms and to discover potential therapeutic targets. The High mobility group box 1 (HMGB1) protein is overexpressed in a wide range of cancers. Likewise, it has been associated to main cancer hallmarks like angiogenesis and metastasis in different tumors as in ovarian and prostate cancer.

Methods: In this work we perform large-scale immunoprecipitations of HMGB1 in epithelial cancer cells, followed by MS/MS analysis to elucidate new interactions of this protein as well as to obtain a whole picture of the functions that are associated to HMGB1 in ovarian cancer.

Results: Our results identify a high number of HMGB1 interacting proteins, many of them related to RNA processes. However, these results also associate HMGB1 to other functions, since interaction of HMGB1 with nuclear proteins like PARP1 and NuRD proteins, cytoplasmic proteins as septins and nucleolar proteins was also detected.

Conclusion: These results associate HMGB1 to different pathways related to tumoral events in cancer cells.

Keywords: HMGB1, cancer, interactions.
A MALDI-TOF MS Comparative Approach for Disclosing a Novel Peptide Biomarker Signature of Gingivitis

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Background: Gingivitis (G) is considered a reversible condition associated with bacterial plaque accumulation and gingival inflammation but, if untreated, it may ultimately progress to periodontitis (1). Therefore, there has been a crucial interest in delineating the mechanism of the still unknown biological processes involved in the progression from health to disease conditions. The discovery and validation of reliable biomarkers, in order to facilitate the early therapeutic intervention of disease, still represents an unmet medical need. Gingival crevicular fluid (GCF), as oral fluid released in the gingival sulcus, is an important diagnostic source of biomarkers for both periodontitis and gingivitis (2,3).

Methods: In the present study, we propose a MALDI-TOF MS comparative approach between a group of G and healthy (H) subjects for disclosing a potential novel peptide biomarker signature of gingivitis in the still underestimate GCF peptidome.

Results: Reproducible signatures of GCF peptidome between a group of G and healthy (H) subjects were detected by MALDI-TOF MS. Specifically, a pattern of five peptides resulted differentially expressed between G and H groups. Interestingly, among these biomarkers we found the C-terminal fragment of alpha-1-antitrypsin (AAT) namely C-36 peptide and two different PTMs of the full length S100A9 protein as indicators of the evolution of the periodontal disease.

Conclusions: It is worth to note that, among proteomic investigations, with the exception of one SELDI-TOF study, only bottom up approaches have been performed with the consequent limitation of loss of information related to potentially important post-translational modifications (PTMs) of the putative biomarkers. On the contrary, our top down MALDI-TOF platform is able not only to extrapolate informations about endogenous peptides and their PTMs, but also to allow the detection of increased levels of C-36 peptide in the GCF of G patients. Furthermore, we highlight the potential of a high-throughput screening MS-based platform in discovering key-peptidic-biomarkers for periodontal diseases.

Keywords: Biomarkers / MALDI-TOF-MS / Peptidomics / gingival crevicular fluid / gingivitis.

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Investigating protein interactions of MALAT-1, a long-noncoding RNA, in brain tumors

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Background: Metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is a long-noncoding RNA described to promote cell proliferation, migration and invasion in various non-brain cancer types. MALAT1 is highly conserved among mammals and known to regulate gene expression, post-transcriptionally modify primary transcripts and directly interact with several proteins. As expression levels are elevated in brain cells and MALAT1 is involved in synaptogenesis, this study aims to shed light into MALAT1s role in tumorigenesis by characterization of protein interaction networks in various brain tumors.

Methods: MALAT1 expression in brain tumor cell lines was confirmed via RT-qPCR. Affinity purification of MALAT1 interacting proteins was achieved through hybridization with respective biotinylated DNA probes in crosslinked cells followed by enrichment via streptavidin coated magnetic beads. Proteins were analyzed applying a label-free bottom-up strategy on a high resolution mass spectrometer (MS).

Results: A sample set consisting of five medulloblastoma and five glioblastoma cell lines exhibiting high MALAT1 expression was processed to identify putative protein interaction partners. Pulldown efficiency determined on basis of RNA level ratios amongst housekeeping RNA and MALAT1 was employed as quality control threshold. After MS analysis, 627 proteins were quantified in the sample set. Out of these, 264 proteins were significantly enriched in the MALAT1 specific samples compared to the control samples. From 20 known MALAT1 interactors and regulators, 11 were enriched in the MALAT1 specific samples. Out of the enriched proteins, 72 % are localized in the nucleus concurrent with MALAT1s predominant localization.

Conclusions: Identification of 11 out of the 20 known MALAT1 interactors and regulators demonstrates the feasibility of the chosen approach. Further analysis of putative interactors will increase the understanding of MALAT1s role in brain tumorigenesis.

Keywords: brain tumor, MALAT1, RNA protein interaction, long-noncoding RNA, mass spectrometry


[3] M. Preianò¹, G. Maggisano¹, M. S. Murfuni¹, C. Villella¹, C. Pelaia², T. Montalcini², N. Lombardo², G. Pelaia², R. Savino¹ and R. Terracciano. A MALDI-TOF MS Comparative Approach for Disclosing a Novel Peptide Biomarker Signature of Gingivitis. Revision pending.
“Cancer Moonshot Center Lund, Sweden”

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The National Cancer Institute (NCI), a part of the National Institute of Health (NIH) identified research opportunities that align with the Cancer Moonshot that is the biggest Cancer initiative ever, as a US & Global Cancer program. These opportunities, which may be supported with existing funds or with funds from the 21st Century Cures Act, mark the beginning of a Cancer Moonshot portfolio that will be expanded on with a 7 year project period and a substantially resourced budget. Ultimately, the aim of the “Moonshot” is to win the war on cancer and to get to a point in the very near future when we are managing cancer the same way we might manage any chronic disease, such as diabetes or asthma. Additional objectives involving drug treatments and therapies are to finally stop the toxic therapies, such as chemotherapy and radiation that decimate the immune system.

In Lund, Sweden, we have partnered with the Cancer Moonshot activities and will work on Melanoma Cancer. Malignant Melanoma is the type of cancer that has the fastest increase in the number of patients in Sweden (3% per year). In 2012, 2800 new malignant Melanoma cases were reported in Sweden. Taking all cancers into consideration, every fifth cancer patient will die in malignant melanoma. The 5-year survival rate is 5%-10% and the median survival is 6-10 months.

The most important risk factors are type-, and number- of pigments, nevi, sun exposure, as well as genetic factors. Melanoma is also a disease that features a cancer type where currently no biomarker or diagnostics is available to safely identify the disease staging, or responders to Personalized Medicine treatment within this patient group.
An outline and status report will be given on the Cancer Moonshot LUND activities.
Serum extracellular vesicles contain protein biomarkers for primary sclerosing cholangitis and cholangiocarcinoma

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Background:

Extracellular vesicles (EV) have emerged as pathogenic players involved in disease development and progression and also as an important tool in the search of biomarkers for different disorders. Cholangiocarcinoma (CCA) includes a heterogeneous group of biliary cancers with poor prognosis which Incidence seems to be increasing worldwide. Conditions such as primary sclerosing cholangitis (PSC) are known risk factors. Accurate non-invasive biomarkers for CCA or PSC are not yet available. In this study, we investigate the potential role of serum EV as carriers of protein biomarkers for PSC and CCA.

Methods:

Serum EV were isolated from CCA (n=43) or PSC (N=30) patients and healthy individuals (n=32) using well established ultracentrifugation/filtration methods. The characterization of EV was performed by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and immunoblot. The proteome of EV was determined by label free nLC MS/MS using Progenesis (Nonlinear Dynamics, Waters).

Results:

Using NTA, serum EV concentrations were found slightly higher in CCA and PSC than in controls. Round morphology (by TEM), size (~165 nm diameter by NTA) and markers (CD9, CD63 and CD81 by immunoblot) indicated that most serum EV were exosomes. Relative quantification analyses revealed 128, 121 and 43 proteins differentially expressed in CCA vs control, PSC vs control, and CCA vs PSC groups, respectively. These proteins showed high diagnostic values [maximum of 92.3% sensitivity (SEN), 90.0% specificity (SPE) and an area under the ROC curves (AUC) of 0.983 for CCA vs control, 100% SEN, 90.0% SPE and AUC of 0.967 for PSC vs control, and 83.3% SEN, 88.9% SPE and AUC of 0.898 for CCA vs PSC].

Conclusions: Novel proteomic signatures found in serum EV of CCA and PSC patients show potential usefulness as diagnostic and prognostic tools. CCA-derived EV contain increased concentration of oncogenic proteins that might participate in tumor progression.

Keywords:

Cholangiocarcinoma (CCA), Extracellular Vesicles (EVs), Serum Biomarkers
Secretomic Analysis of Metastatic Biomarkers in Oral Cancer

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**Background:** Oral cancer is a malignant tumor and it is characterized by poor prognosis and a low survival rate despite of radiotherapy with concomitant and adjuvant chemotherapy. The secreted fractions involve in various physiological processes including cell signaling, immune defense, and cell metastasis. Thus, secreted protein analysis becomes an emerging strategy to discover diagnostic and prognostic biomarkers in cancer detection. Therefore, to discover potential prognostic, diagnostic and therapeutic biomarkers during the process of oral cancer metastasis is beneficial for development of effective strategy.

**Methods:** In this study, we used 2D-DIGE and MALDI-TOF MS to identify the secreted proteins involved in oral cancer invasion. Moreover, to further validate the expression of identified proteins, we performed immunoblotting of the differentially expressed proteins between OC3 and OC3-I5 cells. We used RNA interference technique to investigate the effect of secretion of proteins treated OC3-I5 cells in invasion and migration. The cell proliferation was determined by MTT assay. The levels of cell migration and invasion were examined by wound healing assay and transwell assay, respectively.

**Results:** A proteomic study reveal that invasive properties alter the expression of 151 secreted proteins in OC3-I5 cells comparing to OC3 cells. Many potential proteins have been validated by immunoblotting. We choose Urokinase-type plasminogen activator (uPA) to be our candidate protein. Our results showed that the invasion and migration properties of OC3-I5 cells were significantly decreased by reducing the expression and secretion of potential protein. Furthermore, we used the recombinant protein uPA to investigate the direct effect on OC3 cells metastasis. Our results revealed that the proliferation, migration and invasion properties of OC3 cells were significantly increased through the recombinant protein uPA treatment.

**Conclusions:** To sum up, our secretomic analysis provides a new platform for the effective identification of metastasis-associated secreted proteins in oral cancer metastasis.

**Keywords:** Metastasis, Secreted protein, Oral cancer
Overcoming OCT drawbacks for renal tissue proteomics

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\textbf{Background:} Chromophobe renal cell carcinoma (chRCC) and Benign Renal Oncocytoma (BRO) are two renal cell tumors which, due to their similarity, the accurate diagnosis is sometimes compromised\textsuperscript{1}. Nowadays, proteomic approaches are a growing field with many applications for disease research, enabling the detection of a larger panel of proteins in a high throughput manner. However, some standard methods to preserve the samples collected for clinical researching clash with mass spectrometry analysis\textsuperscript{2,3}. For instance, the use of Optimal Cutting Temperature (OCT) compound, to embed and preserve tissues, hampers the MS analysis by suppressing the peptide signals.

Herein, we present an effective workflow to overcome the OCT problems in OCT-embedded samples to study the proteome of chRCC and BRO carcinomas

\textbf{Methods:} Ultrasonic energy and vortex were assessed to clean OCT from OCT-embedded samples. The methods were firstly developed by eliminating the OCT from mouse kidney tissue, and then the best conditions found were applied to human samples. Thus, biopsies tissues from human kidneys identified with chRCC (n=5) and BRO (n=3) were treated and compared. Non-tumoral kidney specimens (n=5) were used as control. Quantitative proteomics (label free) was carried out by nanoLC-MS/MS.

\textbf{Results:} Priori to trypsin digestion, OCT compound was successfully removed by a series of washing steps involving ethanol and water with the aid of the ultrasonic energy. The proteomes of the human kidney samples were quantitatively compared, and their proteomic profiles were used to cluster the samples with success.

\textbf{Conclusions:} A new method to assess the proteome of OCT-embedded kidney tissues samples has been developed. In addition, chRCC, BRO, and healthy samples were successfully profiled and clustered.

\textbf{Keywords:} Proteomics, OCT-embedded tissue, Chromophobe renal cell carcinoma, Benign Renal Oncocytoma, Diagnosis.

Diagnosing Chromophobe Renal Cell Carcinoma and Renal Cell Oncocytoma by MALDI-mass spectrometry-based profiling

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Background: Different renal carcinomas present similar profiles and are difficult to diagnostic. For instance, Chromophobe Renal Cell Carcinoma (chRCC) presents similar morphological features and radiographic imaging to Renal Cell Oncocytoma (RCO) ¹. This fact makes difficult chRCC differentiation from RCO by non-invasive methods. This problem is overcome by extracting a biopsy whose analysis differentiates chRCC from RCO. However, this method is invasive, causing pain and discomfort to the patient, and does not guarantee a correct differentiation in all cases. Herein it is presented a MALDI mass spectrometry-based method to differentiate chRCC from RCO.

Methods: A MALDI mass spectrometry (MS)-based method was used to profile the proteome of chRCC (5), RCO (3) and healthy (5) biopsies. The method was based on immobilized trypsin digestion of the proteomes plus selective peptide extraction, followed by MALDI-MS analysis ².

Results: The proteomic profiles were conveniently compared using PCA and clustering.

Conclusions: Our preliminary results suggest that MALDI mass spectrometry-based profiling is a promising methodology to differentiate chRCC from RCO.

Keywords: Chromophobe Renal Cell Carcinoma, Renal Cell Oncocytoma, MALDI, Sequential extraction, Immobilized Trypsin Magnetic Nanoparticles, Proteomics.

Ultrasonic-assisted protein extraction from FFPE nasopharyngeal carcinoma samples

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Background: Tissues are an important source of biological information with invaluable applications in the medical and scientific field. A standard procedure in hospitals for long-term preservation of the pathologic tissues is the formalin-fixed, paraffin-embedded (FFPE) method. The FFPE tissues are easy to storage and are an excellent resource for the study and discover of biomarkers with potential diagnostic outcome¹. However, a critical concern in the application of FFPE tissues for proteomics studies is protein extraction since it is a challenging step as a result of the extensive molecular crosslinking due to formalin fixation². We have overcome this drawback with the use of ultrasonic energy.

Methods: Nasopharyngeal carcinoma FFPE samples were deparaffinize using xylene in a dried bath at 63 °C. Tris HCL 20mM with SDS 4% and DTT 0.1M was used as retrieval solution and the samples were incubated for 20 min at 100 °C followed by ultrasonication using an ultrasonic probe (1 min time, 2mm tip, 100% amplitude). The digestion of the extracted proteins was done using the filter aided sample preparation (FASP) method³. Finally, samples were interrogated by mass spectrometry using the label-free protein quantification methodology.

Results: We observe a synergetic effect of temperature and ultrasound to improve the extraction of proteins from FFPE tissues. Quantitative and gel analysis of the extracted proteins revealed that more than 80% of the proteins are extracted in the first extraction step and the remaining content in a second extraction step.

Conclusions: Temperature is a key element in protein extraction from FFPE tissues. Ultrasonication has an important role in improving the quantity of extracted proteins.

Keywords: Proteomics; formalin-fixed, paraffin-embedded (FFPE); ultrasonication; protein extraction.

References:

PINPOINTING NEW BIOMARKERS OF BLADDER CANCER IN LIQUID BIOPSIES

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Background: Bladder cancer is the fourth most frequent neoplasia in men in developed countries [1] and the most expensive one on cost per patient, as the detection method is not cost effective [2]. The stage of bladder cancer is related to patient’s prognosis: Ta comprises papillary carcinomas confined to urothelium and is the stage with the best prognosis; T1 comprehends the invasion of lamina propria and is related to a poorer outcome and T2+ is a set of stages comprehending tumours that had invaded muscle or muscle and beyond, and near half of affected patients die in 5 years [3]. Other stages are not considered in this work. Clinical diagnosis of bladder cancer in early stages remains a major challenge. Herein we present a non-invasive method that allows to classify the above-mentioned stages.

Methods: A biomarker study was conducted using urine samples from: i) individuals with bladder cancer (stages Ta, T1 or T2+), ii) individuals with genitourinary disorders and iii) individuals without any urinary pathology. Using Filter Aided Sample Preparation, urinary proteins were purified and digested. Liquid Chromatography coupled to Mass Spectrometry was used for the identification and quantification of digested urinary peptides.

Results: A number of proteins was found to successfully help in the discrimination of the bladder cancer stages Ta, T1 and T2+.

Conclusions: Our results suggest that the urinary proteome can be used as a tool to diagnose bladder cancer stages Ta, T1 and T2+.

Keywords: Bladder Cancer, Proteomics, Mass Spectrometry, Urinary biomarkers

References:
Exosomes as new tools for searching relevant proteins in colorectal cancer metastasis

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**Background:** Exosomes are extracellular vesicles with a wide range of functions, including metastasis seeding, in most types of cancer. The aim of this study was to find proteins critical for colorectal cancer metastasis.

**Methods:** A non-metastatic murine model of colorectal cancer was compared with the metastatic mouse cell line CT-26 to investigate the exosomal protein content. To optimize the recovery of exosomes, cells were incubated in serum-free DMEM for 48h. Colon tissues were incubated for 2, 4 and 8h in serum-free DMEM. The exosomes were isolated by ultracentrifugation and lysated with different buffers: 7M urea/2M thiourea, methanol-chloroform and RIPA buffer. Exosomal lysates were analyzed in the Q-Exactive using a gradient: 5–35% (90min), 35%-100% (4min) and 100% (8min) ACN with 0.1% formic acid. Mass spectra were acquired using a “top 15” method and searched against SwissProt using Sequest HT search engine through Proteome Discoverer. Identified peptides were filtered using Percolator with a q-value threshold of 0.01 at a FDR of 1%.

**Results:** The largest amount of mouse exosomes from colon was recovered at 2h. The 7M urea / 2M thiourea buffer was the most effective lysis method, with 35% more exosomal proteins detected by mass spectrometry. The identified proteins correlated with: remodelling of the ECM, organotropism, angiogenesis and chemo-atraction, EMT, cellular metabolism, etc. Significant differences were observed between metastatic and non-metastatic exosomes. Proteins of metastatic exosomes are largely related to the interaction and modulation of the extracellular matrix and cell migration, including α3 and α5 integrins, in contrast to the non-metastatic exosomes. Laminins α5, β1 and γ1, which form laminin-511, are major candidates for further studies.

**Conclusions:** Methods for extracting and lysing exosomes from mouse colon and metastatic cells were optimised. A preliminary protein profile involved in metastasis has been obtained and will be further analysed using functional experiments.

**Keywords:** exosomes, metastasis, colorectal cancer, extracellular matrix, mouse models.
Urinary Exosomes from patients with cc-Renal Cell Carcinoma at different stages: a Proteomic Approach

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Background: Renal Cell Carcinoma (RCC) represents 2-3% of all cancers and its incidence is increasing. Clear cell Renal Cell Carcinoma (cc-RCC) is the most frequent type. Although ccRCC are usually diagnosed at early stages, their aggressiveness and clinical outcomes remain heterogeneous within each staging group; thus, novel predictors are needed. A non-invasive test using bodily fluids, like urine, will have a significant impact on patient management, and, in particular, Urinary Exosomes (UE), are proposed as a useful biomarkers source for diagnosis, prognosis, and therapeutic purposes of RCC. The aim of this study is to pinpoint a characteristic UE protein profile of RCC progression.

Methods: UE pools obtained from patients with cc-RCC classified as pT1a (n=6) and pT3a (n=6), and from age- and sex-matched healthy subjects (n=6) were isolated by ultracentrifugation, the corresponding proteome was analysed by label-free nLC-ESI MS/MS, allowing to identify and quantify 524 protein species.

Results: The results show that the UE proteome remained essentially constant among the three groups, considering only the protein identification. However, when we evaluated the protein abundance in relation to tissue origin and biological processes, we noted that as the tumour stage increases, many typical proteins of the urinary system changed their levels in UE, in particular that involved in signalling pathways, suggesting a relationship with the tumour dedifferentiation. Then, a panel of proteins were selected in order to validate their differential content between RCC and Ctrl UE through immunoblotting.

Conclusions: UE comparative proteomics could represent a promising starting point for the identification of RCC biomarkers for prognostic purposes.

Keywords: Exosomes, RCC (Renal Cell Carcinoma), label-free, markers, prognosis.

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IDENTIFICATION OF TUMOR ASSOCIATED ANTIGENS IN COLORECTAL CANCER PATIENTS BY MASS-SPECTROMETRY BASED PROTEOMICS

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Background: Ninety percent of patients suffering from the 8 most common cancer types can be successfully treated if early diagnosed. The humoral immune response has been demonstrated useful for cancer diagnosis, predating clinical symptoms up to 3 years. Therefore, we have here aimed at developing a methodological approach based on the specific immunoprecipitation of seroreactive proteins to IgGs of colorectal cancer (CRC) patients and their identification by mass spectrometry to complete the panel of CRC tumor associated antigens that could help establishing a diagnostic signature of the disease.

Methods: Serum samples from CRC patients at stages III and IV were used to immunoprecipitate specific seroreactive proteins from protein extracts from CRC cell lines with different metastatic properties. Protein extracts were previously clarified with IgGs from healthy individuals to remove non-specific proteins. Eluted proteins with selected reactivity to cancer patients’ autoantibodies were identified by mass spectrometry using a LTQ-Orbitrap Velos.

Results: A total of 1743 peptides corresponding to 645 proteins reactive to CRC patients’ sera and controls were identified, with 79 proteins showing a unique and specific seroreactivity to CRC IgGs. To avoid false positive targets and to select those proteins with a higher potential as CRC diagnostic markers for validation, the dataset was analysed using the CRAPome database and different bioinformatics tools. With the CRAPome, we selected 31 proteins more prone to be actual targets of CRC autoantibodies. Among them, 29 protein showed genetic variation in CRC and 7 showed a statistically significant expression deregulation in CRC. Lastly, one of the identified proteins has already been described as colorectal and renal cancer prognostic marker.

Conclusions: We have developed a methodological approach to identify specific proteins target of autoantibodies of colorectal cancer. We have selected a total of 13 proteins for their validation and evaluation of their inclusion in diagnostic panels.

Keywords: autoantibodies, immunoprecipitation, mass spectrometry, diagnostic signature
Proteome analysis of microdissected tumor cells reveals Annexin A10 as biomarker candidate for differentiation of intrahepatic cholangiocarcinoma and liver metastases of pancreatic ductal adenocarcinoma

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Background:

Intrahepatic cholangiocarcinoma (ICC) and pancreatic ductal adenocarcinoma (PDAC) are highly aggressive cancer types that arise from epithelial cells of the pancreatobiliary system. Owing to their histological similarity, differential diagnosis between ICC and metastases of PDAC located in the liver (mPDAC) frequently proves an unsolvable issue for pathologists. Yet, differential diagnosis of ICC and mPDAC is highly relevant as it leads the patient either to possibly curative resection (for ICC) or to palliation (for mPDAC). Therefore, novel biomarkers capable of improving this task are urgently needed.

Methods:

We performed laser-capture micro-dissection combined with a label-free proteomics approach and analyzed isolated tumor cells from nine ICC and eleven primary PDAC (pPDAC) patients. The resulting biomarker candidates were evaluated in a large independent cohort of 87 ICC and 88 pPDAC tissue samples using immunohistochemistry. Moreover, we also tested biomarker candidates published in the literature (MUCIN 1, Agrin, S100P, MUC5 AC, Laminin, VHL, CK 17, N-Cadherin, ELAC2, PODXL and HSPG2) and applied the biomarker candidates with the most promising results to an independent sample set including biopsies of 27 ICC and 36 mPDAC.

Results:

In the proteome analysis, we found 180 proteins with a significantly differential expression between ICC and PDAC cells of which nine candidate proteins were chosen for the immunohistochemical verification. Annexin A1 and Annexin A10, together with three previously published biomarker candidates (MUC5 AC, CK17 and N-Cadherin), showed the highest AUC values for the discrimination of ICC and pPDAC (between 0.72 and 0.84). These five biomarker candidates were applied to the independent set including biopsies of ICC and mPDAC emulating the challenging situation in clinical practice. Here, Annexin A10 showed the highest diagnostic potential with correctly 75.0% classified mPDAC (sensitivity) and 85.2% correctly classified ICC (specificity), respectively.

Conclusions:

We propose Annexin A10 as a biomarker meeting the challenging task of differentiating ICC from mPDAC. Our results highlight the great potential of proteomics techniques in biomarker discovery when a specific
diagnostic problem is addressed and illustrate possible solutions to overcome technical challenges like sample heterogeneity.

**Keywords:**

Biomarkers, Intrahepatic cholangiocarcinoma, PDAC, microdissection, Annexin A10
Detection of HCT116 mutated proteins by MRM mass spectrometry for preclinical diagnosis of colorectal cancer

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Background:
Colorectal cancer (CRC) is the third most common cancer in men and the second most common cancer in women. Genetic studies on HCT116 tumor line\(^1\) have demonstrated that mutations in DNA were transcribed in identified mutated mRNAs but there is no evidence of the translation of these mutated mRNAs into mutated proteins.
The aim of this work has been the identification of target mutated proteins in HCT116 cell culture samples by single Multiple Reaction Monitoring assay as preclinical exploratory study in cancer biomarkers research.

Methods:
A method based on mass spectrometry in MRM mode has been developed for the identification of mutated C-terminal sequences within the six target proteins, extracted from the HCT116 human colon cancer cell lines. Skyline Software was used to select the best values of collision energy and precursor ion-product ions transitions. A total number of 11 peptides and 40 transitions were monitored in a single analysis.

Results:
The application of MRM method demonstrated the translation of mutated target peptides in HCT 116 samples. The semi-quantitative analysis derived from MRM analysis showed the trend in mutated protein expression in HCT116 samples by evaluating the area under the peaks of the monitored transitions for each peptide, compared to control samples. The analyses were performed in triplicate to evaluate the reproducibility (CV values 10-15 %) and to give greater reliability to the obtained results.

Conclusions:
A MRM method has been developed for the unambiguous identification of mutated C-ter target proteins. The qualitative MRM analysis on HCT116 samples has demonstrated the translation of the mutated proteins with considerable advantages: a *single method* to detect all the target proteins in a single analysis, *high selectivity* and *high specificity* since the monitored transitions are strictly correlated to the C-ter modified amino acidic sequence.
The identification of these mutated proteins can offer a great contribution to the preclinical diagnosis of colorectal cancer and give the molecular basis for the optimization of targeted drug therapies.

Keywords: MRM, Mass Spectrometry, HCT116, Cancer Biomarkers
References

Quantitative targeted proteomic analysis of One-Carbon Metabolism Proteins in human liver cancer

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Background: Primary liver cancer (HCC) is recognized as the fifth most common neoplasm and the second leading cause of cancer death worldwide. Although most risk factors are known, and the molecular pathogenesis has been widely studied the underlying molecular mechanisms remain to be unveiled. This is a central issue to facilitate the definition of novel biomarkers and clinical targets for more effective patient management. We utilize the B/D-HPP popular protein strategy to detect proteins that have been associated to liver cancer in previous studies. Several enzymes highlight the known metabolic remodeling of liver cancer cells, four of which participate in one-carbon metabolism (1CM). This pathway is central to the maintenance of differentiated hepatocytes, as it is considered the connection between intermediate metabolism and epigenetic regulation.

Methods: We designed a targeted selective reaction monitoring (SRM) method to track quantitatively 15 different 1CM proteins in human liver samples (control, HCC and cirrhotic). This method allows systematic monitoring of one-carbon metabolism and could prove useful in the follow-up of HCC and of chronically liver-diseased patients (cirrhosis) at risk of HCC.

Results: Relevant changes occur at the quantitative level when non-diseased and tumor liver samples are compared. A more complex quantitative pattern was found from samples obtained from cirrhotic livers, suggesting that these particular samples are more heterogeneous than previously expected.

Conclusions: Significant and reliable quantitative changes found for several 1CM-specific proteins could be useful for the diagnosis and prognosis of human liver cancer.

Keywords: B/D-HPP; liver cancer; liver fibrosis; NAFLD; one-carbon metabolism; SRM
Immuno-MS assay to monitor LIF protein for tumor stratification and monitoring of LIF-targeted therapy.

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Background
Leukemia Inhibitory Factor (LIF) is a cytokine aberrantly expressed in cancer that acts as a pleiotropic oncogenic factor. It regulates cancer stem cells, the tumor microenvironment and specifically the immune system. LIF has emerged recently as a new therapeutic target in cancer. As a tool for tumor stratification and monitoring of LIF-targeted therapy we have developed a highly sensitive immuno-mass spectrometry method to measure LIF levels in plasma samples.

Methods
Tryptic digest of recombinant LIF protein was used to explore the response of the different peptides observed by liquid chromatography-mass spectrometry (LC-MS) analysis. Rabbit antipeptide antibodies were raised for the best candidate peptide. Isotopically labeled version of the peptide was obtained as internal standard for absolute quantitation.

A method was developed to monitor LIF protein levels in plasma, comprising the following steps:
- Digestion of plasma protein samples with trypsin, followed by spiking with a known amount of internal standard labeled peptide.
- Binding of the antipeptide antibody to protein A coated magnetic beads
- Enrichment of the targeted peptide through incubation of the tryptic digest with the antibody-loaded beads
- Washing of unbound background peptides
- Elution of the endogenous plus the labeled standard forms of the targeted peptide
- LC-MS analysis by Parallel Reaction Monitoring (PRM) on an Orbitrap Velos Instrument.

Results
Optimization of the different steps of the method allowed the accurate quantification of LIF levels in plasma on the range of 0.01 ng/mL. Analysis of paired samples from glioma patients demonstrates a good correlation between tumor and plasma levels of LIF.

Conclusions
The reported immuno-MS method allows reaching the required levels of sensitivity to measure LIF levels in plasma, enabling its potential use as a biomarker in the clinical setting.

Keywords
Immuno-MS / Targeted analysis / LIF / Glioma
Quantitative proteomics reveals proteins and pathways involved in the progression from non-cancerous lesions to gastric cancer.

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Background: Gastric cancer is among the most aggressive human malignancies. It is the fifth in cancer incidence and the third in cancer mortality. The progression of this disease is slow, with prolonged and sequential precancerous stages, including chronic gastritis, intestinal metaplasia (IM), dysplasia and, finally, gastric cancer (GC). The knowledge of premalignant conditions can be considered as ideal stage for the early detection of gastric cancer.

Methods: Here, we used the iTRAQ approach combined with high-resolution mass spectrometry analysis to describe the spectrum of the gastric cancer cascade. Biopsies from three stages: chronic gastritis, intestinal metaplasia and gastric adenocarcinoma, were selected for the quantitative proteomics analysis.

Results: We identified and reported quantitative data for 3,914 different proteins with at least two unique peptides confidently quantified. We uncovered pathways and processes dysregulated between the different stages.

Conclusions: Intestinal metaplasia is characterized by the down-regulation of ribosomal proteins with over expression of cell survival proteins, such as GSTP1 and EPCAM. The transformation to gastric cancer involves the overexpression of proteins corresponding to the DNA replication and the spliceosome pathways. Many proteins involved in ATP production and enzymes that play important roles in maintaining the function of the mitochondrial proteins like SIRT3 and SIRT5 were downregulated. The impairment of mitochondrial pathways was correlated with overexpression of enzymes supporting the glycolytic phenotype such as HK3 and PCK2. Several proteins found dysregulated during the progression to gastric cancer have the potential to be used as specific biomarkers and/or therapeutic targets.

Keywords: Gastric cancer, iTRAQ labelling, Quantitative proteomics, Cancer progression, Intestinal metaplasia.
PROTEOMICS IN AGRICULTURE
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Natural variation and pan-omics approach drove discovery of novel wood quality biomarkers in *Pinus pinaster* Aiton

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Natural variation of the metabolome and proteome of *Pinus pinaster* was studied to improve our understanding of phenotypic diversity, and wood quality. The metabolome and proteome of needles and the apical and basal section of buds were analyzed in three provenances of *P. pinaster* with contrasting growth capacity selected from mountain in the northwest (CDVO) to the coastal region of southeast Spain (ORIA) also considering a provenance from a sandy Moroccan area (TAMR). The three provenances were grown in a common garden for five years and metabolite and protein extraction were performed from the same sample. For metabolite detection two complementary mass spectrometry techniques: GC-MS and LC-Orbitrap-MS were used, while for protein identification GeLC-Orbitrap/MS combined with the development of custom peptide databases was used. Metabolome, proteome and environmental and growth data were integrated employing modelling and statistical tools to provide a comprehensive picture of phenotypic diversity.

A total of 1576 metabolites and 2069 proteins were identified. The characteristic metabolites of each tissue are related to primary metabolism, while provenances were distinguishable when tissues were analysed independently (Meijón et al., 2017) being secondary metabolites, and in particular flavonoid and terpenoid pathways, essential to reach this differential clustering. In relation to proteome, according to the MapMan categories, the proteins that participate in the synthesis of tetrapyrrole, mitochondrial electron transport or in the metabolism of amino acids are overaccumulated in TAMR while in CDVO the metabolism of nitrogen in the apical part of the bud. Integrative studies of metabolome, proteome and physiological parameters showed strong separation of the three population. Key proteins linked by sPLS networks to wood quality traits were identified in this analysis, such as, LIPOXYGENASE related to abscisic and jasmonic acids response according to Melan et al. (1993) and Pauwels et al. (2008). Altogether these results provide a new perspective of how tree metabolism adapt to different environment, and how these adaptions are also reflected in wood quality, providing these results a new set of biomarkers for breeding programs and forest management practices.
The alliance of proteogenomics & multiplexed targeted ecotoxico-proteomics for environmental monitoring of river water quality

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Background: New tools for predicting the impact of contaminants and diagnosing chemical contamination/toxicity of aquatic systems are worth to develop. We proposed omics methodologies on sentinel animals such as invertebrates for defining and monitoring biomarkers to predict the quality of our environment.

Methods: We implemented a two-stage proteomics-based biomarker discovery and validation workflow for a non-model species, the amphipod Gammarus fossarum. First, shotgun differential proteogenomics was performed highlighting candidate biomarkers of exposure to endocrine disrupting chemicals. A quantitative multiplexed targeted proteomics assay based on Selected Reaction Monitoring mass spectrometry was then developed to quantify 25 proteins of interest through male and female reproductive cycles and after exposure to contamination through laboratory and in situ assays.

Results: A protein sequence database was constructed based on RNAseq and proteogenomics measurements. A total of 55 proteins with key physiological functions were identified as potential biomarkers. The SRM quantitative screening of these candidates during the reproductive cycles led to the validation of specific proteotypic peptides. Their sensitivity to toxic contamination was demonstrated both in laboratory and during field monitoring taking into account four reference and thirteen contaminated sites. Laboratory exposures to two heavy metals modulated the levels of proteins involved in immune, antioxidant, and detoxification processes. The large-scale field exposures done in various rivers from South-East France led to strong inhibitions of molt- and reproduction-related proteins (notably chitinase and vitellogenin) in sites identified as having strong contaminant pressures.

Conclusions: Ecotoxico-proteomics is a useful methodology and illustrates the future of chemical risk assessment and environmental monitoring. The data obtained from the field surveys led to a new integrated multibiomarker index covering several key functions susceptible of being modulated by a large spectrum of chemicals.

Keywords: Environmental monitoring, biomarkers, proteogenomics, SRM, non-model species

References


DIFFERENTIATION OF THE GEOGRAPHICAL ORIGIN OF TIGER NUT (CYPERUS ESCULENTUS) THROUGH A PROTEOMIC APPROACH

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Background: Tiger nut or “chufa” is a grass-like plant from the Cyperaceae family with rhizomes ending with hard tubers which can be consumed raw, roasted, or ground to manufacture a milky beverage called “horchata”. The cultivar is mainly harvested in Western Africa and the Valencian community region (Spain) where its cultivation has been protected by the designation of origin “Horchata de Valencia”. In this sense, the characterization of indigenous Valencian tubers and their differentiation from foreign varieties is essential for quality assurance. This research addressed the characterization and discrimination of chufa tubers from Africa (Burkina Faso and Mali) and Valencia through a proteomic approach. Methods: Tubers (300 g) were ground in a laboratory grinder and mixed (1:5) with water, allowed to stand for 10 minutes and sieved (0.3 µm). The resulting liquor was centrifuged, obtaining an intermediate layer that was centrifuged again and filtered. Protein composition of the eluates was assessed by SDS-PAGE, followed by colloidal Coomassie staining and protein band quantification by digitalization and densitometry analysis. Bands showing different intensities between the two analyzed sample groups were trypsin digested and peptides characterized by hyphenated LC-ESI-MS/MS using Mascot, together with Unipot KB and NCBI nr protein databases. Results: Seven protein bands in the MW range 40-120 kDa showed different abundances between the two origins. Of them, six bands showed higher abundance in African samples. Because of harvesting, climate and soil peculiarities of the region, a higher expression of proteins related to drought stress was observed in these African varieties. Conclusions: This preliminary study of the Tiger nut proteome enabled the differentiation of samples with distinct geographical origins and can facilitate the elucidation of specific authenticity markers. Methodology proposed will help in the understanding of cultivation practices on protein expression of chufa cultivars. Keywords: Tiger nut; geographical origin; food authenticity; Proteomics; chufa.
STUDY OF MYOFIBRILLAR PROTEOME FROM PRE-SLAUGHTER STRESSED ANIMALS USING LIQUID ISOELECTRIC FOCUSING AND MASS SPECTROMETRY

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Background: In recent years, Proteomics has been used to elucidate which biochemical mechanisms are involved during meat aging. However, this process could be altered due to pre-slaughter stress conditions (PSC). Meat from animals that have experienced PSC is known as DFD meat (dark, firm and dry), which causes huge economical losses in the meat industry because of its poor quality attributes. Therefore, the study of myofibrillar proteome from pre-slaughter stressed animals in the early post-mortem stages will provide a greater knowledge about myofibrillar protein changes related to stress.

Methods: Proteomic analysis was performed on eight loin samples from Asturiana de los Valles and crossbred beef cattle previously classified as normal and DFD meat at 24 h post-mortem using pH measurements. Myofibrillar sub-proteome of Longissimus thoracis muscle was fractionated by means of liquid isoelectric focusing (OFFGEL) in the pH range of 4 to 7 and the obtained fractions were further analysed by SDS-PAGE.

Results: Six different protein bands showed significant statistical differences (p<0.05) between the two sample groups allowing the comparison of normal and DFD proteome. Proteins present in these bands, which were identified by LC-ESI-MS/MS, were ATP synthase subunit beta, desmin, myosin light chain 1/3, troponin T, adenylate kinase isoenzyme 1, troponin I, myosin-1, myosin-2 and myosin-binding protein C.

Conclusions: The current study introduces a novel approach to study the muscle proteome and to search for proteomic biomarkers related to pre-slaughter stress. OFFGEL technology separates proteins along different liquid fraction according to their isoelectric point. Moreover, proteins contained in each fraction could be directly identified by mass spectrometry avoiding the previous SDS-PAGE separation. This achievement stands out as an alternative to the use of 2-DE.

Keywords: DFD, pre-slaughter stress, OFFGEL, biomarkers, myofibrillar proteins.
CULTIVATION TYPE MAKES A DIFFERENCE: 
THE PROTEOME OF MONOLAYERS AND SPHEROIDS OF 
CANINE OSTEOSARCOMA CELLS

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Background: In dogs, osteosarcoma is an aggressive bone tumor with high metastasis rate in the lungs. Previous literature suggests that three-dimensional tumor cell cultures mimic the in vivo situation of micro-tumors and metastases and are therefore better experimental in vitro models than the often-applied two-dimensional monolayer cultures. The present study aimed at a proteome characterization of cells of both culture types.

Methods: Canine osteosarcoma cells (cell line D17) were cultivated in two different ways, namely as standard monolayer or as three-dimensional spheroid cultures. Harvested at time points where they had established typical cell features, cells were lysed and proteomes compared by 2D-DIGE. Differentially abundant proteins were identified by MALDI-TOF/TOF, and most interesting findings confirmed by independent methods (immunoblotting, PTM detection by LC-MS/MS).

Results: Narrowing down the number of differently represented spots by applying stringent filtering conditions (regulation, spot size, gel position), a selection of 27 unique canine proteins was identified. Most of them were of a functional network including mainly chaperones, structural proteins, stress-related proteins, proteins of the glycolysis/gluconeogenesis pathway and oxidoreductases. The most striking pattern difference in the DIGE image pattern was a noticeable shift to more acidic pI values for several proteins of medium to high abundance in monolayer cells compared to their spheroid counterparts. This could be traced back for two selected proteins (protein disulfide isomerase A3, stress-induced-phosphoprotein 1) to phosphorylation, both by electrophoretic and MS analyses.

Conclusions: Results indicate regulation of stress proteins in the process of three-dimensional organization characterized by a hypoxic and nutrient-deficient environment comparable to tumor micro-metastases. Findings are of importance a) as a pre-requisite to characterize cell systems before future use in specific hypoxia and angiogenesis studies, and b) as osteosarcoma develops in a similar way in dog and human, thus the dog being a suitable model for human studies.

Keywords: canine, cell culture, DIGE, osteosarcoma, proteomics.
Immunoproteomic approaches to solve the proteomic profile of olive pollen and its complex allergogram

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Background: Olive pollen is one of the main causes of allergy in Mediterranean countries. Although its complex allergogram has been extensively studied, some allergens remain unidentified due to restricted proteomic and genomic information. We here aimed to obtain the most complete olive pollen protein profile using two different immunoproteomic approaches and recently reported genomic data.

Methods: First, a tryptic digest of the olive pollen extract was analysed by LC-MS/MS using a Q-exactive. Second, serum IgG from patients allergic to olive pollen were isolated and used in a developed immunoprecipitation technique to restrict the analysis to the most immunoreactive proteins in the extract. We used Blast2GO to get a global perspective of the proteomic data. Thermo Scientific Proteome Discoverer was used to identify new olive pollen potential allergens using the list of all known allergens -Allergome database-. The cDNAs encoding five candidate allergens were cloned and expressed as N-terminal His-tagged proteins in E. coli. Finally, their IgG and IgE immunoreactivity was analysed by ELISA and WB.

Results: Blast2GO analysis showed that most of the olive pollen proteins were related to metabolic pathways. Among them, proteins belonging to more than 20 allergenic families not previously described in olive pollen were identified. A cyclophilin, a pectate lyase, an enolase and two different malate dehydrogenases were cloned and produced in bacteria. All of them were recognized by IgGs from olive pollen allergic patients, and cyclophilin showed IgE reactivity. Thus, it has been defined as a new olive pollen allergen.

Conclusions: We have performed the most complete proteomic analysis of olive pollen, obtaining relevant information about the predominant molecular function of its proteins, while increasing the number of potential olive pollen allergens. These results are relevant for both, diagnosis of olive pollen allergy and to understand the mechanisms through which olive pollen elicits an immune response.

Keywords:

Allergy
Olive pollen
Immunoproteomics
Immunoprecipitation
Cyclophilin
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ChloroKB: A WEB-APPLICATION FOR THE INTEGRATION OF PROTEOMIC KNOWLEDGE RELATED TO CHLOROPLAST METABOLIC NETWORK

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Quantitative proteomics approaches, together with controlled organelle fractionation, allows subcellular localization to be determined. Using such a strategy we provided the subplastidial localization of chloroplast proteins in the stroma, the thylakoids, and envelope membranes compartments (AT_Chloro database). The accurate localization of proteins is a key feature in the context of functional investigations, especially to get insight over metabolic pathways occurring in the chloroplast. Indeed the chloroplast is a complex and integrated network that produces a high number of metabolites (e.g sugars, vitamins, lipids and pigments). One way to improve our knowledge of such a metabolic factory is to build metabolic pathways with highly curated and integrated knowledge. Although structural models (stoichiometry matrices) and pathway databases are extremely useful, they cannot describe the complexity of the metabolic context and new tools are required to visually represent integrated biocurated knowledge for use by both humans and computers. Here, we describe ChloroKB, a web-application (http://chlorokb.fr/) for visual exploration and analysis of the Arabidopsis thaliana metabolic network in the chloroplast and related cellular pathways. The network was manually reconstructed through extensive biocuration to provide transparent traceability of experimental data such as proteomic data. Proteins and metabolites were placed in their biological context (spatial distribution within cells, connectivity in the network, participation in supramolecular complexes, regulatory interactions, quantification obtained by MS) using CellDesigner software. The network contains more than 1100 reviewed proteins with different localizations. The visual presentation is intuitive and browsing is fluid, providing instant access to graphical representation of integrated processes and to a wealth of refined qualitative and quantitative data. Thus, ChloroKB is a significant support for structural and quantitative kinetic modeling, for biological reasoning, when comparing novel data to established knowledge, for computer analyses, and for educational purposes. ChloroKB will be enhanced by continuous updates following contributions from plant researchers.
Ancient tooth proteomes

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Background: Teeth are a remarkable source of information in paleoanthropological research. Investigation of ancient dental proteomes has the potential to provide complementary biomolecular data such as sex and taxonomic determination, phenotype and disease marker identification, or diet composition, that are essential to understand morphostructural signatures of fossils remains and to get insight on human phylogeny and ancient physiopathology.

Methods: In the frame of setting up paleoproteomic approaches, human teeth from the french Neolithic site of Mont Aimé (3500 BC, France) have been analyzed by online nanoflow liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) using Q Exactive™ Plus and Orbitrap Fusion™ Tribrid™ mass spectrometers. At each step, protocols and procedures have been adapted to the particular case of ancient samples. In particular, low protein content and protein contamination by modern or environmental materials have been taken care of by careful manipulation in cleanrooms and by insertion of appropriate blank controls and column washing procedures. Another major issue concerns protein degradation and modification. Therefore, a protein identification workflow using iterative searches with home-build protein databases and Proteome Discoverer™ software has been optimized to take into account several modifications (deamination, oxidation, carbamoylation), missed cleavages, non-tryptic peptides, and the variant peptides containing diagenetic amino-acid substitutions. Finally, PRM-based targeted mass spectrometry approaches have been implemented to detect, identify, and quantify systematically and precisely the sex determination peptides of amelogenin.

Results: Ten Neolithic tooth proteome have been analyzed. The original iterative search workflow allowed to identify a large number of proteins, 500 to 1400 proteins in each sample, except in the negative controls that contained 4-32 proteins (essentially collagens, keratins and serum albumin), indicating a low level of contamination during extraction and MS analysis. The majority (80%) of proteins were identified in steps other than in the classical semi-tryptic step. Among the proteins identified in dental tissues, some of them such as amelogenin, ameloblastin, enamelin, amelotin, and dentin sialophosphoprotein, are very specific to enamel or dentin, thus ensuring the reliability of the analyzed samples. In addition, the detection of the two forms of amelogenin encoded by the chromosomes X (AMELX) and Y (AMELY) potentially offers the opportunity to characterize male specimens, an essential information for paleoanthropologists when confronted to sexual dimorphism.

Conclusions: This work demonstrates that protein identification workflow using iterative searches has the potential to yield more in-depth coverage of proteomes, especially in ancient samples that contain diagenetic random degradation of proteins. Moreover, the finding of a complex proteome in 5000 year old teeth may
Paraoxonase 1 (PON1) as plasma marker to detect illicit treatment with dexamethasone in veal calves

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**Background:** In order to enhance carcasses and meat quality traits, corticosteroids, and dexamethasone in particular, are widely used as illicit growth promoters in veal calves and beef cattle, either alone or in combination with anabolic agents especially at low dosages. The strong pharmacological activity of synthetic corticosteroids makes their residues dangerous for meat consumers. The “omics” techniques represent innovative methods to identify illicit treatments. Our previous proteomic study based on two-dimensional electrophoresis (2DE) carried out on plasma samples collected from Friesian veal calves treated and untreated experimentally with dexamethasone sodium, allowed to identify a significant disappearance of two isoforms of a protein, identified with liquid chromatography-tandem mass spectrometry (LC-MS/MS), as paraoxonase 1 (PON1) only in the treated animals.

**Methods:** To evaluate the performance of this biomarker to identify anabolic treatments in veal calves, further analyses were performed by two-dimensional electrophoresis on a large sample of experimentally treated and not-treated cases (20 and 36 respectively).

**Results:** The statistical analysis estimated a sensitivity of 95% (95%CI: 75.1% - 99.9%) and a specificity of 100% (95%CI: 90.3%-100%).

**Conclusions:** These results reveal that PON1 is a useful plasma marker to identify illegally treated animals already at farm level before they enter the human food chain. Moreover, to exclude other factors that may affect the expression of this biomarker and to assess its applicability in national monitoring plans, a pilot study involving several Italian regions is currently ongoing.

**Keywords:** dexamethasone; anabolic treatments; PON1; 2DE; veal calves.
be explained by a permanent regeneration of the dentine tissue induced by the damages caused by the roughly grinded regimen of the Neolithic populations at the early stage of agriculture. Finally, the ability to detect sex-specific AMEL peptides by targeted MS is promising for sex determination of ambiguous teeth fossils and it is also possible to detect polymorphisms with interest in dental morphiostuctural studies or archaic taxonomy.

**Keywords:** Paleoproteomics, Database iterative searches, Tooth, Palaeoanthropology
Changes in the phosphorylation status of storage proteins linked to potato sprouting

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Background: Influence of phosphorylation in potato (Solanum tuberosum L.) storage proteins during sprouting is unknown. Patatin is the major storage protein in potato tuber which is comprised by glycosylated and phosphorylated isoforms. Mobilization of storage proteins play a key role in sprouting process providing nitrogen supply. Sprouting of potato is a fundamental process within the tuber biological cycle to control vegetative propagation. Another important issue is to extent the tuber storage time in order to avoid large economic losses. Therefore, the aim of the present study is to assess the significance of patatin phosphorylation in the sprouting process.

Methods: In this report, isoforms of patatin present in potato (cv. Kennebec) at partial dormancy and sprouting stages of tuber cycle were studied. Two-dimensional electrophoresis (2-DE) and downstream identification by mass spectrometry (MALDI-TOF and MALDI-TOF/TOF MS) allowed the construction of high-quality maps of the patatin isoforms in both tuber stages analyzed. In addition, chemical dephosphorylation analysis by hydrogen fluoride-pyridine together with 2-DE, were used to estimate relative phosphorylation rate (PR) of patatin isoforms.

Results: There are statistically significant differences in PR between Kuras and non-Kuras patatin isoforms throughout transition from partial dormant tubers to sprouting tubers. In particular, we found that Kuras patatin isoforms are more phosphorylated than the non-Kuras patatin isoforms.

Conclusions: The results suggest that phosphorylation can play a relevant role in the mobilization of the patatin during the transition from partial dormancy to tuber sprouting.

Keywords: Patatin, Potato tuber, Storage proteins, Protein phosphorylation, Tuber sprouting.
Phosphoproteomic analysis reveals that \textit{Salmonella} Typhimurium increases neutrophil survival after infection and decreases their immune response capacity

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\textbf{Background:} Interference with kinase-mediated phosphorylation in host signaling is a beneficial strategy used by many pathogens. \textit{Salmonella} Typhimurium colonizes the mucosal epithelium causing a wide range of gastrointestinal and systemic diseases. The aim of this study was to investigate the phosphoproteomic profile of porcine neutrophils after \textit{Salmonella} Typhimurium infection to better understand how the pathogen interferes with the cellular signal transduction pathways involved in the host innate immune response.

\textbf{Methods:} Neutrophils from four pigs were isolated and \textit{in vitro} infected for 30-minutes with \textit{Salmonella} Typhimurium, and each sample had a non-infected control processed simultaneously. Then, neutrophils were lysed for phosphoprotein enrichment and purification. Label free LC-MS/MS analysis was performed, and phosphoproteins were identified and quantified. Results were compared by a paired T-test, and functional analysis was performed.

\textbf{Results:} A total of 1,450 phosphoproteins were detected, from which 52 were differentially regulated in infected samples (21 up- and 31 down-regulated). These proteins were mainly involved in inflammatory response, cell signaling/interaction, cell death and survival. We found a predicted decrease in development and migration of leukocytes based on CORO1A, DCTN2 and LCP2 loss of phosphorylation. We found decreased phosphorylation of PRKAR2A and PRKAR1A from the MAPK signaling pathway, and up-regulation of PSMC2, PSMC4, SLA-1 and HSPA8 phosphoproteins, involved in antigen presentation. NFkB2 increased phosphorylation affected IL-1, IFN production, and RAR-mediated cell proliferation signaling pathways, predictably inhibiting apoptosis and increasing survival. We also found other differentially expressed phosphoproteins that potentially regulate cell death through inflammatory pathways, such as methyl CpG binding protein MECP and coronin CORO1A.

\textbf{Conclusions:} During neutrophil infection, \textit{Salmonella} Typhimurium interferes with major processes such as cell death and inflammatory response. Our results suggest that bacterial pathogen exploits host signaling pathways to obstruct host defense mechanisms and enhance their own survival and proliferation.

\textbf{Keywords:} Neutrophils, phosphoproteomics, \textit{Salmonella}, host-pathogen interaction, pig
Development of mass spectrometry-based analysis of blood protein glycation in birds

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Background: Glycation corresponds to the (non-enzymatic) covalent bonding of, e.g. glucose, to proteins by an irreversible Amadori rearrangement and further transformations yield a highly heterogeneous group of advanced glycation end products (AGEs), their formation being associated with ageing and metabolic diseases. As glycation rates are proportional to glucose levels, the amounts of glycated hemoglobin are used to highlight the diabetic syndrome. Despite very high chronic glycaemia levels, birds’ lifespan is proportionally 2-3 times higher than mammals. Previous studies looking at glycation in birds [Récapet et al., Biology Letters, 2016] used existing kits lacking specificity and sensitivity. Therefore, we aimed at developing a specific mass spectrometry-based assay, and determining if birds have developed a protection mechanism against glycation of proteins.

Methods: Blood was collected from 12 zebra finches (Taeniopygia guttata) held in captivity. Red blood cells (RBC) proteins were analyzed by nanoESI-MS direct infusion using a nanomate Triversa (Advion) coupled to a Synapt G2 (Waters). Plasma proteins were stacked on a 5% stacking gel, digested with trypsin and analyzed by nanoLC-MS/MS. MS/MS data interpretation was performed using in-house developed Proline software (http://proline.profiproteomics.fr/), searching specifically for glycation events.

Results: Through nanoESI-MS direct infusion of RBC protein extracts, the three globin chains were nicely detected, but no glycated form of hemoglobin was present, whatever glycaemia levels (up to 5 times the normal level in humans). Conversely, major blood proteins were found glycated and a series of AGE modification sites were identified in plasma samples using our proteomic approach.

Conclusion: Absence of glycated hemoglobin could indicate that amine sites are protected, or that some particular mechanism of deglycosylation could exist in bird’s RBC. To gain further insights into glycation events in birds, we will now develop proteomics-based quantitation of glycations level of circulating proteins of the zebra finch.

Key words: glycation, birds, proteomics, AGEs
Title of the abstract (word limit is 15): Proteomic study in *Mus Musculus* under arsenic, cadmium and mercury exposure to evaluate metal toxicity

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Haptoglobin, ovostatin, and albumin as possible salivary biomarkers of canine parvovirosis.

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Background: canine parvovirosis (CPV) is a severe disease that caused nearly 100% morbidity and up to 90% mortality in unvaccinated puppies. The most common clinical signs include acute severe vomiting and diarrhoea, fever, dehydration and lethargy. An early diagnosis of the CPV is essential to provide an adequate treatment and to prevent death and virus spread. In this sense, proteomic approaches of non-invasive specimens such as saliva have been widely employed in the search of biomarkers of diagnosis in canine diseases.

Methods: one-dimensional polyacrylamide gel followed by mass spectrometry (MS) was performed in saliva from 14 client owned-dogs. Dogs (9 males and 5 females) ranging from 2 to 10 months old (5 ± 2.3) were divided into three groups: healthy (control group, n=4), and dogs with CPV that survived (survival group, n=6) or perish due to the disease (dead group, n=4).

Results: Three bands that were differentially expressed between the groups were identified as containing haptoglobin, ovostatin and albumin. The bands containing haptoglobin and ovostatin were upregulated and downregulated in both groups with CPV, respectively, when compared to controls. The one containing albumin was upregulated in dead group when compared to control group.

Conclusions: Haptoglobin, ovostatin and albumin in saliva could be suitable biomarkers of diagnosis, assessment of disease severity, and prognosis of canine parvovirosis.

Keywords: saliva, dog, one dimension electrophoresis, biomarkers.


A PROTEOMIC APPROACH TO UNDERSTAND THE EFFECTS OF ENVIRONMENTAL ENRICHMENT IN THE PIG HIPPOCAMPUS

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Background: Environmental enrichment (EE) produces changes in brain structure and functionality. In rats and mice, EE enhances memory and learning abilities, it is associated to enlarged brain volume and it increases dendritic morphological complexity and neurogenesis, mainly in the hippocampus. Pigs are an interesting animal model due to the resemblance of their brain to the human organ. In porcine production, EE is known to have positive effect on pig welfare.

Methods: In the present study, a total of 44 female pigs (crosses of Large White × Landrace NN sows with Pietrain heterozygous Nn boars) aged 8 weeks were allocated under the same housing conditions, which consisted in a full slatted floor with a density of 1.2 m²/pig. At 15 weeks, the density of two pens was reduced to 0.7 m²/pig with the same floor (barren) whereas on the other two pens the density was maintained, the floor changed to concrete with 700 g of straw/pig (enriched). At 24 weeks of age, pigs were stunned with CO₂ and exsanguinated. The skull was opened, the brain was removed and the hippocampus (HC) was excised and kept frozen at -80 °C. Proteins from the HC were extracted, labelled with iTRAQ and analyzed by nanoLC-ESI-MS/MS on an Orbitrap Fusion Lumos™ Tribrid (Thermo).

Results: The results indicate that 56 proteins were present in differential amounts (p<0.05), 46 upregulated and 10 downregulated (enriched/barren). The main three groups of upregulated proteins correspond to: 1) ribosome components and assembly, 2) tubulins and 3) proteins related to oxidative phosphorylation (ubiquinone, succinate dehydrogenase, cytochromes, ATP synthase).

Conclusions: These results are consistent with an increased hippocampal neurogenesis and higher metabolic activity of brain cellular components.

Keywords: hippocampus, pig, environmental enrichment, iTRAQ
Proteomics of Holm oak (*Quercus ilex*): an orphan and recalcitrant plant species. How to get a confident protein identification and quantification in bottom-up/shotgun approach

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Proteomics of orphan, unsequenced, and recalcitrant organisms, as it is the case of the typical Mediterranean forest tree Holm oak (*Quercus ilex*), is very challenging [1]. Before translating proteomics data into biological knowledge a few questions dealing with the analytical technique itself must be answered. What are we identifying and how confident is the identification? [2] It is important to clearly differentiate a hit from an ortholog and gene product identification, with the difference depending on the database and the confidence parameters (score, number of peptides, and coverage) [3]. Does the identified protein correspond to a protein species, allelic variant, isogene? How confident is the quantification? If the answer to that questions is no clear enough and the approach not optimized and validated our data will be just putative, a matter of low probability, and our research descriptive and speculative, and our conclusions most probably erroneous [4].

We have been investigating different aspects of the Holm oak biology (growth, development, responses to stresses) and the biodiversity within the species [5] by using, classical biochemical approaches, ecophysiology, proteomics, and other –omics, moving ow on the Systems Biology direction [6]. With respect to proteomics we have been mainly used a 2-DE based platform [7-10]. In order to deep into the proteome coverage we are now employing a bottom-up/shotgun approach. For that different protocols have been optimized to be used with Holm oak, in particular, and other orphan plan species, in general. Results of a time dilution experiment with extracts obtained from a mixture of different plant organs (embryo, seed, root, leaf) will be presented discussing on the number of protein species that may be confidently identified and quantified.

References


Keywords: Holm oak, orphan species, plant proteomics, shotgun
Proteomics, and its integration with transcriptomics and metabolomics, allowed the reconstruction of the metabolism as it occurs in *Quercus ilex*

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**Background**: Holm oak (*Quercus ilex*) is the most representative species of the Mediterranean forest and of the Spanish agrosilvo-pastoral “dehesa” ecosystem. Despite its environmental and economic interest, Holm oak is an orphan species whose biology is very little known, especially at the molecular level. In order to increase the knowledge on the metabolism of this tree species (enzymes and chemical composition), the employment of a holistic and multi-omics approach, in the Systems Biology direction, would be necessary.

**Methods**: By using a sample consisting of a pool generated by mixing equal amounts of homogenized tissue from seed, embryo, leaves and roots, protocols for proteome (shotgun LC-MS/MS), transcriptome (NGS-Illumina) and metabolome (GC-MS) analysis have been optimized.

**Results**: These analyses resulted in the identification of around 2380 protein species using a custom-built specific database that contains 62629 transcripts. RNA and protein sequencing favoured each other, increasing the number and confidence of the proteins identified and correcting erroneous RNA sequences.

The integration of the large amount of data reported using bioinformatics tools allows the Holm oak metabolic network to be partially reconstructed (carbohydrate and energy metabolism, amino acid metabolism, lipid metabolism, nucleotide metabolism and biosynthesis of secondary metabolites). The TCA cycle was the pathway most represented with 6 out of 8 protein enzymes and 5 out of 10 metabolites. On the other hand, gaps, missed pathways, included metabolism of terpenoids and polyketides and lipid metabolism.

**Conclusions**: The multi-omics resource generated in this work will set the basis for ongoing and future studies, bringing the Holm oak closer to model species, to obtain a better understanding of the molecular mechanisms underlying phenotypes of interest and to select elite genotypes to be used in restoration and reforestation programs, especially in a future climate change scenario.

**Keywords**: *Quercus ilex*, omics, metabolome, proteome, transcriptome
The recombinant allergen Ole e 7 assembled by de novo mass spectrometry as a tool for diagnosis of olive pollen allergy.

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Topic: Plants and Animal Proteomics

Olive tree is one of the main causes of allergy in Mediterranean countries. Among the thirteen olive allergens identified to date, the Ole e 7 non-specific lipid transfer protein is a major allergen in regions with high olive pollen levels. Indeed, Ole e 7 is associated to severe symptoms such as anaphylaxis. Despite its clinical importance, its cloning by classical approaches has been impossible since the 90’s due to its high polymorphism.

The objective of this work was to obtain the whole amino acid sequence of Ole e 7 by de novo sequencing by mass spectrometry, and determine its molecular and immunological features.

Ole e 7 was separated by 2DE-gel electrophoresis. After Coomassie Blue staining, a predominant but diffuse spot was identified, demonstrating the presence of multiple isoforms of Ole e 7 in olive pollen. The most prevalent allergen isoform was in-gel digested with trypsin and extracted peptides subsequently analyzed by nLC-MS/MS to obtain as much de novo sequenced peptides as possible. From the total of 453 de novo peptide sequences obtained, we were able to complete the full length amino acid sequence resembling Ole e 7 using 13 peptides sequences. Then, the optimized DNA
Proteomic study on advanced glycation end-products treatment in kidney of mice

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Background: Diabetic nephropathy is the most common cause of end-stage renal disease in the world. Advanced glycation end products (AGEs) are heterogeneous cross-linked sugar-derived proteins which could accumulate in glomerular basement membrane, mesangial cells, endothelial cells, and podocytes in patients with diabetes and/or end-stage renal failure. AGEs are thought to be involved in the pathogenesis of diabetic nephropathy via multifactorial mechanisms such as oxidative stress generation and overproduction of various growth factors and cytokines.

Methods: Nε-(carboxymethyl) lysine-conjugated bovine serum albumin (CML-BSA) is a major component of AGEs. To investigate a role of CML-BSA in the regulation of diabetic nephropathy, we performed in vivo animal experiment and proteomic analysis. To confirm the effect of AGEs on mitochondrial dysfunction, HK-2 cells, which are human renal tubular cells, were treated with CML-BSA and examined by electron microscopy.

Results: This study aims to analyze advanced glycation end-products (AGEs)-mediated protein network in mice kidneys. We used mass spectrometry to detect proteome in kidney from streptozotocin (STZ)-induced diabetic mice kidneys. Chronic consumption of AGE induced grade 1 necrosis of renal tubules. In addition, from the proteomic analysis, we identified 981, 1025, and 911 proteins in the control, STZ, STZ+AGEs mouse kidney, respectively. As a result of IPA analysis, AGEs-mediated differential proteins were involved in mitochondrial dysfunction. To confirm the effect of AGEs on mitochondrial dysfunction, HK-2 cells, which are human renal tubular cells, were treated with CML-BSA and examined by electron microscopy to directly observe the mitochondrial dysfunction morphology.

Conclusions: Many of proteins are functionally associated with kidney toxicity and specific mitochondrial dysfunction related proteins were identified in AGEs-treated mice kidney. We found grade 1 metastatic necrosis of renal tubules without inflammation in AGEs-treated mice kidney. AGEs are likely to induce diabetic nephropathy by inducing chronic mitochondrial dysfunction in the mice kidney tubules.

Keywords: Advanced glycation end products (AGEs), proteomics, diabetic nephropathy
codon usage codifying for Ole e 7 for its production in Pichia pastoris yeast was synthesized and directly used to express the recombinant protein using the KM71H yeast strain. We next evaluated the correct folding of the purified recombinant protein and assessed its allergenic and antigenic properties. We confirmed by WB, ELISA and cellular assays that the recombinant protein rendered most of the antigenic and allergenic properties of the natural allergen isolated from pollen.

The recombinant allergen assembled by proteomics will permit its analysis and characterization in detail and might be used for a more effective clinical diagnosis of olive pollen allergic patients.
PROTEOMIC CHARACTERIZATION OF SOYBEAN SEED HULL FOR BIOTECHNOLOGICAL PURPOSES

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Background: soybean crop has a huge development. Its consumption is led by oil and flour, for which the hull must be removed. Hull contains valuable proteins that can be extracted prior to pellet it. The aim of this work is the proteomic characterization of soybean seed hull, as a way to identify candidate proteins for downstream processing, allowing the revaluation of soybean crop.

Methods: hulls were extracted with phosphate buffer pH 6.9. The extract was centrifuged and concentrated further by ultrafiltration (10X). Then it was reduced with dithiothreitol, alkylated with iodoacetamide and digested with trypsin. Peptides were extracted with acetonitrile and analyzed by nano-HPLC-ESI-Orbitrap. Data analysis was performed with Proteome Discoverer. The protein-protein association network was performed using STRING. Gene Ontology analysis was performed using QuickGo. Relative abundance was estimated by Peptide Spectrum Matches (PSM) and by Exponentially Modified Protein Abundance Index (emPAI).

Results: a total of 149 proteins were identified. The interactomic map contains 401 interactions. Proteins were classified by their cellular location (membrane: 32%; cytoplasm: 15%; extracellular region: 10%; nucleus: 6%; cell wall: 5%; endoplasmic reticulum: 4%; cytosol: 3%; others: 7%; n.a.: 18%), by their molecular functions (catalytic activity: 47%; nucleotide binding: 11%; metal ion binding: 11%; others 17%; n.a.: 13%), and by biological processes (metabolic process: 55%; response to stimulus: 14%; regulation of biological process: 4%; transport: 4%; biogenesis: 4%; others: 8%; n.a.: 11%). Approximately 60 proteins with biotechnological applications were identified, of which 15 were selected by its high relative abundance: Glucan 1,3-β-Glucosidase, Glycinin (G1, G2 and G4), Trypsin inhibitor, Protein disulfide-isomerase, Cysteine proteinase, Beta-amylase, β-D-xylosidase, 2S Albumin, Peroxidase, Lectin, GDSL esterase/lipase, Thioredoxin, Chitinase and Lipid transfer protein. 10 proteins were identified as allergens.

Conclusions: high-value proteins of biotechnological interest, expressed in high proportion, were identified, that could be purified allowing the revaluation of soybean crop.

Keywords: PROTEOMIC, SHOTGUN, SOYBEAN, SEED, HULL.
PHYSICOCHEMICAL CHARACTERIZATION OF PROTEIC CONTAMINANTS FROM INSECT LARVAE INFECTED WITH RECOMBINANT BACULOVIRUS FOR PROTEIN EXPRESSION

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Background: the use of baculovirus as an expression vector for the production of recombinant proteins has grown substantially in the last few years, but insect cell lines require aseptic environments, sterile equipment, bioreactors and expensive growth medium. The utilization of insect larvae constitutes an excellent alternative with high expression levels and without the need for the requirements cell lines impose, reducing costs several times. The biggest limitation for this approach is its complex purification. The aim of this work was to study the proteic contaminants present in the system, their chromatographic behavior and their physicochemical characteristics, to elaborate rational and scalable purification algorithms based on this knowledge.

Methods: *Rachiplusia nu* larvae infected with recombinant baculovirus based on *Autographa Californica*, were reared for 4 days at 27°C and fed an artificial diet. Homogenates obtained were clarified and purified using different chromatographic matrixes. The eluates and total protein extract of infected and non-infected larvae were analyzed using 2D gel electrophoresis.

Results: a smaller number of total proteins was detected in infected larvae compared to non-infected ones. Using cationic exchange matrixes, the number of spots detected was scarce. In contrast anion exchange matrixes adsorbed a high number of host and viral proteins. When studying hydrophobic interaction matrixes, most of the proteins detected presented pI near 7 and an average low molecular weight.

To validate these results, horse radish peroxidase expressed with an 8-arginine tag to elevate its pI from 7.0 to 9.5 was purified using CM-sepharose and SP-sepharose matrixes, obtaining high levels of purity with the latter.

Conclusion: in conclusion, the study of chromatographic behavior and physicochemical characterization of the system’s contaminants, allowed us to design and optimize rational purification processes. The predominance of acidic proteins represents an opportunity to easily purify proteins with high isoelectric points using CM and SP-sepharose chromatography.

Keywords: PURIFICATION, CHROMATOGRAPHY, BACULOVIRUS, RECOMBINANT PROTEINS, LARVAE
Proteome variation of the rat liver after simple cold storage using an

*ex vivo* model

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**Background:** Cold storage is a common procedure for liver preservation in a transplant setting. However, during cold ischemia, the liver suffers molecular alterations that may affect its performance. Also, deleterious mechanisms set forth in the storage phase are exacerbated during reperfusion. The aim of this study was to identify liver proteins potentially associated with injury during cold storage and/or normothermic reperfusion using the isolated preserved rat liver model.

**Methods:** Livers from male rats were subjected to either (1) cold storage for 24 hs, (2) *ex vivo* normothermic reperfusion for 90 min or (3) cold storage for 24 hs followed by *ex vivo* normothermic reperfusion for 90 min. Then, livers were homogenized and proteins were extracted. Protein expression between each experimental group and the control (freshly resected livers) was compared by 2D gel electrophoresis. Protein identification was carried out by matrix-assisted laser desorption/ionization time-of-flight spectrometry (MALDI-TOF/TOF) using MASCOT as the search engine.

**Results:** 23 proteins were detected with significantly altered levels of expression among the different treatments, including molecular chaperones, antioxidant enzymes, and proteins involved in energy metabolism. Some of them had already been postulated as biomarkers for liver damage while others had been identified in other organs subjected to ischemia and reperfusion injury.

**Conclusion:** Our results give new evidence in favor of proteins proposed as biomarkers for liver injury. The whole data set will be a useful resource for studying deleterious molecular mechanisms that result in diminished liver function during storage and subsequent reperfusion.

**Keywords**

2D-PAGE, reperfusion injury, cold ischemia, MALDI-TOF/TOF
STANDARDIZATION AND TECHNOLOGICAL INNOVATION
Determination of site specific phosphorylation ratios in proteins with targeted mass spectrometry

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Background
Protein phosphorylation is an important post-translation modification that regulates many biological processes. Increased speed and sensitivity of modern mass spectrometry equipment has made large scale quantitation of phosphorylation sites possible. We developed a parallel reaction monitoring (PRM) assay for exact quantitation of the phosphorylation ratio using stable isotope labelled peptides.

Methods
We developed this method using U87 cells. A number of phosphorylation sites were selected and stable isotopic labeled peptides were purchased for AHNAK S5480-p, CAMK2D T337-p and EGFR S1166-p. We have compared two different PRM methods, namely direct-PRM (tryptic digest measured by PRM without any further sample preparation) and TiO₂-PRM (tryptic digest enriched with TiO₂ cartridges using the Bravo AssayMap platform, followed by PRM measurement). For both PRM methods not only the phosphorylated peptides but also the non-phosphorylated counterpart including their stable isotope labelled standards are targeted and measured using an LC-MS Q Exactive HF Orbitrap system.

Results
Percentages of phosphorylation could be determined in a reproducible way (CV 6-13%) using direct-PRM or TiO₂-PRM. In addition, we tested the two approaches in a cell culture experiment in which U87 cells were deprived from serum. As a “gold standard” we included an immune precipitation of EGFR followed by PRM (IP-PRM) for comparison reason. For EGFR (S1166) and AHNAK (S5480), a statistical significant change in the percentage of phosphorylation could be observed as a result of serum deprivation; for EGFR (S1166) this change was observed for both TiO₂-PRM and IP-PRM.

Conclusions
In conclusion, we show a relative fast and automated approach for absolute quantification of phosphopeptides and their phosphorylation ratio. We demonstrated that the method can be
Trapped ion mobility spectrometry with parallel accumulation – serial fragmentation (TIMS-PASEF): pushing the limits of shotgun proteomics analysis

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Background: The “Parallel Accumulation - Serial Fragmentation” method (PASEF, Meier et al., JPR 2015, PMID: 26538118) for trapped ion mobility spectrometry (TIMS) coupled to a quadrupole time of flight (QTOF) instrument, has been described with the promise of achieving five to ten times faster data dependent acquisition of fragment ion spectra with improvements in sensitivity. Here we present results showing that, with further hardware and software development, the promise of PASEF has been realized.

Methods: The performance of a timsTOF instrument with PASEF for shotgun proteomics has been evaluated by using tryptic digests of human cancer cell lysates (HeLa) spiked or not with an UPS peptide mixture, and separated by 90 min nanoLC gradients. Data were analyzed using DataAnalysis (Bruker), Mascot (www.matrixscience.com), MaxQuant (Cox group, MPI of Biochemistry) and a modified version of the PEAKS™ proteomics processing software suite (Bioinformatics Solutions).

Results: A standard 1,1 second PASEF acquisition cycle, enabling to perform over 1200 MS/MS at high sensitivity, allows to identify over 3500 protein groups from a 10ng injection of an HeLa cell digest separated with a 60min gradient, and over 5000 protein groups from a 100 ng injection with the same gradient. In parallel, the trapped ion mobility separation allows to separate isobaric co-eluted peptides prior to fragmentation, further increasing the ID rate. The label free quantification of the PASEF method has also been evaluated: triplicate injections of 200 ng of a cell digest resulted in more than 5300 protein groups identified at 1%FDR for each injection. 4931 of those proteins were identified in all three replicates, and their corresponding intensities were covering >4 orders of magnitude. Quantitation accuracy has also been evaluated over a similar range of intensities with mixture of Hela Cell and E.Coli proteomes digest mixed in a 1:1 and 1:2 ratio in two different sample sets.

Conclusions: By enabling data dependent acquisition at very high speeds, with improved sensitivity, the PASEF method enables to dig deeper into the proteome, using less sample, and while still performing reproducible quantitation.

Keywords: PASEF, TIMS, SENSITIVITY, SPEED, timsTOF Pro
applied in a multiplexed way. This technology can potentially be used for studies in which the activity of specific pathways is monitored by protein abundance and site specific phosphorylation ratios.

**Keywords**
Phosphoproteomics, PRM, Targeted mass spectrometry, Quantitative proteomics and Phosphorylation ratio
Targeted proteomics method comparison:

SRM, PRM and SWATH-MS

to quantify proteins in bovine muscle tissues

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Background: Targeted proteomics has become an approach of choice to validate and precisely/absolutely quantify protein biomarkers. Besides selected reaction monitoring (SRM), alternative methods have emerged over the last few years, among which parallel reaction monitoring (PRM) and sequential windowed acquisition of all theoretical spectral (SWATH-MS). These approaches perform differently in terms of instrumentation, multiplexing, and performances such as sensitivity, selectivity and accuracy (Borràs and Sabidó, 2017). A careful consideration of these points is required to select the best-suited targeted proteomics method.

In this study, we have evaluated and benchmarked three targeted methods to precisely quantify beef tenderness biomarkers in muscle tissues. Meat tenderness and lipid content (marbling) guide customer choice. Controlling these quality criterions by relying on protein biomarkers presents a high agronomical and economical interest (Picard et al., 2015; Ceciliani et al., 2017).

Methods: Twenty peptides, corresponding to ten previously identified protein biomarkers of beef adiposity and tenderness, were quantified in two muscles, Longissimus thoracis and Rectus abdominis, using a SRM assay on a triple quadrupole mass spectrometer (MS) (TSQ Vantage, Thermo Fisher Scientific), a PRM assay on a quadrupole-Orbitrap MS (Q Exactive Plus, Thermo Fisher Scientific) and a SWATH-MS method on a quadrupole-time-of-flight MS (TripleTOF 6600, Sciex). Analyses were performed on a cohort of 64 samples of bovine muscles, covering a wide dynamic range of adiposity and tenderness. Limits of quantification for each peptide were determined by establishing isotope-dilution calibration curves.

Results and Conclusions: We showed that (i) PRM performs better than SRM and SWATH-MS regarding sensitivity, (ii) SRM and SWATH-MS exhibit similar – and enhanced while comparing to PRM – dynamic range performance, and (iii) PRM and SRM selectivity is better than SWATH-MS one for the low-concentrated peptides. We also highlighted the advantage of PRM and DIA-SWATH over SRM for post-acquisition data refinement.

Keywords: Quantitative Proteomics, Selected Reaction Monitoring, Parallel Reaction Monitoring, Data-Independent Analysis.
Towards absolute and generic top-down quantitative proteomics through combination of elemental and molecular mass spectrometry

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Background: Determination of absolute levels of target peptide/proteins by ESI-MS is hindered by the unavailability and characterization of proper specific standards for each target analyte. In contrast, elemental mass spectrometry (ICP-MS) species-independent detection, together with recent analytical and instrumental developments (tandem ICP-MS, core-shell chromatographic phases), now enables absolute quantification of proteins using a single generic standard, without resorting to pre-HPLC enzymatic digestion. However, it cannot provide structural information or identities. Therefore, combination of ICP-MS with top-down MS workflows appears as a promising step further towards comprehensive quantitative proteomics.

Methods: Protein sample quantitative analysis was carried out with parallel HPLC-ICP-MS and HPLC-ESI-MS. Any certified compound containing the detected element, analysed via Flow Injection before the HPLC analysis of the sample, can be used as generic standard for the absolute quantification of individual intact proteins. Sulphur (present in Met and Cys), phosphorous, and iodine were simultaneously detected for protein quantification and quantitative characterization of the respective modifications. ESI-MS-MS/MS top-down sample analysis provided identification of the proteins corresponding to each HPLC peak.

Results: From HPLC-ICP-MS sulphur analysis and ESI-MS protein information, absolute quantities of intact proteins present in simple (i.e., standards certification) and relatively complex samples (snake venoms containing about 40 protein species) were obtained. Protein phosphorylation and iodination degrees were characterized in a single analysis from P/S and I/S areas ratios measured in the corresponding HPLC peak, respectively. The strategy demonstrated to be extendable to the simultaneous analysis of other elements present in proteins (e.g., Se and As).

Conclusions: Multi-elemental quantitative capabilities of the proposed ICP-MS-based strategy turn up as an excellent and robust alternative in the certification of peptide/protein standards. Moreover, the possibility of combining ICP-MS with “discovery” top-down MS workflows enables quantification of individual proteins present in relatively complex samples in a single analysis, and quantitative characterization of PTMs, using just element generic standards.

Keywords: Absolute protein quantification · Protein standardization · Hybrid mass spectrometry · HPLC-ICP-MS · Post-translational modifications
The combination of protein, peptide and lipid MALDI-IMS analysis allows a deeper insight in Molecular Histology

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**Background:** Matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) is a powerful tool for investigating the distribution of proteins and other molecules within biological systems through the in situ analysis of tissue sections, enabling Molecular Histology. Traditionally, these studies have been performed separately for proteins and peptides on one hand, and for lipids on the other, giving only a narrow vision of the molecular changes present in tissue. In this work we have merged these two fields in a single tissue analysis for a deeper insight of the underlying Molecular Histology.

**Methods:** 10 um tissue slices were fixed onto a ITO glass slides For protein/peptide analysis, samples were delipidized using a modified Carnoy procedure, dried onto a vacuum chamber and sprayed using the SA matrix using Langartech automated sprayer. MALDI-IMS was performed using an Autoflex III Smartbeam spectrometer in linear mode with a mass window of 1-30 kDa, and a shot grid with 75 um spacing. For lipidic analysis, samples were matrix coated by sublimation with MBT matrix. MALDI-IMS was performed using an Orbitrap XL spectrometer with a mass window of 100-1000 Da, and a shot grid with 40 um spacing.

**Results:** The images obtained with both approaches (lipid and peptide/protein) achieve a relative high resolution revealing interesting molecular patterns and structures not seen at optical level. Both types of images are complementary and mutually inclusive, enhancing the amount of underlying information of the tissue.

**Conclusions:** The combination of protein/peptide and lipid MALDI-IMS can give additional information in relation to the traditional single-analyte MALDI-IMS approach, enhancing the knowledge of the underlying molecular histology in tissue studies.

**Keywords:** MALDI-IMS, Imaging, Molecular Histology, Lipidomics, Proteomics
Protocol for Protein interaction screen on peptide matrices (PRISMA) to dissect motif specific binding partners

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Background:

Short Linear Motifs (SLiMs) are stretches of protein sequences involved in transient and dynamic protein interactions. SLiMs often occur within disordered protein regions and function as components in signal transduction and as targets of post-translational modifications (PTM). Unraveling the interactome of SLiMs will help to determine their functionality in protein crosstalk and complex formation. We have established a protocol to systematically decipher the interactome of proteins based on their SLiMs. The method, termed PRotein Interaction Screen on peptide Matrices (PRISMA), permits mapping of protein interactions across the entire protein primary sequences and also considers PTM-specific interactions.

Methods:

We established the method exploring the linear and PTM specific interactome of “CCAAT enhancer binding protein β” (C/EBPβ), as a paradigm intrinsically disordered transcription factor. C/EBPβ is involved in the regulation of metabolism, reproduction, immunity, proliferation, and tumorigenesis. We probed 203 tiled peptide arrays with protein extracts to achieve highly selective binding information for each individual target sequence.

Results:

The datasets were globally integrated across all peptide pull-downs and significant interactions were identified across replicates, neighboring peptides and various PTM-states. Across the linear sequence of C/EBPβ, we identified key proteins involved in transcription, DNA replication, or Chromatin remodeling. We characterise interactions with protein complexes, like mediator complex, nuclear pore complex, or SWI/SNF complex.

Conclusions:
Development of a novel LC concept for clinical proteomics

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Background: Mass spectrometry based proteomics and metabolomics are fast growing and powerful technologies, with the potential to revolutionize health care and precision medicine. However, available separation technology has so far limited throughput and robustness and thereby prevented omics technologies from being fully integrated and routinely used in a clinical setting.

Methods: Here we describe a conceptually novel low-flow chromatography system that delivers the robustness and throughput required for clinical applications while maintaining the sensitivity of current nano-flow LC instrumentation. The new system uses four low-pressure pumps in parallel to elute samples from a disposable trap column while simultaneously creating a final but offset chromatographic gradient with embedded analytes. Initially, two low-pressure pumps form a primary gradient that flows through the disposable trap column thereby eluting the analytes of interest while two additional low-pressure pumps positioned right after the trap column are used to modify the eluent and create an "offset" to the gradient with the purpose of increasing retention of the now embedded analytes at the separation column. The offset gradient with the embedded analytes are moved into a long, narrow holding loop that subsequently is switched in-line with a single high-pressure pump and a separation column. Thus elution from the disposable trap column and gradient formation become de-coupled from the high-pressure separation.

Results: We have characterized the performance of the new system regarding cross contaminations (<0.05%, total TIC), retention time shifts and peak widths (<8.5 sec) in over 1,500 HeLa runs. The short overhead time of approximately 3 min allowed us to measure 60 samples per day (21 min gradient, 3 min overhead time). To benchmark reproducibility, we measured 100 human plasma samples in 40 hours and each resulted in several hundred quantified plasma proteins.

Keywords: LCMS, proteomics, high throughput.
The PRISMA method provides a repository of high molecular resolution data for protein interactions of an intrinsically disordered protein. This approach serves as a basis to explore gene regulatory and PTM-modulated functions, and can assist the rational design of mutant proteins for interaction studies. C/EBPβ acts as a multi-tasking molecular switchboard, integrating signal-dependent modifications and structural plasticity to orchestrate interactions with numerous protein complexes, in directing cell fate and function.

**Keywords:** C/EBPβ, interactome, intrinsically disordered protein, post-translational modifications.
Suitability comparison of EDTA-plasma, heparin-plasma and serum for shotgun proteomics

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Background: Despite the challenge it represents from the analytical point, blood plasma constitutes the preferred body sample for clinical chemistry investigations due to its minimal sampling invasiveness. EDTA-plasma has been recommended and used as the preferred sample matrix for human blood proteomic profiling over the last two decades. Serum has also been employed widely. Only few studies have assessed the difference and relevance of the proteome profiles obtained from plasma samples, such as EDTA-plasma or heparin-plasma, and serum. A more comprehensive evaluation and comparison of these sample matrices appear necessary for optimal deployment of shotgun proteomics in clinical research.

Methods: In this study we compared proteomic profiles from EDTA-plasma, lithium-heparin-plasma, and serum using a scalable automated proteomic pipeline (ASAP²) [1]. Coverage consistency and quantitative performance were established with mass spectrometry (MS)-based shotgun proteomics using pooled commercial samples. Protein measurements in EDTA-plasma and lithium-heparin-plasma samples were compared using matched sample pairs from 20 participants of the Australian Imaging, Biomarkers and Lifestyle (AIBL) Study.

Results: In commercial EDTA-plasma, heparin-plasma, and serum, 342 ± 27 identified proteins (IDs), 341± 25 IDs, and 350 ± 13 IDs were found respectively per liquid chromatography MS analysis; 47.8% of the total IDs were detected in all sample types. Quantitative precision and accuracy were equivalent across the sample matrices using ASAP². A Bland-Altman plot of the 20 matched EDTA-plasma and heparin-plasma samples from the AIBL Study showed comparable proteomic results using both sample matrices.

Conclusions: Shotgun proteomics, and in particular ASAP², can be applied to EDTA-plasma, heparin-plasma, and serum. Those sample types are technically suitable for discovery proteomics in blood. Analyzing two types of plasma, as well as serum, appears attractive as it increases the comprehensiveness of the proteome profiles [2].

Keywords: EDTA; heparin; isobaric tagging; mass spectrometry; plasma; serum; shotgun proteomics


OPTIMIZATION OF A DEPARAFFINIZATION/EXTRACTION PROTEIN PROTOCOL FROM FFPE THYROID SAMPLES TO APPLY PROTEOMIC TECHNIQUES

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Background: The vast majority of tissue biopsies from patients in hospital environments are included in paraffin (FFPE - "formalin-fixed and paraffin-embedded") for long-term storage. In the proteins this fixation produces a modification called "crossed links", which improves protein stability, necessary for its conservation. Therefore, these modifications make the development of a good protein extraction protocol necessary. Due to the specific characteristics of each tissue the same extraction buffers or deparaffinization protocols are not equally effective in all cases, and it is necessary to obtain a specific protocol for each tissue.

The main objective of this work is to establish a deparaffinization/extraction protocol from paraffin thyroid tissue samples, which allows us to obtain enough protein of optimum quality, for the application of proteomic techniques.

Methods: We use FFPE samples from the CHUS Biobank. We first cut the paraffin block into 3.0-4.0 mg sample pieces. Then, deparaffinization was carried out using xylene and water at different temperatures. To extract the protein, we used different buffers to verify which was most effective. Finally, we submitted these protein samples to proteomic techniques by means of LC – MS/MS analysis.

Results: Our results show that it is possible obtain protein from FFPE samples to carry out proteomic studies. We tested two deparaffinization methods, one using water and another with xylene. In both methods we obtained thyroglobulin as one of the proteins identified. We also tested two fraction methods, one using Amicon® Centrifugal Filter Devices, and the other with "in gel" band fraction. Applying these techniques we improved protein identification.

Conclusions: Both deparaffinization methods are capable of extracting protein from FFPE samples. The xylene extraction method allows identification of more proteins with fewer peptides. Our results suggest that applying this protocol to different thyroid tumor samples, we could find differences in protein expression as well as post-translational modifications that could be used as biomarkers.

Keywords: FFPE samples, deparaffinization, xylene, TripleTOF, LC-MALDI MS/MS.
Sensitivity and specificity of mass-spectrometric proteomic technologies as factors determining the prospects for the CHPP project.

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Background: Chromosome-centric "Human Proteome Project " (CHPP) is a functional continuation of the "Human Genome project". In its implementation, proteomic technologies are used. They are fundamentally different from genomic sequencing, which is based on the polymerase chain reaction (PCR). The reaction has no limit of sensitivity (LOD) and has absolute specificity. There are no analogous reactions in analytical chemistry. The sensitivity and specificity of proteomic technologies can be estimated using the widely used FDA equations for medical diagnostic tests. They are based on ROC-analysis, which combines these two concepts.

Methods: To analyze the shotgun and SRM-technologies, two "golden" standards were chosen: UPS1 and UPS2. Both of them contain 48 proteins. UPS1 is a mixture of proteins taken in identical molar concentrations. UPS2 – is a mixture of proteins with different molar concentrations. In our case protein concentrations range from $10^{-6}$ to $10^{-11}$ M were investigated. Both pure protein solutions and mixtures of the biomaterial (E. coli extract or human plasma) with protein solutions were analyzes.

Results: The obtained results demonstrate that both technologies have high specificity, while sensitivity is a function of the biomaterial used as a noise source and protein concentration in the standard.

When the concentration of proteins is $10^{-8}$ - $10^{-9}$ M, SRM reveals all 100% of the proteins of the UPS1 and UPS2 standard. When it decreases to $10^{-11}$ M, the number of detected proteins found reduces to 50%. The sensitivity of panoramic mass-spectrometric technology is even lower.

Conclusions: The SRM and shotgun technologies have high specificity and low sensitivity, which does not allow them to be used both to detect the complete proteome of full exome or individual chromosomes.

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Keywords: HPP, sensitivity, specificity, proteome.
NEW PROTEIN EXTRACTION PROTOCOL FROM FORMALIN-FIXED PARAFFIN EMBEDDED KIDNEY TISSUE SAMPLES

Background: The majority of tissue sample biopsies obtained from patients are embedded in paraffin (FFPE-formalin-fixed and paraffin-embedded) for a long-term storage. Nowadays, due to the chemical modifications induced it is difficult to obtain intact proteins from this tissues, and these samples are only routinely used in clinical practice for immunohistochemical techniques. This is the reason why, for each type of tissue, it is necessary to tune up a specific deparaffinization and extraction protein protocol. The aim of our work was to establish a deparaffinization and protein extraction protocol from paraffin embedded kidney samples, to obtain high quantity and quality protein, suitable for proteomic techniques.

Methods: we used sections of FFPE healthy kidney to make a initial standardization. These sections were cut in 3 mm pieces and used 4-5 mg of sample per tube in the first approach and in a decreasing amount in a second approach in order to pinpoint the sensibility of the process. We tested 3 methods of paraffin extraction and 6 different buffers for protein extraction. Finally, we submitted these proteins to LC-MALDI-TOF/TOF.

Results: we have seen that, out of the six buffers tested, the two most adequate are those with SDS percentages (Tris-SDS-LB and SDS-LB), since we were able to obtain higher quantity and better quality protein for LC-MALDI-TOF/TOF. Our deparaffinization method using xylene at RT, rehydration with decreasing alcohol percentages and a final step in H2O allows an adequate paraffin removal and consequently the protein extraction was more efficient. Moreover, including the use of the TissueLyser, which highly improves the tissue rupture, facilitates the buffer penetration, which means an improvement in the obtaining of good condition protein.

Conclusions: Our results suggested that it would be possible to apply our proteomic techniques and protocols to FFPE samples from a range of kidney diseases, to find possible biomarkers and therapeutic targets from diverse pathologies.

Keywords: FFPE kidney samples, deparaffinization, protein extraction, LC-MALDI-TOF/TOF.
Pillar based HPLC: The next big thing?

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Background: It is well-known that latest generation packed-bed nano-HPLC columns have reached their limits in terms of length and particle size, due to the high backpressure that is generated. Therefore, only a paradigm shift in column design could allow for a considerably higher peak capacity and better LC-resolution. One of the latest innovations in column design is the micro pillar array column (µPAC) manufactured by Pharmafluidics. It is a chip-based micro-HPLC column filled with perfectly ordered pillars etched out of a silicon wafer. This unique structure gives a many-fold reduction in backpressure and less peak dispersion, otherwise caused by the random particle distribution, enabling the vendor to enlarge the column up to 2 metres in length. The column is divided into 5 cm pieces connected to each other with a flow distribution system at each turn. This unique design gives rise to a pressure of about 90 bar with 300nL flow.

Methods: The µPAC has been tested on a nano-acquity/Synapt G2-Si system to assess the actual gain in peak capacity and resolution compared to a regular 25cm 1.7µm bead size M-class packed bed column from Waters. To ensure a useful comparison, triplicate runs of a standard human protein digest were analysed with five different gradients. The gain was assessed in terms of peak capacity, peak width and resolution throughout the gradient, as well as the number of peptide identifications extracted from the UDMSE data using the Ion Accounting algorithm from Waters.

Results: A significant gain in peak capacity and peptide identifications was observed using longer gradients starting at 120’.

Conclusions: The µPAC column proves its added value compared to packed bed columns on complex samples analysed with long gradients.

Keywords: nano-HPLC, Pillar-based, peak capacity
Ultra-fast protein digestion for label-free protein quantification

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Background: Protein digestion is a critical step in bottom-up mass spectrometry-based proteomics, which is generally done in-solution. This approach is very robust and reproducible; however, it is common to observe peptides resulting from auto-digestion of trypsin, creating background interference and in some cases ion-suppression. In addition, the standard method of protein digestion is time-consuming, lasting as much as 12 hours. To overcome the aforementioned problems trypsin immobilized in Nano-spheres accomplished to ultrasonic energy has been applied for the first time.

Methods: E. Coli samples were used for proof-of-concept. The digestion time (overnight vs ultrasonic) and the type of particle (nanoparticle vs commercial particle) were optimized and compared. Samples were analysed by nano-ESI-MS/MS using a label-free approach.

Results: Four times more proteins were identified with the nano-trypsin. The digestion time with ultrasonic energy was successfully shortened from 12 h to 5 min. This methodology can be used for protein quantification.

Conclusions: Combining these new magnetic nanoparticles of immobilized trypsin and the ultrasonic method for protein digestion, we discover a method that is ultra-fast and ultra-efficient for protein digestion resulting in an outstanding capability for protein identification and protein quantification.

Keywords: Proteomics, protein digestion, immobilized trypsin, ultra-fast digestion.
Proteomic analysis of silica hybrid sol-gel coatings: a potential tool for predicting the biocompatibility of implants in vivo

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Background:

The interactions of implanted biomaterials with the host organism determine the success or failure of an implantation. Normally, their biocompatibility is assessed using in vitro tests. Unfortunately, in vitro and in vivo results are not always concordant; new, effective methods of biomaterial characterisation are urgently needed to predict the in vivo outcome.

Methods:

The silica hybrid coatings were obtained using the sol-gel route. The surface topography and specific surface area of the coated titanium discs were characterised employing atomic force microscopy (AFM) (BrukerMultimode 8). To evaluate the histological response to the selected coatings, dental implants were surgically placed in the tibia of New Zealand rabbits. Samples for histological examination were processed following the method described by Peris et al. (1993). Ti discs coated with different sol-gel compositions were incubated with human blood serum as described in Romero-Gavilan et al (Biofoulin 2017 b). Label Free protein quantification by nLC MS/MS was performed Progenesis IQ.

Results:

Four distinct hybrid sol-gel biomaterials were tested. The in vitro results were similar for all the materials examined here. However, in vivo, the materials behaved differently. Six of the 171 adsorbed proteins were significantly more abundant on the materials with weak biocompatibility: CRP, SAMP, C1QB, C1QC, C1S and CO7; these proteins are associated with the complement pathway.

Conclusions:

A cluster of proteins mostly associated with the activation of an acute inflammatory response differentially adhered to the coatings with poor biocompatibility. The formation of a fibrous connective tissue surrounding the 50M50G and 50V50G materials in vivo might be correlated with the adsorption of these proteins. Regardless of the experimental limitations of the study, this correlation might be the basis for the development of new methods to detect biocompatibility problems and, therefore, reduce the number of experimental studies in vivo. The proteins in this group (CRP, SAMP, C1S, C1QB, C1QC, C7 and VTN) should be useful as biomarkers in the evaluation of material biocompatibility.

Keywords:

Biocompatibility, Osteoimmunology, fibrous capsule, bone regeneration, dental implants.
OPTIMIZATION OF SERUM EXOSOME ISOLATION METHODS AND PROTEIN CARGO ANALYSIS BY SHOTGUN PROTEOMICS

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POSTER

Background: Exosomes are extracellular nanovesicles of complex and heterogeneous composition including proteins, lipids and RNA that are secreted by cells both in physiological and pathological conditions and that are released in different biofluids such as blood. Numerous studies have pointed out the potential value of exosomal proteins as biomarkers for diagnosis and prognosis, based on qualitative and quantitative protein changes found in exosomes derived from healthy and diseased subjects. Therefore, serum exosomes could be an excellent source of putative protein biomarkers associated to preeclampsia, a pregnancy-related pathology. Due to the overlapping physico-chemical and structural characteristics present in the different types of extracellular vesicles, currently there is no universally accepted exosomal enrichment method that meets all the quality standards required.

Methods: Several isolation methods based on precipitation and on size-exclusion chromatography (SEC) using qEV columns (Izon), and a combination of both, were tested. A variety of parameters like sample volume or lysis buffer composition were optimized.

Results: Following SEC or precipitation-based isolation, exosomes were characterized by shotgun proteomics and compared to exosomal protein markers included in ExoCarta (http://www.exocarta.org) and EVpedia (http://www.evpedia.info) repositories. 371 and 472 proteins, respectively, were identified and label-free quantified from two different and independent cohorts of samples. Exosomal characterization was also carried out by Transmission Electron Microscopy and Nanoparticle Tracking Analysis.

Conclusions: SEC provided the highest isolation efficacy in terms of exosome-sized vesicles purification with a minimum number of serum contaminants.

Keywords: Exosomes, Size-exclusion chromatography, Biomarkers, Shotgun proteomics, Preeclampsia
FAST PROTOCOLS FOR THE ENRICHMENT OF LOW-ABUNDANCE PROTEINS IN PLASMA SAMPLES

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Background: Numerous biomedical studies have demonstrated that plasma protein levels reflect human physiological or pathological states and can be used for disease diagnosis and prognosis. Sample preparation and handling is critical for the plasma proteome analysis. Depletion of plasma highly abundant proteins (albumins, immunoglobulins (IgG), alpha-1-antitrypsin (A1AT), fibrinogen, and haptoglobin (HG)) is often desired prior to proteome analysis.

The objective of this work is to develop rapid, cheap and effective protocols for the elimination of the majority proteins of plasma samples.

Methods: Two different protocols for the removal of high abundance proteins have been compared: a) the plasma sample was passed through an affinity column, b) the plasma sample was mixed with a special denaturing buffer (200 mM NaCl/10% AcN in 200 mM AmBic) and passed through Amicon® (Millipore) with decreased nominal molecular weight limit (NMWL). In both protocols the proteins were identified with a Triple-TOF 6600 (AbSciex).

Results: In both cases, high number of proteins was identified with an error of 1%. It has even been possible to compare the different fractions obtained in the case of the size separation method with Amicons®. Both methods are fast, low cost and are easy to implement in proteomic platforms.

Conclusions: Two valid protocols have been obtained, which provide a good pre-treatment and enrichment of plasma samples in order to make a proteomic analysis and give good results.

Keywords: enrichment, low-abundance proteins, plasma samples.
Plasma has re-emerged as the primary bio-fluid evaluated for biomarker identification. The experimental protocols, however, have changed from small targeted protein panels measured exclusively using SRM/PRM assays to global proteome profiling across large cohorts. Consequently, experimental methods must balance comprehensive proteome coverage with sample throughput and instrument ruggedness to maintain analytical accuracy. To address the challenges of the plasma proteome, we have incorporated trapping columns packed with PS/DVB particles to the UHPLC separation methods to significantly increasing the loading and peak capacities for high resolution LC-MS analysis. Chromatographic separations were performed using a Vanquish UHPLC system. The sample injection cycle consisted of the analytical method, cleaning injection, and fast quality control/carryover analysis totaling 65-minutes. All HRAM MS and MS/MS data was acquired using a tribrid mass spectrometer operated in DDA mode. The combination of the trapping and analytical columns packed with very different particle chemistries (PS/DVB vs. silica-based C18) enables increased protein loading amounts without altering the chromatographic performance. To evaluate the workflow, a loading curve was analyzed ranging from 7 to 220 µg of plasma digest on column. The number of proteins confidently identified and quantified increased from 326 to 745 and the number of peptides increased from 1233 to 1963. The ruggedness test utilized 336 plasma samples. A total of 90 µg of plasma digest was injected per digest samples and the instrument was operated for over 21 days with only changing the ion transfer tube following 48 samples. An average of 596 proteins and 2296 peptides were profiled across the 32 different groups. The average retention time variance was 0.5% and the calculated variance for peptide area values were less than 25%. The new method demonstrated exceptionally stable chromatographic performance over large dynamic protein loading amounts as well as increased ruggedness and robustness across large-scale studies.
Hands-free sample preparation for proteomics using universal chemistry and a microfluidic benchtop instrument

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While this bottom-up proteomics approach provides comprehensive peptide and protein identification, poor sample preparation technique can significantly decrease protein identification or cause the LC-MS to fail. Most sample preparation is performed manually using non-standardized protocols and thus is prone to user error and makes comparing results among labs impossible. We have engineered a hands-free microfluidic device that performs the entire sample preparation workflow within 1 hour. The microfluidic device, was assembled using a combination of peristaltic pumps, solenoid valves, microcontrollers, and 3D printed parts. A UV detector and capillary flow cell was placed in the fluid path to quantify peptide concentration. HeLa cells were used to evaluate the precision and accuracy of the system in preparing samples for bottom-up analysis with LC-MS. Following processing in the device, samples were briefly dried down in a speed vacuum and reconstituted in 0.1% formic acid. HeLa digest were then injected into a Thermo Scientific™ EASY-nLC™ 1200 system coupled to a Thermo Scientific™ Q Exactive™ HF MS and analyzed using Thermo Scientific™ Proteome Discoverer™ 2.2. We first compared the total peptide amount recovered from 1 million HeLa after processing with our device and following a traditional manual sample preparation. Interestingly, we obtained 150 µg and 108 µg respectively. We explained these differences by the effective agitation in the presence of beads in the small fluidic path of the device. We further evaluated other quality control metrics and we obtained similar amounts of missed cleavages, alkylated peptides, deamidation, and oxidation with our device compared to manual sample preparation and a HeLa standard. Furthermore, we did not observe differences when compared the number of peptide and protein identifications. Finally, average CVs for the peptide intensities among five different sample preparation batches were below 10%, thus validating this technology to perform quantitative proteomics experiments.
Title: Development of a Quality Control Standard for Tandem Mass Tags (TMT) Workflows.

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Introduction: Quantitative proteomics strategies using Tandem Mass Tags (TMT) enable sample multiplexing and precise measurement of protein abundance. However, successful execution of this workflow includes multiple steps that may require optimization including chromatography, mass spectrometry (MS), and data analysis. Paulo et al. (JASMS, 2016) created a yeast triple gene knockout (TKO) TMT9plex standard to access ratio distortion using different instrument methods, as co-isolated ion interference can suppress accurate ratio quantification and thereby mask true differences in protein abundance across biological systems. Therefore to be able to detect and diagnosis co-isolation interference, enable MS method optimization and validation, we extended above mentioned TKO sample and developed the Thermo Scientific™ TMT11plex™ labeled peptide reference sample.

Methods: Lysates from four strains of Saccharomyces cerevisiae (a parental line and three lines respectively lacking the non-essential proteins MET6, HIS4, or URA2) were reduced, alkylated and digested with LysC and trypsin. Samples were then labeled with TMT11plex reagents according to manufacturer’s instructions and mixed in equimolar ratios. Peptides from each knockout strain were labeled in triplicate, while the parental line was labeled in duplicate. The samples were then analyzed on a range of Thermo Scientific™ Orbitrap™ instruments using LC-HCD MS2 or MS3 fragmentations. Data analysis was performed with Thermo Scientific™ Proteome Discoverer™ 2.2 software. Additionally, we benchmarked the proteomic standard using High pH fractionation, and evaluated the effect of modulating the advanced peak determination (APD) algorithm to optimize instrument methods.

Preliminary Data: To develop a widely applicable TMT11plex standard, we modified the selection of yeast strains from Paulo et al. (JASMS, 2016) to select met6Δ and ura2Δ and his4Δ, as the original pfk2Δ line exhibited slower growth rates, and included the parental strain BY4742 as reference channels. We show the yeast triple knockout (TKO) standard has less than 20% variability with lot to lot reproducibility.
Dried blood spot sampling for clinical metabolomics: effects of different papers and sample storage stability

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Background: The dried blood spot (DBS) sampling has a lot of advantages in comparison with the "standard" venous blood collecting: small collection volume; painless and easy sample collection with minimal training required; stable and transportable at ambient temperatures etc. The aim of this study was to determine comparability of four different types of DBS sampling (HemaSpot™-HF Blood Collection Device (Spot on Sciences); Whatman® 903 Protein Saver Snap Apart Card (GE Healthcare); card ImmunoHealth™ (analogue of Whatman®) and glass fiber strip ImmunoHealth™) for analysis of the global metabolites profile.

Methods: All the samples were collected from the same person (male) at the same time and stored at room temperature during 4 weeks in order to exclude all possible deviations deriving from biological variances between patients and to evaluate sample storage stability. Metabolome profiling by direct injection of a deproteinized capillary blood DBS sample into an electrospray ion source of a hybrid quadrupole time-of-flight mass spectrometer was used.

Results: Differences in the metabolomic profile were found between the different DBS collection materials, especially for glass fiber strip ImmunoHealth™. However for most clinically relevant compounds the differences in analytical performance are of minor incidence.

Conclusions: Our results indicate that the analytical performance of all tested DBS sampling showed consistent results overall detected metabolites and no distinguished changes in the metabolic composition with increase of the storage time. Based on our data it is recommended to select an appropriate DBS sample collection device depending on the design of the study.

Acknowledgments: Measurements were performed using the equipment of “Human Proteome” Core Facilities of the Institute of Biomedical Chemistry (Russia) which is supported by Ministry of Education and Science of the Russian Federation (agreement 14.621.21.0017, unique project ID RFMEFI62117X0017).

Keywords: Postgenome medicine, mass spectrometry, dried blood spot, metabolomics
LC/MS mAbs quantification in human serum after protein-A tips or nSMOL sample preparations

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Background:

Monoclonal antibody (mAb) is the first class of biotechnological products followed by recombinant proteins and vaccines. Bevacizumab (AVASTIN) product by Roche, is able to neutralize Vascular Endothelial Growth factor (VEGF), a regulator of angiogenesis and surexpressed in a lot of human tumors. By combining a step of Protein-A capture and a LC-MRM analysis, Bevacizumab can be quantified on patient serum during cure (cycle 1, 3, 5, 7, 9 and 11).

Methods:

The analytical strategy was designed to quantify the total fraction of bevacizumab kinetics in human serum. Sample pretreatment was necessary for the quantitation of mAb due to the complexity of human plasma matrix.

Two approaches were developed: (i) an automated Prot-A purification workflow (Bravo Assay map Agilent) followed by a trypsin digest and LC-MRM analysis; and (ii) the use of a commercially available kit based on nano-surface and molecular-orientation limited (nSMOL) proteolysis. Both approach have been validated and compared. 10 min LC-MRM runs were performed on a 8060 TQ from Shimadzu. Data were normalized using SILuTMMAb as internal standard and compared with 23 patients’ serum dosed using ELISA assay.

Results:

Unique proteotypic peptide (FTFSLDTSK) was selected for the two approaches and for the quantification in agreement with previously published work (1; 2). Both methods were validated on linear range 1.957 to 766.667 µg/ml for the automated Prot-A purification workflow, and 0.269 to 766.667 µg/ml for the nSMOL workflow. The two workflows were analytically validated in agreement with international guidelines. nSMOL workflow gave a 10-fold lower LLOQ, a more linear calibration curve but is a little bit less accurate (96.33% against 100.59%) than the automated Prot-A purification workflow. The use of nSMOL workflow was finally less time consuming and easier to perform. The 2 workflows were compatible with high throughput LC-MRM analyses of mAbs.

Conclusions:

The two workflows exhibited similar quantitative performance considering the low number of patient samples analyzed. Both methods used a limited sample volume (5µL) and are suitable for clinical application.

Keywords:
mass spectrometry, therapeutic drug monitoring, automation, protein A purification, nSMOL
MULTI-LABORATORY ASSESSMENT OF PERFORMANCE OF SWATH® ACQUISITION MASS SPECTROMETRY

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Background: It has been demonstrated that within an individual laboratory, SWATH® Acquisition can quantify 1000s of proteins across many samples, with high data completeness and quantitative accuracy, approaching that of the MRM approach. The question not yet answered is whether SWATH® Acquisition is robust enough to support the generation of reproducible larger scale proteomic datasets. The aim of this multi-site study using data from 11 sites around the world is to demonstrate that SWATH® Acquisition is a reproducible and accurate MS technique that can be confidently deployed for large-scale protein quantification in life science research.

Method: Digested protein extracts from HEK293 cells spiked with synthetic peptide dilution series were shipped to the 11 sites. Samples were acquired in a specified order to include both intra-day and inter-day replicates. SWATH MS analysis was performed on TripleTOF® 5600+ systems (SCIEX) equipped with NanoSpray® Source. After data acquisition, the 229 SWATH acquisition files were assembled centrally and processed using two strategies, allowing for the determination of linear dynamic range and peptide LLOQs, as well as the determination of intra- and inter-day CVs.

Results: In general ~4000-5000 proteins detected by all sites with a linear dynamic range ~4.5 orders of magnitude. The CVs were 5-20% within sites and ~25% between sites. The XIC based quantification at the MS/MS level from SWATH® Acquisition data is ~1 order of magnitude more sensitive than quantification at the MS1 level due to higher specificity.

Conclusions: This Multi-site study is benchmarking performance demonstrates the robustness of SWATH® Acquisition for performing large scale, multi-instrument / multi-site quantitative proteomics studies. It allows consistent detection of large set of proteins across labs and the accumulation of new proteins is very low. This suggests that SWATH acquisition could be used to analyze large proteomic datasets across multiple instruments in multiple sites.

Keywords: standardization, Multi-laboratory, SWATH, TripleTOF
STANDARDIZING AND HARMONIZING MULTIPLE TRIPLETOF® SYSTEMS FOR DDA AND DIA USING A DEDICATED PERFORMANCE KIT

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Background: To get consistently high quality, reproducible quantitative proteomics data, proper controls are required. This is particularly important as the proteomics field begins to embrace larger and larger studies where the integrity of the entire workflow must be ensured over many samples, multiple instruments, multiple users, and over long periods of time. A validated kit has been assembled to assess the performance of the TripleTOF® systems for IDA and SWATH® proteomics workflows to aid in performance monitoring and instrument harmonization.

Method: A series of standards and tests have been developed to test the performance of the various LC-MS modes. A set of 20 synthetic peptides of varied mass and retention time attributes has been developed for use as an infusion and LC-MS standard. Once confirmed, a standard complex tryptic digest (human) is used to test the performance of the instrument in both IDA and SWATH® modes.

Results: The SWATH® Acquisition showed good instrument harmonization, with only a 5% variation in the number of proteins and 12% variation in the number of peptides quantified across all eight machines.

Conclusions: The Stoller Biomarker Centre in Manchester has eight TripleTOF® 6600 systems for IDA and SWATH® Acquisition operating in microflow mode to help discover novel biomarkers for many different diseases. The eight systems were benchmarked using the newly developed kit in microflow after the systems were installed and also after 6 months of operation. With the current setup over 20 samples can be run per day per instrument.

Keywords: standardization, SWATH, TripleTOF
Implementation of proteomics as a quality control tool in the fashion and food industries

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Background: The major efforts in the field of proteomics have been focused mainly in studying the huge amount of different proteins in the human body and their interactions and roles, leading to new valuable diagnostic and therapeutic tools. However proteins are also found in other living organisms many of which are used as raw materials to produce the food we eat and the garments and footwear we wear. The leather materials widely used in fashion industry are mainly keratin and it is well known that the proteins present in many food products are determinant for their nutritional and organoleptic properties.

Methods: We have developed an HPLC-MS/MS analytical method for the assessment of the fiber composition in garments made with animal or plant fibers through protein identification. Also the current demand for vegan products has driven us to develop a HPLC-Q-TOF methodology for testing the presence of animal origin materials in vegan clothes and footwear allowing the manufacturers to demonstrate that those products are indeed animal-free as claimed. In the field of the food industry, we are currently using a similar approach for studying casein peptides contained in milk derived products and find a correlation with some organoleptic defects found in those products.

Results: The analytical methods developed in our company allow detection of fraud in those cases where microscopy and chemical tests fail to distinguish between the fibers due to their same chemical nature or in so claimed vegan products where there is no other methodologies to roll out the presence of animal residues.

Conclusions: Our investigations show that Mass spectrometry (MS)-based proteomics methodologies can be applied successfully to solve several problems faced by food and fashion industry nowadays and that those techniques should be included among the quality control regular tests performed to the processes and the items manufactured.

Keywords: fashion, food, proteomics, fibers, vegan
speed meets performance

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A TWO-STEP PRM STRATEGY IDENTIFIES A NEW BIOMARKER SIGNATURE IN CEREBROSPINAL FLUID FOR MULTIPLE SCLEROSIS PROGNOSIS

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Background:
Multiple sclerosis (MS) is an inflammatory disease characterized by an initial demyelinating event (CIS), followed by remission periods and relapses occurring at irregular intervals. Clinical symptoms and brain imaging allow diagnosis with a good sensitivity. However, there is still a need for prognostic biomarkers of conversion to MS and disease progression after a CIS. Using quantitative proteomics, we previously identified ~60 candidate MS biomarkers exhibiting differences in abundance in the cerebrospinal fluid (CSF) from patients with MS and symptomatic controls or from rapid and slow converters. These proteins were combined with previously described biomarker candidates to generate a list of 87 CSF proteins that were quantified by Parallel Reaction Monitoring (PRM) in different cohorts.

Methods:
A first PRM analysis monitored 226 peptides corresponding to the 87 proteins in a first cohort comprising symptomatic controls and MS patients at different disease stages (n=60). Proteins that passed this qualification step were further verified in a new PRM assay using a shorter gradient and high-purity, heavy isotope-labelled peptides (AQUA Ultimate, ThermoFisher), in a larger cohort (n=189) comprising patients with MS at different disease stages or other inflammatory and non-inflammatory neurological disorders.

Results:
These studies identified a combination of five biomarkers allowing i) positive diagnosis of MS at any disease stage, ii) discrimination between MS and the other neurological diseases and iii) prognosis of conversion to MS for patients with radiologically-isolated syndrome (RIS), a presymptomatic form of MS. Elevated CSF concentration (>154 ng/mL) of Chitinase 3-like protein 1, one of these biomarker candidates, also improves the prognostic value of spinal cord lesions or positive CSF (presence of oligoclonal bands and/or elevated IgG index) in RIS patients.

Conclusions:
Combined with existing CSF biomarkers and MRI, the identified biomarker signature should improve MS prognosis and MS patient follow-up, especially at early disease stages.

Keywords: Multiple sclerosis, biomarker, cerebrospinal fluid, PRM
An update on the Human Plasma Proteome

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Background: The Human Plasma Proteome Project (HPPP) is among the first major initiatives started by HUPO in 2002. Continuing advances in technology and data annotation have called for an update of the previous collections of 1,900 proteins in 2011 (Farrah et al, MCP).

Methods: In a recent update (Schwenk et al, 2017, JPR), we gathered data from hundreds of shotgun MS experiments derived from Human plasma samples into the Human Plasma PeptideAtlas Build 2017-04 to describe the current composition of the plasma proteome. We compared the current and old builds with RNA abundances, estimated protein concentration, and cellular location. In addition, we extended our analysis to targeted MS as well as collected targets addressed by different immunoassay platforms.

Results: The collective analysis for human plasma currently suggests that > 3,500 proteins can be reliably identified by two peptides in > 170 studies conducted with mass spectrometry. This is based on 43 million peptide spectrum matches with > 120,000 distinct peptide sequences at a 1% protein-level FDR and application of the HPP data interpretation guidelines. Immunoassay based methods from different providers cover > 2,000 proteins of which ~ 1,000 were in common with MS-based identifications.

Conclusions: The current list of proteins detected in the plasma proteome by shotgun MS is about 17% of the predicted human proteome. New methods and approaches will certainly lead to a further expansion of that list > 5000 proteins in the near future. However, considerations regarding sample collection and integrity as well as study design are needed to annotate the plasma proteome in relation to variations of and heterogeneity among the studied subjects.

Keywords: Plasma, Proteomics, Mass Spectrometry, Affinity Assays, Study Design
Olfactory Proteomic Survey unveils a Stage-dependent Proteostasis Imbalance in Parkinson’s disease

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Background: Olfactory dysfunction is one of the earliest features in Lewy-type alpha-synucleinopathies (LTS) such as Parkinson’s disease (PD). However, the underlying molecular mechanisms associated to smell impairment are poorly understood. Objectives: To characterize the magnitude and chronology of the olfactory bulb (OB) proteome modulation in parkinsonian subjects with different LTS staging respect to elderly controls, and search common olfactory substrates between PD and Alzheimer’s disease (AD) phenotypes. Methods: We have applied mass spectrometry-based quantitative proteomics and protein interaction networks in postmortem OBs across limbic, early-neocortical, and neocortical LTS stages (total n=21). A cross-disease study of selected olfactory molecules was performed in sporadic AD cases (n=14) and controls (n=8). Results: A stage-dependent proteostasis impairment was observed, identifying 268 differentially expressed OB proteins between controls and PD phenotypes. In addition to the widespread metabolic imbalance observed across stages, network-driven proteomics revealed a LTS stage-dependent modulation in OB Extracellular signal–regulated kinase (ERK1/2), upstream Mitogen-activated protein kinase kinase 3/6 (MKK3/6), and Phosphoinositide-dependent protein kinase 1 (PDK1)/Protein kinase C (PKC) signaling axis. Furthermore, olfactory protein derangements like the modulation of Secretagogin (SCGN), Calcyclin binding protein (CACYBP), and Glucosamine 6 phosphate isomerase 2 (GNPDA2) levels, were differentially detected in PD, and AD. Interestingly, an inverse correlation between GNPDA2 and alpha-synuclein protein levels was also reflected in PD cerebrospinal fluid (CSF) (n=25). Conclusion: Our stage-dependent proteome resource provides important avenues for understanding the chronology of the OB proteostasis imbalance in PD, deciphering mechanistic clues to the equivalent smell deficits observed in AD, and PD pathologies.

Keywords: olfactory bulb, Parkinson’s disease, neurodegeneration, mass-spectrometry
Background: Cardiovascular (CV) diseases are the leading cause of death and present high prevalence in developing countries. Nevertheless, there are no biomarkers available to detect the causal physiopathological process of the clinical illness. The current study is focused on CV risk stratification attending to oxidative stress for the discovery of new markers for early detection of the physiopathological process.

Methods: 24 blood samples from a cohort of patients in average age of life (30-50 years old) classified in 3 groups of study according to their CV risk were analyzed using FASIXOX, a novel strategy for analysis of the dynamic thiol redox proteome. We have also analyzed irreversible oxidation by targeted searches in databases. To our knowledge, this study constitutes the first description of Cys oxidation in human plasma according to CV risk stratification.

Results: We identified that reversible oxidations are decreased in CV events compared with healthy subjects, while irreversible oxidations follow the opposite trend. Only proteins involved in coagulation cascade have reversible oxidations increased in CV events, biologic function directly linked with CV pathology, whose alteration could result in a CV event. Moreover, these functions match with the functions of proteins with irreversible oxidation. We have also showed down-regulation of proteins involved in antioxidant respond, which is in line with the loss of redox homeostasis in CV events.

Conclusions: CV events present a severe oxidative stress, exceeding antioxidant mechanisms as reversible oxidations of Cys, generating irreversible oxidations that with the down-regulation of redoxins, generate an increase of molecular damage. The present draft of redox targets together with the quantification of protein may help to better understand the role of oxidative stress in CV diseases and also, in the stratification based on its stage/severity beyond traditional prognostic and diagnostic markers.

Keywords: Cardiovascular diseases, young population, thiol redox proteomics
Protein aggregates enriched from blood as new and promising sub-proteome for biomarkers discovery in neurodegeneration

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Background: Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disorder in which the accumulation of protein aggregates leads to motor neuron death. To date, the lack of effective treatments and difficulties in diagnosis highlight the need for reliable biomarkers detectable in accessible biofluids.

Methods: Protein aggregate enrichment from plasma was performed by ultracentrifugation, using a high detergent concentration and a sucrose cushion. Aggregates from 6 ALS patients and 6 healthy controls (HC), age and gender matched, were compared by the TMTcalibrator™ workflow, using brain lysates from 2 ALS patients to enhance detection of brain-derived proteins. Following data integration and statistical procedures to remove technical variance, a LIMMA-based modified t-Test was applied to identify regulated peptides and proteins.

Results: Principal Component Analysis, based on a subset of significantly-regulated proteins, showed a clear separation (41%) of ALS and HC groups along the first principal component. Almost 5000 proteins were shared between brain and plasma aggregates, of which 285 were significantly regulated. Filtering for protein groups identified with at least 2 unique peptides, 190 protein groups were differentially regulated between ALS and HC, with 44 proteins showing at least a two-fold increase or decrease. Pathway enrichment analysis confirmed the relevant role of lipoprotein-related pathways in this condition. In addition, the enriched pathways included: synaptic vesicle; neurotransmitter release cycle; glycosaminoglycan metabolism; lysosome; and synthesis of phosphatidic acid. References in literature corroborate the association of these pathways with ALS.

Conclusions: Protein aggregates are a common hallmark of neurodegeneration and it is important to shed light on their role in pathogenic processes. Here, we provide evidence that proteomic profiling of aggregates from plasma represents a useful means to identify ALS biomarkers. In addition, this study demonstrates the potential of using blood-circulating aggregates to reveal systemic mechanisms involved in the development of ALS.

Keywords: protein aggregates; ALS; biomarkers; TMTcalibrator™;
Deep-Dive in the Proteome of Human Cerebrospinal Fluid: A Valuable Data Resource for Biomarker Discovery and Missing Protein Identification

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Background:
Cerebrospinal fluid (CSF), although it is a body fluid of choice for the discovery of biomarkers of brain disorders, remains relatively understudied compared to other biological fluids such as blood plasma, partly due to the invasiveness of its sample collection. However, it represents a very informative and useful matrix for the diagnosis of various neurological diseases such as Alzheimer’s disease and Parkinson’s disease. The further characterization of the CSF proteome appears valuable for the identification of missing proteins.

Methods:
A commercial pool of human CSF samples was depleted from 14 proteins. After immuno-affinity depletion, the sample was submitted to alkylation, reduction, digestion, and isobaric labelling. It was then fractionated with off-gel electrophoresis and analyzed with the last hybrid generation of Orbitrap mass spectrometer, i.e., the Orbitrap Fusion Lumos Tribrid.

Results:
We obtained a deep profiling of human CSF proteome through the analysis of a unique pool of normal CSF samples. Our workflow allowed the identification of 3379 proteins and 20689 unique peptides; it represents, to the best of our knowledge, the largest single-shot dataset of human CSF proteome reported. Among the identified proteins in CSF, more than a half were annotated as brain proteins; 34% corresponded to genes defined as elevated in the brain in the Human Protein Atlas. The experimentally-established CSF protein list contained the principal Alzheimer’s disease biomarkers (e.g., tau protein, amyloid β (Aβ), apolipoprotein E, and neurogranin). In addition, this dataset significantly fed the Chromosome-Centric Human Proteome Project (HPP). Thirteen proteins considered as missing were identified, in accordance with the HPP guidelines.

Conclusions:
We have established a comprehensive resource of the proteins present in normal human CSF using mass spectrometry. This dataset contributes to the detection of thirteen missing proteins, making significant contribution to the Chromosome-Centric HPP. Such a work constitutes a valuable basis to support clinical studies of brain health and disorders.

Keywords:
cerebrospinal fluid, deep proteome, missing proteins, Human Proteome Project
ELUCIDATION OF EARLY-ONSET MOLECULAR DERANGEMENTS IN ALZHEIMER´S DISEASE BY OLFACTORY PROTEOMICS: FROM MICE TO HUMANS

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Background: The olfactory bulb (OB) is the first processing station in the olfactory pathway. Despite smell impairment is considered an early event in Alzheimer’s disease (AD), little is known about the initial molecular disturbances that accompany the AD development at olfactory level. Methods: We have interrogated the time-dependent OB molecular landscape in Tg2576 AD mice prior to the appearance of neuropathological amyloid plaques (2-, and 6-month-old), using transcriptomics, quantitative proteomics, and network biology. Results: The human mutated amyloid precursor protein (APP) induced an inverse regulation of downstream extracellular signal-regulated kinase (ERK1/2), and p38 mitogen-activated protein kinase (MAPK) routes in 2-month-old Tg2576 mice respect to wild-type (WT) mice. In contrast, Akt and mitogen-activated protein kinase Kinase 4 (SEK1/MKK4)/ stress-activated protein kinase/Jun-amino terminal kinase (SAPK/JNK) axis were parallel activated in the OB of 6-months-old-Tg2576 mice. Furthermore, a survival kinome profiling performed during the aging process revealed that olfactory APP overexpression leads to changes in the activation dynamics of protein kinase A (PKA), and SEK1/MKK4-SAPK/JNK between 6 and 18 months of age, when memory deficits appear and AD pathology is well established in transgenic mice. Interestingly, both olfactory pathways were differentially activated in the OB derived from human AD subjects with different neuropathological grading. Conclusions: Taken together, our data reflect the early impact of mutated APP on the OB molecular homeostasis, highlighting the progressive modulation of specific signaling pathways during the olfactory amyloidogenic pathology.

Keywords: Olfactory bulb, amyloid precursor protein, Alzheimer
NEUROANATOMICAL QUANTITATIVE PROTEOMICS REVEALS COMMON PATHOGENIC BIOLOGICAL ROUTES BETWEEN AMYOTROPHIC LATERAL SCLEROSIS (ALS) AND FRONTOTEMPORAL DEMENTIA (FTD)

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Background: Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are neurodegenerative disorders with an overlap in clinical presentation, and neuropathology, the former characterized by loss of motor neurons in the spinal cord and the latter characterized by atrophy of frontal and temporal lobes. For that, we consider appropriate to deeply map the neuroproteomes derived from spinal cord and non-motor cortex across the ALS/FTD spectrum.

Methods: We carried out a differential proteome-wide analysis of both brain areas derived from ALS subjects (n=8), ubiquitin-positive frontotemporal lobar dementia (FTLD-U) subjects (n=9) and neurological intact controls (n=8).

Results: At spinal cord level, 248 differentially-expressed proteins were detected among ALS cases versus controls, while 33 proteins were deregulated among FTLD-U cases and the control group. Interestingly, 20 out of 33 differential spinal proteins were shared between both neurological syndromes. In contrast, 56 and 50 differentially-expressed cortical proteins were respectively detected in ALS and FTLD-U respect to control group. At non-motor cortical level, a proteome subset of 16 proteins was co-deregulated between ALS and FTD. Data mining of generated proteomic data emphasized the involvement of these proteins in cell survival, mitochondrial homeostasis, and neuron-specific functions.

Conclusions: ALS and FTLD-U share molecular and functional alterations at spinal and cortical level, although part of the proteostatic impairment is region- and disease-specific. These results shed new light on the search for potential diagnostic and prognostic biomarkers, as well as for the identification of the underlying molecular mechanisms involved in the pathogenesis of ALS and FTD.

Keywords: Amyotrophic Lateral Sclerosis (ALS), Frontotemporal dementia (FTD), Motor neuron
Unravelling phenotypic heterogeneity in ALS using quantitative proteomics: from animal models of the disease to human pathology.

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Background: Clinical heterogeneity and a lack of prognostic biomarkers hamper any therapeutic development in Amyotrophic lateral sclerosis (ALS) a fatal neurodegenerative disease. Objectives: Finding new prognosis and diagnosis biomarkers to address this challenge and to compare potential therapeutic targets across species (SOD1 mouse models and human) Methods: A novel tissue-enhanced bio fluid mass spectrometry. Methods: TMTcalibrator™ technology has been applied here to characterize the ALS plasma proteome, using peripheral blood mononuclear cells (PBMCs) as reference. In this approach for each TMT® 10plex, a pooled tryptic digest of ALS PBMCs was labelled with four different TMT® tags and mixed to form a calibration curve (ratio 1x:4x:6x:10x). This reference was mixed with tryptic digests from slow ALS and fast ALS albumin depleted plasma samples (n=6) individually labelled with the remaining six TMT® tags from a TMT® 10plex kit. All the TMT® 10plexes were fractionated and subsequently analysed using an Orbitrap Fusion Mass Spectrometer. We have tested fast and slow progressing ALS patients at an early and late stage, as well as slow and fast progressing SOD1G93A mouse models at a pre-symptomatic and symptomatic stage. Results: Biological processes associated with senescence, RNA processing, cell stress and metabolism with altered glucose uptake (late stage) and major histocompatibility complex-II linked immune-reactivity (early stage) were enriched in fast progressing ALS patients. The markers of progression differentiating fast from slow progressing ALS mouse models were largely different from those identified in human pathology, with only processes encompassing membrane trafficking, acute phase response and cytoskeleton organization showing enrichment in both species. Conclusions: These findings implicate cell senescence and metabolism as targets of novel treatment strategies and suggest that immunomodulation may be effective as an early intervention. Differences in the plasma/PBMC proteomes between humans and animal models may explain the poor reproducibility of treatment response between species.

Keywords: Amyotrophic lateral sclerosis (ALS), TMT®, TMTcalibrator™, plasma.
Proteomics analysis of the peritoneal dialysis effluent by HiRIEF and TMT: a longitudinal study

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Background: Peritoneal membrane (PM) failure in end stage renal disease patients submitted to peritoneal dialysis (PD) cannot be predicted and does not occur in every patient in the same sequence and to the same extent. Moreover, long-term PD leads to morphological and functional alterations in the PM, reducing the lifespan of dialysis to five years, and forcing the replacement of PD by other renal replacement therapies. Peritoneal dialysis effluent (PDE) represents an underestimated biochemical window into the peritoneum and a useful reservoir of potential clinical biomarkers. This work aims to unravel the evolution of the PDE proteome with time, to identify specific molecular changes that can be particularly interesting for the understanding and early detection of long-term PM alterations.

Methods: PDE samples from patients were taken at several timepoints (follow-up study). Firstly, samples for each patient and timepoint were concentrated using centrifugal filters. After protein concentration measurement, the proteome fractions (>10KDa) were analysed as follows; (i) depletion using Multiple Affinity Removal System Column, (ii) the depleted and bound fractions were again concentrated (iii) Clean-up and digestion using SP3 magnetic beads; (iv) peptide labelling by tandem mass tags (TMT); (v) High-resolution peptide isoelectric focusing (HiRIEF) followed by (vi) LC-MS analysis.

Results: After TMT labelling and PDE sample complexity reduction by HiRIEF it was possible to identify and quantify proteins that could be of great value regarding the longitudinal study. The omics data obtained is integrated, analysed and functionally categorized (using Gene Ontology terms) in order to shed light in the underlying biological pathways to PM failure.

Conclusions: Our analysis may enable to identify proteins involved in the peritoneal changes in patients on long-term peritoneal dialysis, helping the early detection of such alterations and delaying ultrafiltration failure.

Keywords: Peritoneal membrane failure, Peritoneal Dialysis effluent, Longitudinal study, Proteome analysis, Biomarkers Discovery
IMPROVING DIAGNOSTIC OF DEGENERATIVE AORTIC STENOSIS: FROM RABBIT TO HUMAN SAMPLES

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\textbf{Background}: Using animal models for the study of degenerative aortic stenosis (DAS) is crucial to avoid the differences between the analyzed subjects, something common in these patients due to its age and the associated pathologies. Specifically, rabbit models are ideal since this animal has many similarities to humans, including valve histology and lipid metabolism. Our purpose was to characterize proteins involved in the first stages of DAS, searching for candidates for early diagnostic suitable to be transferred to the clinic.

\textbf{Methods}: In this work, we have analyzed aortic valves (AV) from healthy and mild stenotic rabbits. Male New Zealand White rabbits were divided in control and pathological group (cholesterol-enriched diet plus vitamin D2). After the sacrifice, AV were harvested and their proteins were analyzed using 2D-DIGE. Differentially expressed proteins were measured in plasma from the same rabbits to corroborate their potential as diagnostic indicators and in plasma from human subjects to confirm their feasible translation to the clinical field.

\textbf{Results}: Fifteen spots were found differentially expressed corresponding to 8 unique proteins. Of them, 5 proteins were measured in plasma samples from rabbit and 3 were also altered in human plasmas: transitional endoplasmic reticulum ATPase, tropomyosin alpha-1 chain and L-lactate dehydrogenase B chain. ROC curves were performed for these proteins, separately and as a panel, in order to establish its sensibility and specificity. In all cases, the area under the curve was higher than 0.73 and the p-value lower than 0.037. The diagnostic power of the three proteins as a panel was much better than the proteins alone (AUC=1, p-value=6.28e\textsuperscript{-56}).

\textbf{Conclusions}: Here we provide a molecular panel composed by 3 proteins related to the osteoblastic differentiation, which may be really interesting as putative CAS indicator and/or therapeutic target in the future.

\textbf{Keywords}: 2D-DIGE; cardiovascular; aortic valve stenosis; rabbit model; calcification
PROTEOMIC EFFECTS OF PAP THERAPY ON RED BLOOD CELLS IN MALES WITH OBSTRUCTIVE SLEEP APNEA

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Background: Obstructive Sleep Apnea (OSA) syndrome, a common public health concern, is characterized by recurrent arousals from sleep and intermittent hypoxemia that can lead to metabolic and cardiovascular diseases. OSA is treated with positive airway pressure (PAP) but its beneficial effect at molecular level is still to be elucidated. We recently demonstrated that OSA can cause alterations in the red blood cells (RBC) proteome that may lead to systemic inflammation. Here we intend to investigate whether the beneficial effect of PAP can revert or modulate the OSA-induced RBC proteome alterations.

Methods: RBC samples from Snores (controls) and patients with severe OSA before and after six months with PAP treatment (n=10/condition) were depleted of hemoglobin, using HemovoidTM system, and analyzed by 2D-DIGE NUPageTM 4%-12% Bis-Tris ZOOMTM mini gels. The generated 2D maps images were statically compared by Progenesis SameSpots v4.5 and the differentially abundant proteins identified by MALDI-TOF/TOF MS. Protein annotation properties were acquired using the “Database for Annotation, Visualization and Integrated Discovery” (DAVID) v6.8.

Results: Ten of 104 protein spots analyzed exhibited significant differences in abundance (Anova p<0.05) among patients groups. The identified proteins are associated with cell death, protein oligomerization and response to stress. Three proteoforms of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), probably as consequence of PTMs events, were identified decreased in OSA RBC samples compared with Snore controls (Anova p<0.05). Six months of PAP treatment reversed this alteration by increasing those GAPDH proteoforms abundances to the control levels.

Conclusions: OSA induces modulation in RBC proteome that can be reverted by six month of PAP treatment. PTMs events on GAPDH, the most important targets of the oxidative stress, seemed to be modulated in RBC by treatment with PAP. Further research is needed to validate GAPDH as candidate biomarker for PAP therapy monitoring in OSA.

Keywords: OSA, Positive-airway-pressure (PAP), GAPDH.
MOLECULAR CHANGES IN RESPONSE TO CELL THERAPY IN CRITICAL LIMB ISCHEMIA A PROTEOMIC APPROACH

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Background: Critical limb ischemia (CLI) is a pathology derived from peripheral arterial disease, it starts by severe obstruction of blood vessels and is commonly caused by atherosclerosis. The high rates of amputation and mortality of the patients who suffer it make necessary the search for alternative therapies. Cell therapy mediated by endothelial progenitor cells (EPCs) is postulated as a good alternative due to their angiogenic as well as revascularization properties. Nevertheless, the molecular mechanisms by which they exert the regenerative effects remain still unclear.

Methods: In our work, we have evaluated the initial effects produced by human EPCs on mice that were induced an ischemic process by ligation of the femoral artery, serving as CLI models. The initial response was evaluated on the CLI mice during three days after cell administration. Thus mice were sacrificed by day 1, 2 and 3 after cell administration and the femoral adjacent tissue was extracted for further proteomic analysis.

Results: By applying a label free approach, we aimed to identify proteins differentially expressed in those days within the ischemic tissues in presence/absence of cells. After identification of the injected cells by immunohistochemistry, the nature of the differentially expressed proteins as well as the biological meaning of such changes will be discussed.

Keywords: critical limb ischemia, endothelial progenitor cells, cell therapy
Identification of new diagnostics biomarkers of Alzheimer Disease's patients by Phage Display and protein microarrays.

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Background: Alzheimer’s disease (AD) is a progressive, chronic and neurodegenerative disorder that affects wide areas of the cerebral cortex and hippocampus. It is the most common cause of dementia worldwide with a 10-30% prevalence in ageing population (>65 years of age) and a high socioeconomic impact. Because the definitive diagnosis of AD requires post-mortem verification, new approaches to study AD are necessary to identify new diagnostic biomarkers and therapeutic targets of intervention. We have here aimed to identify AD-specific autoantibodies and their target proteins as blood-based biomarkers of the disease using a combination of phage display and protein microarrays.

Methods: Two T7 phage display libraries displaying the cDNA repertoire of AD patients and healthy individuals’ brain were biopanned to enrich in phages recognized by IgGs from AD patients. After the biopanning, phage microarrays containing 1920 unique phages and controls were printed and screened with serum from AD patients and controls.

Results: After phage microarray analysis, we identified a collection of 25 peptides target of autoantibodies from AD patients and healthy individuals’ brain were biopanned to enrich in phages recognized by IgGs from AD patients. After the biopanning, phage microarrays containing 1920 unique phages and controls were printed and screened with serum from AD patients and controls.

Results: After phage microarray analysis, we identified a collection of 25 peptides target of autoantibodies from AD patients as potential biomarkers of the disease. For validation, we expressed 13 of them as 6xHis-Halo fusion proteins and analyzed their seroreactivity using a different cohort of sera from AD patients and controls than the used in the microarrays or biopanning (71 sera from AD patients and 52 sera from controls) by WB and a luminescence beads-based test. A significant differential seroreactivity in AD patients in comparison to controls was observed for 8 peptides.

Conclusions: Our results suggest that these peptides may be AD specific autoantigens non-previously described that might be used as blood-based biomarkers of the disease. However, complementary immunological approaches are needed as well as a further validation using a higher cohort of sera from AD patients and controls to determine their actual usefulness as biomarkers of the disease.

Keywords: Alzheimer’s disease, protein microarrays, humoral response, biomarkers.
Secretome analysis of leukocyte-platelet rich fibrin membranes used in wound healing

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Background: Leukocyte-platelet rich fibrin (L-PRF) membranes are extensively used in dentistry and in other clinical scenarios to accelerate wound healing and tissue regeneration. Active platelets secrete growth factors and other proteins that contribute to wound healing. The goal of the present study was to investigate the secretome of L-PRF membranes to better understand their tissue regeneration properties.

Methods: Fresh blood samples were collected from healthy volunteers into glass-coated plastic tubes and centrifuged at 400g during 12 minutes to obtain L-PRF membranes. Membranes were cultured in DMEM medium with 1% penicillin/streptomycin following an established procedure and the secretome collected and concentrated at days 3 and 7 (after membranes extraction). Following precipitation, protein samples were either concentrated in one gel band or separated in a 12% Bis-acrylamide gel. Bands were cut off from the gel and proteins in-gel trypsin digested and identified in a Sciex Triple TOF. A systems biology analysis (IPA) was also carried out.

Results: Overall, over 400 proteins were identified, which were related to the following principal canonical pathways: acute phase response signaling and clathrin-mediated endocytosis signaling. The majority of proteins identified are implicated in cellular movement, cell death and survival and cell-to-cell signaling and interaction, linking to inflammatory and immunological response. In a SDS-PAGE-based differential analysis, four main proteins bands varied between the secretome at days 3 and 7; 399 proteins were identified in those bands at day 3 and 305 at day 7. Between these two conditions, 260 proteins were common, 139 only present at day 3, and 45 proteins only present at day 7. Many of these proteins were related to the canonical pathways mentioned above, although proteins such as epidermal growth factor and complement proteins are only present at day 3.
Conclusions: This highlights differences in the secretome over time, which is of interest for tissue regeneration procedures.

Keywords: Leukocyte-platelet-rich-fibrin, secretome, proteomics, tissue regeneration
ASSESSING EPCs POTENTIAL BIOMARKERS FOR CARDIOVASCULAR REGENERATIVE MEDICINE

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Background: Endothelial progenitor cells (EPCs) are known to play a pivotal role in vascular homeostasis and repair, due to their angiogenic and vascular regenerative functions, which they exert in a paracrine fashion, as well as by replacement of damaged endothelial cells. In atherosclerotic conditions ex vivo, healthy EPCs change their normal function, as our group recently described(1).

Methods: In order to characterize differences in EPCs protein secretion in response to atherosclerotic factors, two sets of EPCs (n:3) were incubated ex vivo with atherosclerotic plaque secretome (AP) or left untreated respectively, and a label free proteomic approach was carried out. Thus, protein analysis was performed using an Orbitrap (ThermoFischer) and quantification was done with MaxQuant and Perseus platforms (2). The up-regulated proteins in the AP treated set secretome were submitted to databases such as DAVID or String for enrichment analysis, which allowed us to make a selection of some candidates.

Results: In total, 1514 proteins were identified, 92 up-regulated and 103 down-regulated in the EPC secretome in response to the atherosclerotic factors. Further validation of these changes has been made by western-blot for some of the potential candidates selected after database analysis. Herein, the potential role of these potential biomarkers within the paracrine effect of EPCs in atherosclerosis will be discussed.

Keywords: atherosclerosis, EPCs, secretome, proteomics.


A Proteomics Study on Septic Shock Patients: an Overview on Sepsis-Induced Myocardial Dysfunction

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Background: Sepsis is the most frequent complication in surgical patients and a potentially fatal disease resulting from a dysregulated host response to infection that induces organ dysfunction. Sepsis and shock are the main causes of death in the ICUs and recent studies suggest that myocardial dysfunction is associated to increased mortality in septic patients.

Methods: A TMT-10plex quantitative approach was employed to analyze blood plasma samples from 28 Septic Shock (SS) patients taken at three time points: 16h (T1), 48h (T2) and 7 days after ICU entry, 7 control patients (sepsis at T1) and 9 healthy controls (N TOTAL = 90). SS patients were grouped according to response to therapy (Non responders, defined as having SOFA score T2 > 8 and ΔSOFA T1-T2 < 5) and to 28-day survival. Fourteen major plasma proteins were immuno-depleted. Each of the ten TMT-10plex set of experiments was offline fractionated by basic reverse-phase into eight fractions that were analyzed in an Orbitrap Fusion Lumos.

Results: ANOVA test was performed to evaluate differences among SS groups at T1 and controls. The only significant differences were found in the comparisons against the healthy group, although some proteins showed clear trends across septic groups. The patients were also classified according to survival and differences were evaluated using two-way ANOVA. We found 63 proteins influenced only by outcome. STRING analysis of the 35 proteins showing FC>1.5, showed enrichment in regulation of immune system, protein activation cascade, complement activation and cell adhesion. Moreover, we observed deregulation in actin, myosin and gelsolin, which support previous reports explaining changes in the cytoskeleton during sepsis.

Conclusions: Although few proteomics studies have been done on sepsis-induced myocardial dysfunction, our preliminary results are in accordance with the literature. Some of the proteins we detected are correlated with outcome.

Keywords: Septic shock, Proteomics, Sepsis-induced myocardial dysfunction
EXPLORING THE PROTEOME ALTERATIONS IN PARK2-MUTATED PATIENTS.

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Background: PARK2 mutations are the most frequent cause of a familial form of Parkinson’s disease (PD). This gene encodes for Parkin, an E3 ubiquitin ligase involved in several cellular mechanisms, e.g., the mitophagy and the unfolded protein response. Given its role in PD pathogenesis, this research project aims at clarifying the whole cellular and the mitochondrial proteome alterations in primary skin fibroblasts of PARK2-mutated patients.

Methods: All whole cell pellets and mitochondrial-enriched fractions from five PARK2-mutated patients and five control subjects were analysed by quantitative shotgun proteomics (Synapt G2, Waters). Differential protein expression was evaluated with a data-independent acquisition (DIA) of the shotgun proteomics analysis by high definition Expression mode (HDMS®). Qualitative and quantitative analysis were performed using the software ProteinLynx Global Server v. 3.0.2. (PLGS, Waters Corp.). The altered biochemical pathways involved were identified using a systems biology approach based on protein networks.

Results: Quantitative shotgun proteomics analysis revealed the alteration of several mitochondrial proteins. In particular, proteins involved in energy production, mitochondrial quality control, and mitochondrial network maintenance quantitatively changed in PARK2-mutated patients. Moreover, the analysis of the whole cellular proteome highlighted the impairment of many pathways that might be involved in PD pathogenesis.

Conclusions: We obtained a definition of the PARK2-related molecular signature and defined which proteins are altered by the lack of Parkin.

Keywords: Parkinson’s disease, PARK2, fibroblasts, quantitative proteomics, systems biology.
COMPUTATIONAL IMMUNOMICS REVEALS AN ANTI-\textit{Candida} SURFACE ANTIBODY-RECTIVITY SIGNATURE FOR INVASIVE CANDIDIASIS

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\textbf{Background:} Invasive candidiasis (IC) is associated with high morbidity and mortality in cancer, post-surgical and intensive care patients if not diagnosed and treated early. However, IC is difficult to diagnose at an early stage, leading to delayed therapeutic interventions and ensuing fatal clinical outcomes. Here, we examined whether profiling of the serologic response to the \textit{Candida albicans} cell surface-associated proteome during dimorphic switching might uncover an accurate molecular discriminator for IC.

\textbf{Methods:} We explored serum IgG antibody-reactivity patterns to the \textit{C. albicans} cell surface-associated proteomes from yeast and hyphal growth forms in IC and non-IC patients by serological proteome analysis and data mining tools. Capture ELISA assays were used to validate the obtained data.

\textbf{Results:} Unsupervised two-way hierarchical clustering and principal component analyses of these serum IgG antibody-reactivity profiles discriminated IC patients from non-IC patients. Pairwise correlation mapping across serum specimens further revealed discrete biological changes underlying commensal-to-pathogen and yeast-to-hypha transitions. Supervised classification analyses with leave-one-out cross-validation unveiled a serum 3-IgG antibody-reactivity signature as the best molecular predictor for IC. A positive association between this molecular fingerprint and IC risk was observed using multivariate logistic-regression models. This association was unbiased by conventional clinical risk factors for IC and other variables related to patients. Receiver-operating-characteristic curve analysis highlighted that this serum antibody-reactivity signature showed a good ability to differentiate between IC and non-IC patients. Its discriminatory power was confirmed by capture ELISA assays in an independent group of IC and non-IC patients.

\textbf{Conclusions:} Our study has brought to light a serum 3-IgG antibody-reactivity signature that may be useful for early and accurate diagnosis of IC. In addition, our findings further provide new insight into pathogenic processes triggered during IC pathogenesis and dimorphic transition. \textbf{Keywords:} Invasive candidiasis, diagnosis, antibody signature, cell surface, computational immunomics. \textit{This work was supported by BIO-2015-65147-R, IPT17/0019-ISCIII-SGEDI/ERDF and RD16/0016/0011.}
Proteomic and metabolomic analysis of molecular processes involved in clinical peritoneal dialysis

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Peritoneal dialysis effluent (PDE) of patients undergoing renal replacement therapy with peritoneal dialysis (PD) represents a rich source of molecular markers as novel tools for predicting clinical outcome and monitoring the therapy. Novel PD-fluids (PDF) may enable patient-tailored interventions, such as peritoneal immunomodulation. High performance chromatographic and mass spectrometric methods allow assessment of molecular changes and therefore detection of candidate biomarkers from the abundant effluent. The combination of proteomic and metabolomic methods is particularly attractive for a better understanding of the related processes.

In a bioinformatics workflow, we combined proteome data obtained from our recently established CPLL-FASP-TMT-LC/MS workflow, which depletes high-abundant plasma proteins and enriches low abundant cellular proteins in PDE, with metabolomic profiles in a multi-omics analysis for PDE from the same randomized clinical trial samples. Data from 2506 individual proteins were surveyed in combination with 188 small molecules and correlated to patient characteristics, such as time on treatment and occurrence of previous peritonitis events. Treatment with alanine-glutamine supplemented PDF in the cross-over trial was also investigated. Co-regulated biological processes on the proteome and metabolome level such as oxidative stress responses were identified. Treatment with the novel PDF rescued processes thought to be involved in decline of PD treatment efficiency and failing adequate immune response. Metabolomic profiling of PDE revealed recently unknown small molecule related changes during PD therapy. The intervention with alanine-glutamine as a potentially cytoprotective PDF does not only restore important immune processes, it further reflected on small molecule responses such as indicators of oxidative stress in accordance with processes on the protein level. The exploitation of PDE information on multiple omics levels in our bioinformatic approach improves understanding of molecular processes in the peritoneal cavity and their role in development of complications and allows identification of biologically relevant small molecule surrogate parameters for patient outcome.
Cross-omics analysis of proteome and transcriptome dynamics during clinical peritoneal dialysis therapy

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Peritoneal dialysis (PD) is not only an important renal replacement therapy, it also represents a modality of scheduled repeated exposure to PD fluids (PDF) which disrupt peritoneal immune defenses and cause increased sterile inflammation. Alanyl-glutamine (AG) has recently been shown to restore cytoprotective stress responses in experimental PD and improve patients’ survival and immune function of leucocytes in critically-ill patients. Addition of AG may therefore restore adequate responses of peritoneal cell populations to stress and infectious stimuli.

Samples from a randomized clinical pilot-trial were analyzed in a cross-omics approach of peritoneal effluent cells (transcriptomics using RNAseq) and soluble proteins (proteomics using LC-MS) to investigate the effect of AG on the interplay of peritoneal cell populations and fluid transport in PD.

In the clinical trial stable patients on chronic PD were treated in a cross-over design with standard PDF or with AG-supplemented PDF. Peritoneal immune-competence was analyzed by functional \textit{ex-vivo} stimulated cytokine release of effluent cells. From each PD dwell unstimulated cells and cell-free PD-effluent was collected at multiple time-points of the dwell. Cellular responses were assessed on the RNA level and soluble proteins in cell-free PD-effluent were analyzed by a recently established depletion and enrichment workflow based on combinatorial peptide ligand library (CPLL) beads, filter-aided sample preparation (FASP) and tandem mass tag (TMT) based LC/MS.

We were able to quantify approximately 10,000 transcripts in PD effluent cells and 2,500 proteins in the PD effluent proteome. Interestingly, differential changes in the proteome could in part be explained by co-regulated biological processes observed on the transcript level. The remaining effects on the proteome are likely due to changes in transport characteristics, supported by clinical findings in patients treated with AG added to PDF. These results correlated with increased \textit{ex-vivo} stimulated cytokine release reflecting restoration of suppressed peritoneal immune responses by AG.
TOP-DOWN AND CHEMICAL PROTEOMICS

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Structural elucidation of proteoforms from Actinobacteria by Top-down MS

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Background:

Post-translational modifications (PTMs) are essential processes conditioning the biophysical properties and activities of the vast majority of mature proteins. However, occurrence of several distinct PTMs on a same protein dramatically increases its molecular diversity. The comprehensive understanding of the functionalities resulting from any particular PTM association requires a highly challenging full structural description of the PTM combinations. Top-down mass spectrometry (TD-MS) is emerging as a dedicated tool to characterize such combinations of PTMs, truncations, splicing events and/or mutations. The corresponding proteoforms can indeed display different activity, half-life, immunogenicity, hence the importance to fully characterize them.

Methods:

Here, we report the in-depth exploration of two systems located at the membrane of Actinobacteria: the Mycobacterium tuberculosis (Mtb) lipoglycoprotein LpqH, and outer membrane protein (OMP) from Corynebacterium glutamicum (Cglu), using a TD-MS approach.

Results:

TD-MS analyses of the purified Mtb LpqH protein allowed the identification of about 130 distinct proteoforms resulting from the intrinsically heterogeneous combination of acylation and glycosylation, together with some truncations (Parra, Marcoux et al, 2017).

Investigation of the partitioning of endogenous and recombinant porins PorA, PorH, PorB and PorC between bacterial compartments revealed that they were both imported in the mycomembrane and secreted in the extracellular medium of Cglu. TD-MS combined with some others approaches enabled to identify and localize the molecular features that allow precursors of such proteins to be targeted to the mycomembrane (Carel, Marcoux et al, 2017).

Conclusions:

The direct view on the co-occurring PTMs stoichiometry of LpqH, together with the identification of novel unsuspected proteoforms, provide new insights into the biogenesis and functional determinants of lipoglycoproteins in Mtb. We also found that such modifications were essential for OMP targeting to the mycomembrane in Cglu. Collectively, our results demonstrate the crucial role played by PTMs in the organisation of the Actinobacteria membrane.

Keywords:

Top-down MS; Post-translational Modifications; Proteoforms; Tuberculosis.
A systematic map of protein-metabolite interactions reveals principles of chemical communication

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Background:
Metabolite-protein interactions control a variety of cellular processes, thereby playing a major role in maintaining cellular homeostasis. Different types of functional interactions between proteins and metabolites have been reported and involve binding of metabolites to the active site of enzymes as substrates, cofactors, or products of enzymatic reactions. Metabolites comprise the largest fraction of molecules in cells, but our knowledge of the metabolite-protein interactome lags behind our understanding of protein-protein or protein-DNA interactomes, since most characterized protein-metabolite interactions have been discovered via hypothesis-driven experiments that rely on in vitro activity assays.

Methods:
Here we present LiP-SMap a chemoproteomic workflow for the systematic identification of metabolite protein-interactions directly in their native environment. We devise an approach that combines limited proteolysis (LiP) with DIA (Data Independent Acquisition) mass spectrometry in the presence of unmodified metabolites to enable a systematic analysis, unbiased with regard to both metabolites and proteins.

Results:
With LiP-SMap we identified a network of known and novel interactions and binding sites in Escherichia coli, and we demonstrated the functional relevance of a number of newly identified interactions. Our data enabled identification of new enzyme-substrate relationships and cases of metabolite-induced remodeling of protein complexes. Our metabolite-protein interactome consists of 1678 interactions and 7345 putative binding sites. Moreover the dataset reveals functional and structural principles of chemical communication, shed light on the prevalence and mechanisms of enzyme promiscuity, and enable extraction of quantitative parameters of metabolite binding on a proteome-wide scale.

Conclusions:
We envision that LiP-SMap will set the standard for future studies of protein-ligand interactomes and the discovery of new drugs.

Keywords:
Metabolite-protein interactome, allosteric regulation, ligand binding sites, chemoproteomics, structural proteomics.
An integrated workflow for cross-linking/mass spectrometry

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Background: Cross-linking/mass-spectrometry (CLMS) has become a standard tool for the topological analysis of multi-protein complexes and has begun delivering high-density information on protein structures, insights into structural changes and the wiring of interaction networks in situ. Advances in the methodology have been made due to the development of new cleavable cross-linkers and the introduction of new mass spectrometers that resulted in a considerable amount of protocols and software that can’t be integrated. Furthermore, challenges like cross-linked peptide identification due to size and low abundance need still to be further addressed.

Methods: We present a 12-fraction concise workflow using standard cross-linkers and fragmentation methods and introduce a sequential digestion step to decrease the size of cross-linked peptides and enhance their detection avoiding over-digestion. We used our software Xi to perform cross-linked peptide search as it supports any type of cross-linker, protease or protease combination and fragmentation methodology. QCLMS was also performed to demonstrate the applicability of the combined protocols.

Results: We benchmarked our protocol in samples that ranged from low to high complexity. We increased cross-linked peptide identification by 1.5 fold without compromising data quality. QCMLMS was successfully combined with sequential digestion suggesting a bottom to bottom orientation for the C3b dimerization. We were able to differentiate, in solution, between different states of the proteasome and point towards the existence of more alternative states and found data that support the existent cryoEM-based model. Finally, we identified an essential interaction for the OCCM complex formation that could represent an ideal target for the development of inhibitors with potential as anti-cancer therapy.

Conclusions: This simple and concise 12-fraction approach, in solution, increases cross-link coverage, reveals dynamic protein-protein interaction sites, which are accessible, have fundamental functional relevance and are therefore ideally suited for the development of small molecule inhibitors.

Keywords: cross-linking/mass spectrometry, sequential digestion
Top-down proteomic approach for the study of basic proteins in the human sperm nucleus

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Background: The protamine 1 (P1) and protamine 2 (P2) family comprises the most abundant basic proteins in human spermatozoa and packs 85-95% of the paternal genome. P1 is synthesized as a mature form, whereas P2 components (HP2, HP3, and HP4) are generated from the proteolysis of the precursor. The particular physical-chemical properties of protamines hinder their identification by the standard bottom-up mass spectrometry (MS) strategy. A top-down MS approach is therefore proposed to identify human protamines and their post-translational modifications (PTMs) at the molecular level.

Methods: The intact protamine-enriched fraction from a single man was isolated from 15 million purified spermatozoa after histone removal and disulfide bonds reduction. Protamine enrichment was verified through acid-urea PAGE. The protamine-enriched fraction was analyzed by nano-liquid chromatography coupled to tandem MS (nanoLC-MS/MS) using a chip-based Advion nanoelectrospray source and an Orbitrap Fusion Lumos (Thermo Scientific) mass spectrometer. The latter was operated in data-dependent acquisition (DDA) mode, and the most abundant ions were selected for fragmentation by Electron Transfer Dissociation (ETD). Data analysis was performed using Proteome Discoverer 2.1 with Prosight PD 3.0 and Sequest HT nodes and TopPIC software.

Results: Preliminary results of the top-down proteomic MS approach allowed identification of the intact naïve P1, HP2, and HP3. Furthermore, multiple protamine proteoforms containing different combinations of PTMs on the same polypeptide chain were detected. However, HP4 was not detected, most likely due to its physiological low abundance. Remarkably, this technology revealed proteoforms with no previously reported experimental evidence and thus corresponding to missing proteins.

Conclusions: The top-down MS approach allows the characterization of protamine PTM profiles at the intact level and helps to identify human missing proteins that have gone undetected experimentally by the well-established bottom-up MS approach. These results will shed new light on male infertility pathogenesis and the potential role of sperm in embryogenesis.

Keywords
Top-down proteomics, electron transfer dissociation, protamines, sperm, missing proteins.

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**Advances in Orbitrap™ instrumentation for native top-down analysis of non-covalent protein complexes**

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**Introduction**

Native mass spectrometry has emerged as a powerful technique to study protein-ligand interactions and elucidate the structure of macromolecular assemblies, including both soluble and membrane protein complexes. Top-down studies of intact protein complexes have been reported since the early 1990’s, but their characterization using MS3 have only recently been reported and most work has been done on homomeric assemblies. However, poor fragmentation into subunits and stripped complexes in the front end of the MS limits the use of current MS instrumentation for native top-down analysis using a pseudo-MS\(^3\) approach.

**Methods**

Experiments were performed using a modified Thermo Scientific™ Q Exactive™ Plus MS in which the ability to perform pseudo-MS\(^3\) scans for native top-down analysis and transmission of high m/z ions were improved by implementing several hardware and software modifications, the most important being pulsed trapping of ions in the injection flatapole region (‘in-source trapping’) and reduction of the frequency of RF voltages applied to bent flatapole, quadrupole, transfer multipole, C-trap and HCD cell. Other modifications include gas pressure control, adjustment of the voltage ramp rate of the Orbitrap mass analyzer and the increase of the maximum HCD energy from 200 to 300V. Pyruvate Kinase, GroEL, rabbit 20S proteasome and LmrP membrane protein were used as model systems.

**Preliminary Data**

Advances towards native MS and native top-down analysis of heteromeric protein complexes became possible only after addressing several major technical challenges. First, implementation of ‘in-source trapping’ capability addressed the insufficient or poorly controllable desolvation issue and allowed significantly improved fragmentation into subunits and stripped complexes in the inject flatapole region. Second, the reduction of RF frequencies on all RF guides and the mass filter was aimed at increasing the transmission of high m/z ions. Third, the adjustment of the voltage ramp rate on the central Orbitrap electrode facilitated successfully transmitting the high m/z ions from the C-trap into the Orbitrap analyzer.
DEVELOPMENT OF IMMOBILISED AFFINITY BASED PROBES FOR SELECTIVE ENRICHMENT OF GLYCOSYLTRANSFERASES IN TRYpanosoma BrucEi

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Background: Trypanosoma brucei (T. brucei) is an extracellular parasite endemic to sub-Saharan Africa, capable of infecting mammals causing sleeping sickness in humans and nagana disease in animals. Infection occurs via the bite of infected tsetse flies (Glossina sp.). These parasites rely on a dense coat of variant surface glycoproteins (VSG) to evade the mammalian host immune system. Many of the glycans decorating these VSG are built by unidentified glycosyltransferases (GTs) forming unique linkages. These distinct GTs could make promising therapeutic targets. However, their uniqueness eliminates the possibility of using homology to identify the enzymes involved in their synthesis. The majority of these unique linkages require a UDP-Galactose donor.

Methods: We developed novel synthetic routes and methodologies to identify galactosyltransferases (GalTs) through the use of immobilised activity-based probes. These probes are immobilised on a resin to allow ease of purification and identification of bound proteins through subsequent use of mass spectrometry based quantitative proteomic approaches.

Results: We developed a newly developed synthetic route, that allowed the attachment of UDP-galactose and UDP-(4F)-galactose tentagel resins. To our knowledge this is the first example of resin bound sugar nucleotides. After initial method development with a commercially available galactosyltranferase along with mixtures of proteins, the resins proven to bind with the expected selectivity. They were then used in an assay to enrich galactosyltransferases from T. brucei lysates. Through the use of the resin containing UDP-(4F)-Galactose we were able to identify several other putative GTs.

Conclusions: The characterisation of these enzymes is instrumental to the development of novel drug targets and to understand the evolutionary significance of these linkages in the context of host-pathogen interactions. Once, fully optimised, the methodology proposed can be applied to any organism.

Keywords: Affinity based probes, chemical proteomics, mass spectrometry, glycosyltransferases
Differentiation between fresh and frozen–thawed curled octopus using two-dimensional gel and shotgun proteomics

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Background: The substitution and sale of frozen-thawed fish labelled as fresh is a widespread and difficult to unmask commercial fraud and a potential danger for the consumer’s health. Differentiating between fresh and frozen-thawed fish is not easy and different methods can be used but not for all type of seafood products, in particular for cephalopods. Freezing and thawing process causes protein denaturation, so proteomics methods could represent a valid approach to find differences between fresh and frozen-thawed samples.

Methods: Eighteen samples of certainly fresh Eledone cirrhosa, were divided into two aliquots, the first was immediately processed to extract the proteins, while the second was frozen at -20 °C for 72 hours and subsequently thawed before protein extraction. Samples were subjected to two-dimensional electrophoresis (2DE) to detect qualitative and quantitative differences between the samples. For proteins identification, spots from the 2DE maps were cut out, digested and analysed with high-resolution liquid chromatography tandem mass spectrometry (LC-MS/MS).

Six samples, three fresh and three frozen-thawed, were subjected to the SWATH-MS acquisition. Analyses were performed in triplicates for each sample and imported into the PeakView software to perform the label-free quantification. Peptides with FDR lower than 1.0% were exported to MarkerView for the statistical analysis by a t-test.

Results: The 2DE analysis showed a significant reduction in two protein spots (p.I. ~ 6.5-7, 45-50 kDa) identified as transgelin with LC-MS/MS. At shotgun analysis, nine proteins resulted modulated and transgelin was confirmed as down-regulated.

Conclusions: This study provides a 2DE and shotgun comparative analysis to differentiate fresh from frozen-thawed curled octopus. This work opens the way to future studies to develop a test, providing a useful tool for producers and official authorities.

Keywords: 2D-electrophoresis, frozen-thawed, curled octopus (E. cirrhosa), shotgun proteomics, seafood safety
Optimisation of protein extraction for in-depth profiling of the cereal grain proteome

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Background: Characterisation of the proteome obtained from grains such as wheat, barley, rye and oats that have been implicated in gastrointestinal diseases *i.e.* coeliac disease (CD) or non-coeliac gluten sensitivity (NCGS) provides the foundation for studies examining the pathology of these disease. Moreover, the grain proteome can provide valuable information regarding plant growth, nutritional status and adaptation to the environmental and/or abiotic stresses.

Methods: In this study, six protein extraction protocols that employed different buffer compositions, with and without defatting and protein precipitation steps were compared. LC-MS/MS was employed to comprehensively profile a single barley cultivar. Subsequently, two cultivars of wheat, barley, rye and oats were investigated under three different extraction protocols. The data generated were interrogated for gluten proteins that are linked to CD, and also for the α-amylase/trypsin inhibitors (ATIs) that have been implicated as elicitors of the poorly characterised condition NCGS. Bioinformatic analyses were conducted on the protein complement to gain insight into the effect of altering the extraction buffer.

Results: Using Tris-HCl and urea-based buffers 1,433 and 1,769 proteins were extracted from the barley cultivar cv Sloop respectively. Inclusion of a hexane-based defatting step prior to protein extraction was of no benefit, whilst protein precipitation employed post-extraction negatively impacted protein recovery, a likely result of problems associated with protein re-solubilisation. The use of an alcohol-based extraction protocol yielded a lower number (645) of proteins but was noted to co-extract the ATIs. Gene Ontology (GO), protein-protein interaction network, GO enrichment, and pathway analysis showed that the choice of extraction buffer impacts on the classes of proteins extracted.

Conclusions: The protein extraction protocol used directly influences the proteome composition thus affecting the downstream bioinformatic analyses and biological interpretation of data.

Keywords: Cereal grains, protocol, protein extraction, proteome, GO enrichment
Characterization of short digestion-resistant peptides of cow’s milk allergens by digestomics approach

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Background: Extensive hydrolysis of allergenic milk proteins to the fragments less than 3 kDa is accepted as a safer alternative for introducing milk into children’s diet. The aim of this study was to investigate and identify digestion products of major milk allergens and to examine IgE reactivity and allergenicity of short digestion-resistant peptides (SDRP) released by pepsin digestion of whole milk.

Methods: Raw milk was subject to simulated gastric digestion. Peptic digests were analyzed by electrophoresis and Western blotting. SDRP were fractionated from digesta and identified by high-resolution mass spectrometry. SDRP of milk were evaluated for aggregability, propensity to compete for IgE binding with individual milk allergens and the mixture of the milk proteins.

Results: Alpha-lactalbumin and beta-lactoglobulin were present in gastric digests mostly as intact proteins. Majority of the SDRP originated from caseins (97% of peptides) and overlapped with the known IgE epitopes of cow’s milk allergens. The SDRP competed with milk proteins to bind to human IgE and readily formed aggregates. With the average peptide length of 10.6 ± 3.5 amino-acids, induction of allergic reaction is attributed to the peptide ability to form functional non-covalent complexes by aggregation of hydrophobic patches.

Conclusions: SDRP corresponding to the mainly continuous epitopes of milk may induce functional allergic response by aggregation.

Keywords: allergy, cow’s milk, digestomics, short-digestion resistant peptides, IgE
The *Antrodia Cinnamomea* Reduce Obesity by Regulating Lipid Metabolism and Biosynthesis in ob/ob Mice

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**Background:** Obesity is defined as excessive body weight and fat accumulation, usually associated with many diseases such as diabetes, cancer and cardiovascular. Therefore, obesity prevention become more important; *Antrodia cinnamomea* (AC) is an aboriginal medical fungus in Taiwan, has multiple functions such as anti-cancer, anti-inflammatory, anti-diabetic, prevent chronic liver damage and obesity fed high fat diet. However, the effects of AC on haptic metabolic mechanism and lipogenesis are unknown; we utilize C57BL/6 mice (WT) and genetic obese ob/ob mice to study whether AC could protect them from developing obesity.

**Methods:** In present study, we fed WT mice and ob/ob mice with AC in dosage of 1666.67mg/kg for four weeks, and use two-dimensional differential gel electrophoresis (2D-DIGE) to analyze the differentially expressed proteins in intestine and liver. Quantitative real-time reverse-transcription polymerase chain reaction (qPCR) analyze the RNA level expression and utilize western blot to elaborate the mechanism.

**Results:** The liver of WT mice fed with AC proteomic analysis indicated AC promotes lipid degradation and glycolysis; otherwise, AC inhibits cholesterol biosynthesis and transport. The qPCR results showed AC could restore increased lipid uptake pathway that caused by leptin-deficient and reduce liver lipid storage. Simultaneously, AC activates AMP-activated protein kinase (AMPK) and inhibits fatty acid synthesis pathway, leading to decrease liver fat accumulation. Moreover, the intestine proteomic analysis demonstrated that AC promotes glycolysis and citric cycle; in contrast, AC inhibits vesicle transport to reduce excessive energy absorption. Notably, AC reduces lipid accumulation also promotes beta-oxidation that could be a reason why AC significantly decreases adipose tissue content.

**Conclusions:** AC can reduce genetic obese by increasing metabolism and inhibiting lipogenesis in liver and affect intestinal permeability. In the future, we will further investigate the effect of AC on lipid mobilization in WAT.

**Keywords:** *Antrodia cinnamomea*, two-dimensional differential gel electrophoresis, obesity
Integration of Hepatic SWATH-based Proteomics and Metabolomics to better understand Diet-induced metabolic disorders

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Background: The present study integrates SWATH-based Proteomics and Metabolomics of the liver in mouse models of obesity and hyperglycemia to better comprehend functional connection between genes, diet and obese phenotypes.

Methods: Mouse models of obesity-hyperglycemia (wild-type mouse fed HFHS diet), obesity-normoglycemia (PPAR-alpha null mouse fed HFHS diet) and healthy body weight-normoglycemia (wild-type mouse fed chow) were analysed by a SWATH data-independent acquisition (DIA) strategy to evaluate changes in the hepatic whole proteome. Several analytical platforms based on mass spectrometry were applied to evaluate changes in metabolites and lipids relevant for the metabolism.

Results: The results are encouraging in terms of the number of identified and quantified proteins and metabolites in liver. Proteomics data indicated that no cellular components were specifically over-represented when assessing the effect of diet in wild-type mice, while proteins from peroxisome, microbody and mitochondria were strongly altered in PPAR-alpha KO mice fed HFHS diet compared to wild-type mice under the same diet. HFHS diet in wild-type mice induced changes in proteins involved in metabolic pathways and PPAR signalling, meanwhile the deficiency of PPAR-alpha in animals subjected to HFHS caused higher effect on PPAR signalling, but also pathways related to fatty acid metabolism were affected. Metabolomics also clearly discriminates the effect of diet and genotype. The integration of metabolomic and proteomic datasets in the pathway maps suggests that certain metabolic differences in the synthesis and accumulation of lipid species in the liver, amino acid metabolism and production of eicosanoids may contribute to explain the development of obesity and hyperglycemia.

Conclusions: The combination of new quantitative proteomics and metabolomics techniques that address the massive quantitation of the components of the proteome and the metabolome have a great potential to provide a more comprehensive understanding of the molecular complexity that is inherent in obesity and other diet-induced metabolic diseases.

Keywords: SWATH, proteomics, metabolomics, obesity, nutrition and metabolism

Topic: Food&Nutrition proteomics or Disease proteomics
A proteomic approach to study the neuroprotective effect of oleocanthal in SH-SY5Y cells

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Background: Olive leaves and virgin olive oil contain many phenolics effective against aging and several lifestyle-related diseases, including neurodegeneration, both in animal models and in humans. Oleocanthal is a secoiridoid, one of the most represented class of phenols in olive oil, and it is responsible of the stinging effect at pharynx level perceived after extra virgin olive oil ingestion. Recently, different studies demonstrated that oleocanthal possesses anti-aggregation activities on tau protein and Ibuprofen-like activity thanks to its ability to inhibits COX-1 and COX-2. The aim of this work is to investigate the neuroprotective effect of oleocanthal in neuron-like SH-SY5Y cells before and after oxidative stress induced by H₂O₂.

Methods: Using 2DE coupled to mass spectrometry the protein maps for different conditions of treatment have been obtained and analyzed by Same spots (TotalLab). PCR analyses were performed to validate proteomic results.

Results: Seventeen spots resulted significantly differentially expressed with respect to control after treatment with hydrogen peroxide, twenty-seven after treatment with oleocanthal (10 µM) followed by hydrogen peroxide, while two spots for direct effect of oleocanthal. Spots of interest were excised and identified by LC/MS/MS. Oleocanthal significantly reverted the down-regulation induced by hydrogen peroxide of 26S proteasome non-ATPase regulatory subunit 1, proteasome subunit beta type-4, Ubiquitin carboxyl-terminal hydrolase and Pyruvate kinase, moreover it increased the expression of Heat shock protein HSP 90-beta and Protein DJ1. Moreover, 10 µM oleocanthal was able to counteract oxidative stress induced by H₂O₂ in SH-SY5Y as measured by MTT viability assay and to increase reduced-GSH level both in the absence and in the presence of H₂O₂ as measured by monochlorobimane assay.

Conclusions: Our findings suggest that oleocanthal may have beneficial health effect in counteracting neurodegeneration.

Keywords: oleocanthal, SH-SY5Y cells, oxidative stress
Proteomics to overcome precautionary labeling: a translational approach to food

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Background: There is currently no cure for food allergy and the most effective way to prevent symptoms is to remove the offending food from diet. Food labels, reporting allergenic ingredients according to local legislation worldwide, must be correctly read. A fraction of food-allergic patients are sensitive to minute amounts of foods, therefore some industries have also spontaneously added some precautionary allergen labelling (PAL), indicating the possible contamination of foods by allergenic traces.

Methods: A prospective, double-blind placebo, mono-centric controlled study was conducted to assess the hypoallergenicity of a specific brand of biscuits (called here MGF) in children with cow’s milk and hen’s egg anaphylaxis. The primary outcome was assessed by double blind placebo controlled food challenge with MGF. The presence of milk and/or egg allergen traces in MGF was assessed by liquid chromatography-tandem mass spectrometry. The binding of patients’ serum with MGF proteins was assessed by Western Blot.

Results: None of the 29 enrolled children reacted to MGFs. Patient’s sera showed no reactivity with proteins extracted from MGFs. The initial proteomic analysis was negative for egg and milk proteins in MGFs. Unique signature peptides specific to cow’s milk and hen’s egg are being analysed, and a quantitative analytical method (by Multiplex Reaction Monitoring, MRM) on latest generation triple quadrupole mass spectrometer is being developed to assess allergens at very low concentration (below 10 ppm).

Conclusions: MGFs seem to meet the American Academy of Pediatrics criteria for hypoallergenicity. Proteomic techniques may be useful to evaluate, by a rapid and multiplexing screening, the exact amount of allergens within the food. Should their sensitivity be pushed up to dose amount of proteins lower than those that can theoretically determine an allergic reaction, they could help in avoiding unnecessary PAL.

Keywords: food, allergen, hypoallergenicity, MRM
DISEASE PROTEOMICS III

XII EUPA CONGRESS
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Multi-omics and functional investigations in hepatic and skeletal muscle mitochondria of pre-diabetic mice

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Background: Mitochondria, although being the major site for energy production in most tissues, are molecularly tailored to meet tissue-specific needs. Disentangling the linkage between mitochondrial molecular protein and lipid fingerprints between tissues and in turn their impact on mitochondrial function provides an important tool for the understanding of tissue-specific mitochondrial alterations upon external stimuli such as diet-induced excess substrate flux causing insulin resistance (IR) and diabetes.

Methods: We performed quantitative LC-coupled proteomic/lipidomic analyses (Q Exactive HF) of ultracentrifugation-isolated mitochondria (4 groups, n=8 each) from skeletal muscle and liver of a pre-diabetic mouse model (6 weeks of control (10 fat/20 protein/70 carbohydrates [kJ%]) vs high-energy diet (HED; 45 fat/20 protein/35 carbohydrates [kJ%])). Mitochondrial proteome was covered by non-targeted DIA (spectral library from DDA of fractionated pooled samples). Mitochondrial function was investigated by respirometry and also confirmed in human samples.

Results: Lipidomics revealed lipid profiles (~280 species/50 µg mitochondria) distinctly different between tissues, reflected especially in the acyl chain composition of phospholipids including the mitochondrial signature lipid cardiolipin (28 species) and phosphatidylethanolamine, key regulators of OXPHOS. Despite this tissue-specificity, their response to HED was comparable on lipid level. However, functional analysis revealed that insulin resistant muscle oxidized more of the complex 1 substrate pyruvate whereas liver respired more of the complex 2-associated substrate succinate, indicating a routing of pyruvate towards carboxylation. Both tissues showed a higher fat oxidation capacity in the IR state. DIA identified ~1000 proteins in liver and ~600 in muscle mitochondria (Ø92% protein abundance with mitochondrial evidence), showing a slight separation in liver profiles by diet.

Conclusions: We linked the molecular composition of mitochondria with tissue-specific mitochondrial (dys)function generating a novel tool for investigation of mitochondrial adaptations e.g. HED-induced insulin resistance.

Keywords: Mitochondria, Omics, Functional Analyses, Insulin Resistance, Mass Spectrometry
Exosomes as new players in dedifferentiation and cartilage remodelling in osteoarthritis

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Background: Chondrocytes in articular cartilage undergo phenotypic changes and senescence, restricting cartilage regeneration and favoring osteoarthritis (OA) progression. Chondrocytes from OA patients (OAc) show a chronic increase in the transmembrane channel protein connexin43 (Cx43), which through the exchange of factors or recruitment/release of signalling factors to the membrane regulates signal transduction. Extracellular vesicles (EVs), including exosomes, have been show to play important roles in many biological functions and to promote disease progression in OA. However the role of these vesicles and exosomal-Cx43 in dedifferentiation and senescence is not known.

Methods: In this study exosomes were isolated from chondrocytes derived from OA and healthy donors, and the protein content was analysed by LC-MS/MS using a 6600 triple TOF. We have also investigated the components of exosomes released by chondrocytes treated with an inflammatory mediator which upregulates Cx43 levels.

Results: Our results indicated that exosomes from OAc had higher Cx43 levels than healthy chondrocytes, and those levels were increased after oligomycin treatment. We have found 141 proteins exclusively presented in exosomes from OAc compared to healthy donors. Among them, proteins related with extracellular matrix remodelling and dedifferentiation processes such as TGF-ß regulators. When OAc were treated with an inflammatory mediator, we identified another set of 202 proteins exclusive of EVs derived from OAc, including Cx43, and several Cx43-interactors, dedifferentiation markers such as CD166, and various matrix-degrading enzymes such as MMP-1 and MMP-3. Interestingly, several factors of the complement system and innate immunity were identified in the EVs containing Cx43.

Conclusions: These results indicate that exosomal Cx43 may be involved in dedifferentiation of osteoarthritic chondrocytes and ECM remodelling by facilitating the cargo delivering of these factors to the nearby cells and at distant sites. The results suggest that further understanding of the role of exosomal Cx43 in osteoarthritis will help to halt disease spread and progression.

Keywords: osteoarthritis, connexin43, exosomes, dedifferentiation
An optimized protocol for the analysis of laser microdissected muscle tissue samples for mass spectrometry

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Background: Muscle tissue is comprised of diverse cell types, resulting in a heterogeneous sample. Utilization of laser microdissection (LMD) combined with mass spectrometry (MS) enables the analysis of defined tissue areas. Therefore, precise and optimal processing of the generated samples is of utmost importance. Here we present an optimized workflow for the analysis of muscle tissue via LMD-MS.

Methods: LMD samples from mouse muscles were used to establish an optimized workflow for label-free quantitative proteomic analysis including: 1) sample lysis with formic acid or rapigest; 2) tryptic digestion using standard in-solution method or via Single-Pot Solid-Phase-enhanced Sample Preparation (SP3); 3) peptide separation applying different high performance liquid chromatography (HPLC) conditions.

Moreover, data dependent (DDA) and data independent acquisition (DIA) were used for MS-based analysis. DDA, relies on the fragmentation of the n most intensive precursor ions in the sample. This method limits the identification and quantification of low abundant or coeluting peptides. DIA overcomes this limitation by fragmenting every precursor in a defined m/z range.

Results: For LMD generated samples lysis with formic acid was the most simple and effective method identifying more than 4500 peptides. Digestion with SP3 showed no beneficial effect regarding protein identification after MS measurements. Gradients varying from 120 min up to 180 min were tested and percentage of organic solvent was adjusted and optimized, leading to the conclusion that a 120 min gradient sufficiently separates the sample resulting in optimal elution profile for DIA. For DIA measurements different spectral libraries were designed and shall be compared regarding peptide identification, quantification and accuracy.

Conclusions: We established a fast, simple and cost effective sample processing workflow for the label free quantitative analysis of laser microdissected samples using DIA.

Keywords: Mass spectrometry, Laser microdissection, Muscle, Data dependent acquisition, Data independent acquisition
Background: Rheumatoid arthritis (RA) is a long-lasting inflammatory autoimmune disorder that ultimately leads to the destruction of joint architecture. The activity of this disease is measured by the assessment of clinical symptoms. The aim of this study was to apply a proteomic strategy to find plasma biomarkers able to discriminate patients with different RA activities.

Methods: 80 plasma samples from the IMID (Immune-Mediated Inflammatory Diseases) Consortium, classified according to the DAS28 score into low (40) and high (40) activity were randomly selected to be analyzed by shotgun mass spectrometry (MS). Four independent pools of each condition were firstly albumin-depleted, digested and differentially labelled with iTRAQ 8-plex reagents. Subsequently, the 8 labelled pools were combined, cleaned using StageTips-C18, fractionated by HPLC and analyzed by nanoLC-MS/MS using three different MS equipments, leading to the identification of 186 proteins. In this screening stage, the abundance of 11 proteins was found to be significantly different between patients with extreme RA activities. This panel of proteins was verified conducting a Multiple Reaction Monitoring (MRM) strategy with synthetic heavy-labelled peptides on a QTRAP 5500 on independent samples.

Results: The results obtained from the verification phase showed a significant increase (p<0.05) in four proteins, discriminating 80 patients with different RA activities, in accordance with that observed in the screening. Moreover, these proteins are related with the RA process and effects (inflammation and immune disorder in joints), giving significance to the results obtained. This protein panel is being validated in a larger cohort of samples of similar characteristics (including healthy and disease controls) to qualify them for clinical applications.

Conclusions: A panel of four RA-related plasma proteins has been verified by MRM, discriminating patients with extreme RA activities. Further validation experiments in a larger cohort are being conducted to assess its usefulness in clinics for the measurement of the disease activity at the molecular level.

Keywords: Rheumatoid Arthritis, Disease Activity, Targeted Proteomics, MRM, Plasma.
Unravelling a knee Osteoarthritis-associated Autoantibody profile for early diagnosis: Data from the Osteoarthritis Initiative

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Background: During Osteoarthritis (OA), changes involving the joint could lead to the production of immunoglobulins against self-proteins (Autoantibodies, AAbs) through a humoral response, even at asymptomatic stages. Therefore, the discovery of an AAb profile might improve the diagnosis of OA, allowing the identification of patients with an increased risk for the disorder.

Methods: We used a Nucleic Acid-Programmable Protein Array (NAPPA) platform to screen 2200 human proteins in sera from two different subcohorts of the Osteoarthritis Initiative: Non-exposed subcohort and Incidence subcohort. Patients in the non-exposed group did not have symptomatic knee OA or any risk factor at the beginning of the study. Participants in the Incidence group had not developed OA at baseline, but all of them developed the disease after 72 months of follow up. We analysed 100 sera at baseline from each subcohort, and also sera from the incidence group at 72 months (to evaluate if AAbs showing different reactivities were specific of asymptomatic OA). Quantitative data were normalized following the Biodesing Institute criteria. Proteins showing reactivities over a cutoff ≥1.1 were analysed by Wilcoxon test and the Partial Area Under the Curve (pAUC) at 95% specificity was analyzed with a p value <0.05.

Results: AAbs against six proteins showed different reactivities between incidence and non-exposed groups at baseline. This panel is composed by Ankyrin repeat and SOCS box protein 7 (P_{AUC} = 0.049), UDP-glucuronosyltransferase 1-7 (P_{AUC}=0.048), Diphosphomevalonate decarboxylase (P_{AUC} = 0.042), Ras-related C3 botulinum toxin substrate (P_{AUC} = 0.049), Vacuolar protein sorting-associated protein 4B (P_{AUC} = 0.047) and Methionine adenosyltransferase 2 subunit beta (P_{AUC} = 0.049), regulatory subunit of the enzyme that catalyzes the formation of S-adenosylmethionine (dietary supplement for OA treatment).

Conclusions: A differential AAbs profile was found in serum samples from OA patients and healthy controls, which could be useful to facilitate early diagnosis.

Keywords: High-throughput, proteomics, immune system.
Integrating Serum Proteomics, Metabolomics and Lipidomics to Study the Effect of Sport Activity

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**Background:** It is well known that physical activities improve overall health and counteract metabolic pathologies. In the last years many studies have investigated the effects of stressful physical activities but the integrated study of serum proteomic, metabolomic and lipidomic profiles is still lacking.

**Methods:** Untargeted shotgun proteomics of serum proteins was performed after the immunodepletion of the most abundant serum proteins. The samples were analysed using a microLC system coupled to an high resolution mass spectrometer (Sciex 5600+). Serum metabolites and lipids were quantified using a derivatization followed by gas chromatography mass spectrometry analysis through a GC-TOF system (LECO). The results were analysed with monovariate statistics and multivariate algorithms in order to integrate the multi-omics data.

**Results:** The purpose of this study was to compare pre and post exercise blood samples in order to provide both qualitative and quantitative information, to understand the effect of sport activity on human health and to integrate multi-omics results. The multi-omics approach allowed the identification of several modulated proteins, metabolites and lipids, but also the enriched pathways after the sport activity. In particular, detoxification pathway as well as immune response, lipid transport, and coagulation were affected by physical activity. The multivariate analysis and bioinformatics allowed the identification of the correlated proteins, metabolites and lipids, shedding new light on serum molecules dynamic.

**Conclusions:** Many researches suggest physical activity as a natural antidote against aging drawbacks and metabolic diseases. Here, using a multi-omics approach, for the first time, we showed the specific behaviour of serum proteins, metabolites and lipids during sport activity.

**Keywords:** Multi-omics; Serum Proteomics; Human Health; Data integration; Metabolomics
Proteomics of Obstructive Sleep Apnea: new insights in patients with diabetes mellitus

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Background: We previously showed that Obstructive sleep apnea (OSA), a common public health concern causing deleterious cardiometabolic dysfunction, induces alterations in red blood cell (RBC) proteome. Alterations in the redox/oligomeric states of PRDX2 correlated with severity and metabolic status of OSA and six month of positive-airway-pressure (PAP) treatment increased PRDX2 redox/oligomeric state associated with chaperone protective function (Feliciano et al 2017). Herein, we aimed to investigate this protein and its relationship with PAP response in OSA patients with diabetes mellitus to better understand the basic mechanisms associated with OSA and OSA outcomes.

Methods: RBC samples from control snorers (n=22 being 3 diabetics) and OSA patients before and after six month of PAP-treatment (n=29 being 8 diabetics) were analysed by non-reducing western blot using antibody against PRDX2 or PRDXSO2 to measure the total and overoxidized levels of monomeric/dimeric/multimeric forms of PRDX2. Groups were statistically compared and correlated with clinical/biochemical data and significance set up at 5% (p value < 0.05).

Results: Monomeric forms of PRDX2 were higher overoxidized in OSA non-diabetic RBCs that decreased after PAP treatment followed by an increase of multimeric-overoxidized forms associated with chaperone protective function. In OSA diabetic RBCs, the level of PRDX2 monomers although higher abundant its overoxidation level was much lower and did not significant change after treatment. Moreover, the level of PAP-induced PRDX2-overoxidized-multimers was also lower in these patients. In diabetic patients, the steady-state level of PRDX2 monomers and dissulfidic-dimers associated with peroxidatic function positively correlated with OSA severity and glycemic status, respectively.

Conclusions: The redox/oligomeric state of RBC PRDX2 were differentially modulated in OSA diabetic patients compared to OSA without this comorbidity. PAP-induced PRDX2 chaperone protective function showed decreased in OSA patients with diabetes. The clinical impact of these findings needs further investigation.

Keywords: Diabetes, OSA, positive-airway-pressure (PAP), PRDX2
Differential proteomic analysis of endometrial fluid reflects increased inflammation and extracellular matrix remodeling in non-implantative IVF cycles

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Background:

Endometrium plays an important role during implantation. A number of proteins have been described in endometrial fluid aspirate (EFA). However their role in human embryo implantation is not well known. Their knowledge could allow the improvement embryo transfer strategies.

Methods:

In this work we have compared the EFA protein patterns of implantative and non- implantative cycles in 38 women undergoing in-vitro fertilization (IVF). A sample of EFA was obtained immediately before the embryo transfer. Filter-aided sample preparation (FASP) was used for the in-solution tryptic digestion of the samples, followed by label-free analysis. In order to unravel the molecular features of receptivity, the list of differential proteins was thoroughly analyzed using bioinformatic tools such as GSEA, IPA and ANN analysis, among others.

Results:

A total of 716 proteins were considered in the differential analysis, of which 212 were significantly different. Bioinformatic analyses pinpointed the deregulation of important processes governing receptivity, such as extracellular matrix remodeling, proteolytic activity or inflammatory-signaling within the set of differential proteins.

Conclusions:

This work provides insights into the molecular features of non-implantative IVF cycles using non-invasive methods. It reveals that the EFA may mirror an increased inflammatory state of non-implantative endometrium. Additionally, it contributes with a list of deregulated proteins in the context of this alteration, whose potential as putative biomarkers should be further addressed. Their knowledge opens a possibility of improving embryo transfer strategies and improving pregnancy rates.

Keywords:

IVF, Endometrial fluid, Label free, Inflammation
Background: Tobacco smoking kills 7.2 million people each year worldwide and near 900,000 are deaths resulting from exposure to Second-Hand Smoke (SHS). SHS exposure can lead to cancer, respiratory disease, cardiovascular disorders and diabetes mellitus type II (DM2) through pathological and molecular mechanisms not yet understood. In Portugal, the partial tobacco smoking ban legislation in public venues allows smoking in restaurants with designed rooms larger than 100m$^2$. After our previously evidence of both SHS air contamination in non-smoking areas and tobacco smoke inhalation by non-smokers workers, we aimed to investigate the SHS effects on airway proteome in exposed workers to uncover predictive biomarkers for potential health risks related to occupationally SHS exposure.

Methods: Nasal epithelia was collected from hospitality workers (non-smokers=40; smokers=12), long-term exposed and non-exposed to SHS at the workplace. Samples were analysed by shotgun proteomics using Orbitrap MS. The generated MS raw data was submitted to ‘PatternLab for Proteomics’ and the differentially expressed proteins analysed by ‘Database for Annotation, Visualization and Integrated Discovery” (DAVID) platform.

Results: “Immune-System” and “Metabolism-of-proteins” were the most enriched pathways identified as associated with SHS-induced proteome alterations in airways from smokers exposed compared with non-exposed ones. “Tobacco-use-disorder” was one of the top-5 diseases enriched by the SHS-induced modulated proteins identified either in non-smokers or smokers exposed. The identified top-1 enriched diseases was ‘DM2’ in non-smokers exposed and ‘cardiovascular disorders’ in smokers exposed.

Conclusions: Our findings strongly support that in non-smokers, prolonged exposure to SHS can lead to airway proteome modulation associated with tobacco smoking-related pathological mechanisms. When validated, the uncovered proteins can be promising candidate biomarkers for clinical assessment of SHS exposure and prevention of tobacco smoke-induced diseases.

Keywords: Second-Hand Smoke (SHS), Nasal epithelia, Proteomics Biomarkers
SEX BIAS ON THE SALIVARY PROTEIN BAND DISTRIBUTION AFTER A MAXIMUM AEROBIC EXERCISE TEST

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Background: A study on gender differences in the normal range of exercise biomarkers in saliva samples had the scope for further attention. In the present study, the salivary protein band distribution of healthy men and women after a maximum aerobic exercise test were compared.

Methods: A exercise test consisting on a continuous maximal indirect multistage run test was performed in 16 men and 14 women to study the gender effect on the levels of salivary exercise biomarkers. Saliva samples were collected at different time points (prior to test (T-1), at the end of the test (T0), 15 min after (T15) and 30 min after (T30)). Biomarkers of inflammation, muscle damage and antioxidant activity were quantified in saliva samples. Moreover, the overall salivary protein distribution was compared in the different time points to search for any possible specific protein band modifications that could be related to gender.

Results: Statistically significant differences were observed for measurements between men and women in antioxidant activity. Although the total salivary protein content was not different between the sexes, the salivary protein distribution analysis showed specific gender differences in 1 band. Moreover, 5 protein bands appeared differentially regulated according to gender 15 min after of the exercise test (T15) and 2 proteins 30 min after the exercise test (T30).

Conclusions: The results of this study reveal a possible gender influence on the discovery of salivary exercise biomarkers. As differences have been reported in the salivary protein distribution in women compared to men, the normal-range values of the studied biomarkers in saliva should be explored and defined according to gender prior to its application in the exercise sciences.

Keywords: saliva, SDS-PAGE, exercise, gender.
Title: Proteomic analysis of cerebrospinal fluid of patients with neuropsychiatric lupus: looking for biomarkers

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BACKGROUND: Neuropsychiatric systemic lupus erythematosus (NPSLE) refers to the neurological and psychiatric manifestations of systemic lupus erythematosus (SLE). In clinical practice, it is often difficult to reach an accurate diagnosis, as the disease presents a variable spectrum in those patients, and the diagnostic tests are not specific enough. Therefore, the aim
of this study was to search for proteomic biomarkers in cerebrospinal fluid that could be proposed as possible diagnostic tools for this disease.

**METHODS:** Cerebrospinal fluid samples of forty-six Mexican and Colombian patients were recruited. The proteomic profiles of 19 patients with NPSLE, sub-classified by neuropsychiatric manifestation (American College of Rheumatology in 1999), 12 patients with SLE and no neuropsychiatric manifestation, 6 patients with neuropsychiatric symptoms but no SLE, 5 with other autoimmune disorders without neuropsychiatric manifestations, and 4 healthy controls, were obtained by two-dimensional gel electrophoresis and compared using ImageMaster Platinum 7.0 software. A defined protein differential expression was considered when it showed a fold change above 1.5 or when it presented with $p < 0.05$ (Graphpad Prism Version 6.0). These proteins were identified by mass spectrometry (MALDI-TOF/TOF) with scores greater than 590.

**RESULTS:** Three spots with differential expression were observed comparing patients with NPSLE against the other study groups. The spots 16, 160 and 161 have a molecular weight below 25 kDa and an isoelectric point ranging from 5 to 6.5. Particularly, the spot 160 was mainly expressed in an acute confusional state (ACS), while spot 161 was in psychosis lupus-related (Psy).

**CONCLUSIONS:** The three proteins with increased expression in NPSLE patients allow us to consider the idea of potential protein biomarkers for the diagnosis of this disease. Interestingly, spots 160 and 161 could be considered specific markers for the diagnosis of ACS and Psy, respectively.

**KEYWORDS:** Biomarkers; Cerebrospinal fluid; Neuropsychiatric systemic lupus erythematosus; Proteomics; Mass Spectrometry.
QUALITATIVE AND QUANTITATIVE PROTEOMICS ANALYSIS IN PLACENTA FOR PREECLAMPSIA-ASSOCIATED BIOMARKER DISCOVERY

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Background: Preeclampsia is a multisystem disease that affects 5-7% of human pregnancies, and that is defined mainly by gestational hypertension and proteinuria. Although preeclampsia aetiology remains unknown, numerous studies have pointed out the key role of the placenta. Abnormal trophoblast invasion into the maternal decidua leads to an incomplete remodelling of maternal spiral arteries, causing placental hypoperfusion, oxidative stress and endothelial dysfunction. Despite the extensive mass spectrometry-based proteomics research on preeclampsia, no unequivocal protein biomarkers have been described for clinical diagnosis.

Methods: In this study, proteome characterization of placenta tissue by previous fractionation and subsequent analysis of each of the 10 fractions by tandem mass spectrometry (2D-LC-MS/MS) was performed. In addition, iTRAQ labelling-based proteomic analysis of 8 placental tissues (4 from patients with preeclampsia and 4 from normal pregnant women) by 2D-LC-MS/MS was also carried out. Finally, differential phosphoproteomics analysis by label-free quantification was also developed, using 4 biological replicates per preeclamptic and normal condition.

Results: Qualitative proteomics analysis resulted in 3059 proteins identified for FDR<1% at peptide level. Among these, 426 are expressed in placenta according to Gene Ontology annotations. iTRAQ labelling experiment revealed 13 differentially regulated proteins for q-value<10%, such as alpha, beta and gamma fibrinogen, haptoglobin and pappalysin-2, reported in bibliography as involved in preeclampsia.

Conclusions: The different qualitative and quantitative proteomics approaches performed in this study allows in-depth placenta proteome characterization and the achievement of a set of potential candidate protein biomarkers associated to preeclampsia.

Keywords: iTRAQ labelling, Label-free quantification, Phosphoproteomics, Preeclampsia, Placenta
IDENTIFICATION OF A CIRCULATING PROTEIN SIGNATURE THAT CORRELATES WITH SYNOVIAL PATHOTYPES IN RHEUMATOID ARTHRITIS PATIENTS

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Background: Rheumatoid arthritis (RA) is characterized by high clinical variability and underlying cellular and molecular heterogeneity. Efforts to find useful tools for the classification of the different disease phenotypes are essential to develop tailored therapies. According to this, specific pathological phenotypes of synovial tissue (pathotypes) have emerged as associated with diverse clinical evolution and response to therapy. The objective of this work was to identify signatures of circulating proteins associated with these pathotypes.

Methods: The study was performed using 54 serum samples from the Pathobiology of Early Arthritis Cohort, classified into three groups: lymphoid (L), myeloid (M) or fibroid (F), according to the phenotype of the synovial tissue. Sera were analysed by reversed-phase nLC-MS/MS using a SWATH strategy in a tripleTOF MS (Sciex). Data were processed using ProteinPilot 5.0.1 and PeakView 2.1 (Sciex). A two-stage support vector machine (TSSVM) with RBF kernel and 10 cross-fold validation for each meta-model was applied using the Classyfire, e1071 and caret R packages.

Results: A screening analysis was performed on the data obtained from a first group of 30 samples (Train set: 10 L, 10 M and 10 F). Data were pre-processed by PCA for dimension reduction. Application of machine learning tools led to the identification of a panel of 11 proteins whose abundance is associated with a specific phenotype of the RA synovium. A very high accuracy (0.9667) and Kappa (0.95) were achieved with this classification tool. Results were confirmed on an independent validation set of 12 L, 8 M and 4 F, with also good performance (accuracy 0.875 and kappa 0.7907). This signature of 11 proteins allowed the correct classification of the samples with high sensitivity and specificity (0.85-1/0.91-1).

Conclusions: A 11-protein signature is associated with specific synovial pathotypes in RA patients. Its correlation with the clinical evolution and response to therapy of the patients remains to be elucidated.

Key words: rheumatoid arthritis, synovium, pathotypes.
COMBINING CLINICAL VARIABLES AND OMICS DATA FOR OSTEOARTHRITIS PATIENT STRATIFICATION


ABSTRACT (300 words)

Background: The prediction of drug response based on the analysis of multiple clinical variables and omics data is mandatory for accomplishing the promise of precision medicine in rheumatology.

Objectives: In the present study we explored potential protein biomarkers useful to predict the therapeutic response of osteoarthritis (OA) patients treated with pharmaceutical grade Chondroitin sulfate/Glucosamine hydrochloride (CS+GH; Droglican, Bioiberica) or the selective cyclooxygenase-2 (COX-2) inhibitors Celecoxib, in order to optimize therapeutic outcomes in OA.

Methods: A shotgun proteomic analysis by iTRAQ and LC-MS/MS was performed on sera from 80 patients enrolled in the Multicentre Osteoarthritis interVEntion trial with Sysadoa (MOVES). A panel of 10 serum proteins potentially useful to predict OA patient's response was clinically validated using commercially available ELISA Kits in the whole MOVES cohort at baseline (n=506). Logistic regression models as well as receiver-operating-characteristics (ROC) curves were used to analyze the contribution of the measured proteins to our prediction models of symptomatic drug response in OA.

Results: In the discovery phase of the study, two different panels of putative predictive biomarkers were identified by shotgun proteomics. In the verification phase, the panel of 6 proteins specific for Droglican treatment (APOA2, APOA4, APOH, ITIH1, C4BPa and ORM2), and the panel of 4 proteins specific for Celecoxib treatment (α2HS, SHBG, CD5L and TSP1) were verified in a larger cohort of OA patients. In the qualification phase, the sensitivity and specificity of a panel of 4 validated proteins (ORM2, APOA2, ITIH1, and TSP1) were blinded tested in the whole MOVES cohort.

Conclusions: Combining clinical and analytical parameters, we qualified 2 panels of biomarkers that could efficiently predict OA patients' response to CS+GH with an accuracy of 82.1% or to Celecoxib with an accuracy of 71.5%.

Keywords: predictive biomarkers, Chondroitin sulfate/Glucosamine hydrochloride, COX-2 inhibitors, proteomics, osteoarthritis.
Semi-quantitative analysis of protein abundance in exosomes from peritoneal exudates in an experimental lupus model

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Background: Intraperitoneal injection of pristane to WT mice provokes a strong inflammatory reaction with a massive influx of pro-inflammatory Ly6C hi monocytes and neutrophils to the peritoneum (experimental lupus model). The inflammation is milder in CD38 deficient (Cd38−/−) mice. The aim of this work has been to isolate exosomes from peritoneal exudates (PE-EXO) of mice treated with pristane and to identify/quantify the proteins by mass spectrometry.

Methods: PE-EXOs were isolated from pristane-treated mice by qEV size exclusion column methodology. Protein extracts were analyzed by LC-MS/MS and/or MALDI-TOF-MS/MS. Protein identification was performed with ProteinScape 4.0 (Bruker) and MASCOT data searching using Swiss-Prot database. For relative quantification the emPAI-based method was used. In addition to emPAI and molar fraction percentages, the fold change in the abundance of proteins identified was also calculated by dividing the molar percentage value for an individual protein in pristane-treated Cd38−/− mice with the cognate value in WT mice. The log2 value of the Cd38−/−/WT ratio was calculated and finally the absolute fold change calculated as 2^log2 value.

Results: Two weeks after pristane treatment 76 proteins were modulated by 1.3-fold or more in PE-EXO from Cd38−/− mice versus WT mice, representing 73% of the proteins that were common in PE-EXO from both types of mice: 26 proteins were significantly up-regulated, whereas 39 proteins were significantly down-regulated. Some of the down-modulated proteins in the Cd38−/− PE-EXOs, such as S100A9, may contribute to the migration of Ly6C hi monocytes and neutrophils to the inflamed peritoneum. Conclusions: emPAI is a reliable method for the semi-quantitative analysis of changes in protein abundance in exosomes from mice subjected to inflammatory/autoimmune challenges. These changes in protein abundance reflect the extent of the inflammatory reaction and may contribute to a better diagnosis/prognosis of autoimmune diseases such as lupus. Keywords: exosomes, inflammation, emPAI, relative quantification, expression proteomics.
Targeted proteomics to detect endogenous peptides differentially released from human knee and hip cartilages

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Background: LC-MS/MS targeted proteomics has been used to identify and quantify candidate molecular biomarkers in a diverse range of samples. The aim of this work was to verify by an MRM-based targeted method the differential release of a panel of endogenous peptides from human healthy (N) and osteoarthritic (OA) hip and knee cartilages, which have a potential biomarker use.

Methods: Proteins secreted from human articular cartilage (secretomes) were obtained by tissue explants culture. Secretomes were obtained separately from the wounded (WZ) and unwounded (UZ) zones of OA cartilage. Peptides were separated by nano-LC, and relative quantitation was performed by Multiple Reaction Monitoring (MRM) in a 5500 QTRAP using stable isotope-labeled (SIS) peptides as an internal control. Data analysis was performed using Skyline software.

Results: Secretome samples from hip (n=10) and knee (n=12) cartilage explants were analyzed, including OA patients and healthy donors (n=12, 5). Peptides belonging to OA-related proteins, as Cartilage Oligomeric Matrix Protein (COMP), Cartilage Intermediate Layer Protein (CILP), Prolargin (PRELP), Clusterin (CLUS) and Matrix Gla Protein (MGP), were differentially detected and relatively quantified in the cartilage secretomes. Three peptides were found with a significant higher release (p<0.05) in the WZ of knee cartilage compared to healthy tissue (fold change >5), two belonging to PRELP and one to CILP. Four peptides, three from CLUS and one from COMP, were significantly decreased (p<0.05) in hip OA samples from the WZ compared to healthy cartilage donors (fold change <0.28). Finally, 1 peptide from MGP was found to be increased in both OA cartilage joints compared to healthy tissue (fold change=3, p<0.05).

Conclusions: We have verified the significantly different release of a panel of eight endogenous peptides from OA articular cartilages compared to healthy tissue. The joint-specific biomarker value of these peptides should still be validated.
Searching for Biomarkers between SLE with and without renal involvement
By Proteomics

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Background: Lupus nephropathy (NL) is an important cause of morbidity and mortality in patients with Systemic Lupus Erythematosus (SLE). Considering that it is a specialized technique and not risk free, a proteomics study is proposed to determine biomarkers that help us to differentiate patients diagnosed with SLE with and without renal involvement.

Methods: We selected 12 patients with SLE and renal involvement and 14 patients with SLE without renal involvement. There were no differences between groups according to race, gender, and age. The patients were classified as moderate (<500), mild (150-500) or normal (<150) level of proteinuria in the urine. A 24-hour urine sample was obtained for analysis.

Results: The Principal Component Analysis (PCA) revealed differences between samples from patients who have high level of proteinuria in 24 hours and patients who do not have renal involvement. Interestingly, patients with mild proteinuria correlated better with patients without renal involvement than with the severe proteinuria group. A total of 292 proteins (identified with at least two peptides with a FDR<1%) were quantified and further considered in the analysis. Consistent with the nature of the sample, the Gene Ontology (GO analysis) of the whole list of identified proteins revealed the presence of extracellular (277 proteins, p = 2.25E-171) and secretion-related proteins (49 proteins, p = 1.1E-09), among others. Proteins related to defensive processes were prominent among them.

Interestingly, clear differences were detected between the three subgroups of samples. The Student’s T-test analysis reflected the differential presence of 147 proteins (p<0.01) between patients with and without renal involvement, being 17 more abundant in the urine of the patients with renal damage, whereas 130 showed the opposite pattern. The subset of proteins whose abundance increases upon renal damage is comprised of typical highly-abundant serum proteins. In addition, differences between most closely related groups (mild proteinuria and no renal affection) revealed differences that may be useful for a better stratification of patients.

Conclusions: A different protein pattern is observed between the groups of patients, so in a more detailed study we can indicate if some of these can serve as prognostic markers for this type of patients.

Keywords: Lupus nephropathy (NL), Systemic Lupus Erythematosus (SLE).
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