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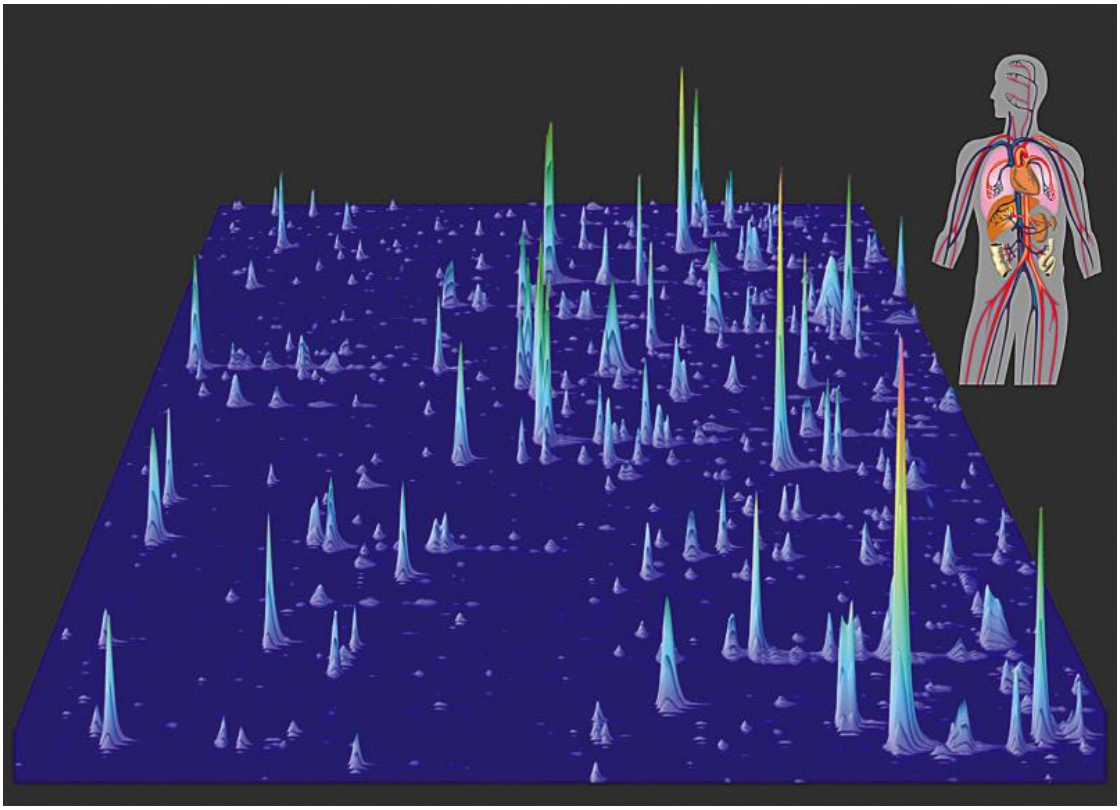
Analytical Biochemistry & Interfaculty MS Center

Annual Report 2021

Prof. Dr. Peter Horvatovich
Prof. Dr. Rainer Bischoff
Dr. Hjalmar Permentier

January 24, 2022



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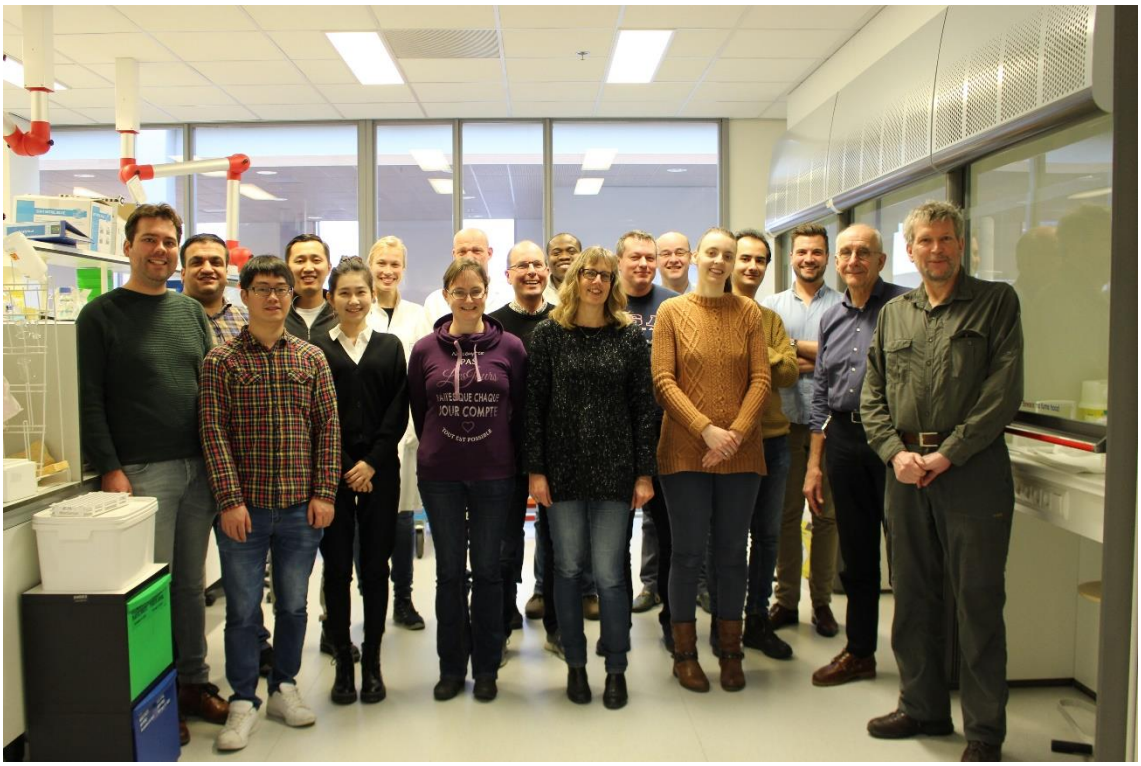

Figure 1a. Group photo from 2019.

From left to right and from back to front:
Bas Sleumer, Walid Maho, Xiaobo Tian, Baubek Spanov, Ydwine van der Veen, Karin Wolters, Marcel de Vries, Nico van de Merbel, Oladapo Olaleye, Jolanda Meindertsma, Peter Horvatovich, Hjalmar Permentier, Janine Stam, Ali Alipour, Rik Beernink, Dirk-Jan Reijngoud, Rainer Bischoff.

Figure 1b. Group photo from September 2021.

From left to right and from back to front:
Amika van Willenswaard, Marjolein Korenhof, Karin Wolters, Jolanda Meindertsma, Baubek Spanov, Xiaobo Tian, Janine Stam, Marcel de Vries, Yanick Paco Hagemeijer, Bas Sleumer, Oladapo Olaleye, Jos Hermans, Mats Nitert, Peter Horvatovich, Hjalmar Permentier, Ali Alipour

Not on the photos in Figure 1a and 1b:
Alejandro Sánchez Brotons, Julia Aresti Sanz, Natalia Govorukhina, Thomas Cremers, Sara Russo, Jennifer Le.

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1 Members of the Research Groups

Staff

Prof. Dr. Peter Horvatovich (Full Professor)
Prof. Dr. Rainer Bischoff (Full Professor)
Prof. Dr. Nico van de Merbel (by special appointment, ICON)
Prof. Dr. Thomas Cremers (by special appointment, CAN Holding)
Dr. Karin Wolters (UMCG, Pediatrics)
Dr. Natalia Govorukhina
Dr. Jennifer Le
Jos Hermans
Jolanda Meindertsma (secretary; 0.4 fte)

Interfaculty Mass Spectrometry Centre (IMSC)

Dr. Hjalmar Permentier (head IMSC)
Marcel de Vries (UMCG)
Walid Maho (until September 2021)
Ydwine van der Veen (UMCG, until May 2021)

Ph.D. students

Peter Bults (ICON)
Bas Sleumer (ICON)
Xiaobo Feng (UMCG)
Yang Zhang
Xiaobo Tian
Ali Alipour
Alienke van Pijkeren (UMCG, University of Innsbruck)
Julia Aresti Sanz (shared PhD with Microbial Physiology, GBB/RUG)
Baubek Spanov
Oladapo Olaleye
Alejandro Sánchez Brotons
Saskia Sokoliova (shared PhD with the Stratingh Institute of Chemistry/RUG)
Sara Russo
Janine Stam
Rik Beernink (IQ Products)
Yanick Paco Hagemeijer

Research Students

Rob Fredericks (MPS master student)
Victoria Aboagye (MPS master student)

Guests

Prof. Dr. Dirk-Jan Reijngoud (UMCG)

2 Overview 2021

2021 was a challenging year due to the continuation of the SARSCOV-2 pandemic and several lockdowns in the winter and spring periods. However, there is hope to fight the coronavirus pandemic, due to the availability of mRNA- and adenoviral vector-based vaccines and mass vaccination of the population in the Netherlands and worldwide in spring/summer 2021. Despite the arrival of the Delta variant in the beginning of 2021 being more infectious and dangerous, vaccination allowed to avoid relatively high hospitalisation. The situation in summer allowed to ease the lockdown restrictions and to get more access to the laboratory, allowing more productive laboratory work. The end of 2021 was the hallmark of the emerging and fast spreading of the Omicron variant, which is apparently less dangerous and will hopefully be better controlled due to the vaccinations. We hope that the last year that our academic activities are affected by the pandemic will be 2022 and that we will meet our students and colleagues in onsite activities as much as before the pandemic.

Despite the challenges due to the second year of the SARSCOV-2 pandemic, the Analytical Biochemistry and Interfaculty Mass Spectrometry Center had a very successful year indicated by 35 accepted peer reviewed papers, 1 book chapter and a large number of other academic contributions. We are very proud that these scientific results summarized in section “3. Research projects” originate from highly diverse interdisciplinary projects with many industrial and scientific collaborations comprising diverse research areas such as clinical research, basic biology, bioinformatics, statistics, signal processing and computer science domains.

The other important change in the life of Analytical Biochemistry (AB) and the Interfaculty Mass Spectrometry Center (IMSC) is the retirement of Prof. Rainer Bischoff in 2022, which started with a pre-retirement period from September 2021. Rainer had a very productive and successful career with industrial and academic periods. He founded and built, upon his arrival from AstraZeneca in 2001, a highly effective interdisciplinary academic group in the field of proteomics and metabolomics, secured during the last 20 years stable funding and established collaborations with many national and international academic and industrial research groups. His scientific activities resulted in more than 300 scientific publications and book chapters, as well as several patents. However, the most precious and valuable aspect of Rainer’s personality was to create a very inspiring and open atmosphere, which is the major contributor to the academic success of the research group. I (Peter Horvatovich) have taken part in the activities of the research group since my arrival as postdoctoral researcher in 2005 as an analytical chemist. The open atmosphere and curiosity-driven research environment contributed significantly to me becoming a mature scientist, ready to take over the group. Rainer will stay with the group after his retirement and will take care of the successful graduation of his PhD students and we are sure that he will be around with his expertise and as scientific advisor supporting further the AB and IMSC groups.

In 2022, our colleague Dr. Natalia Govorukhina will also benefit from a well-merited retirement. Natalia has contributed over the last 20 years to the scientific work and output of the group with high quality scientific expertise in mass spectrometry and biomarker discovery.

The group has recently obtained substantial investments in the infrastructures, which consist of the purchase of two computational servers with a large amount of RAM, a high number of CPUs and storage, a Bravo Assay Map automated liquid handling robot from X-Omics funding, and an Exploris 480 Orbitrap Instrument with an EvoSep One liquid handling system from a UMCG investment. We have an ongoing tender to purchase a high-resolution mass spectrometer for metabolomics. Our challenge next year is to secure continuous funding to use this high-quality instrument park as efficiently as possible.

Peter Horvatovich, Rainer Bischoff and Hjalmar Permentier

3 Research Projects

3.1 Biomarkers

3.1.1 *Proteoforms of Biomarkers*

In 2021, we continued the collaboration with the Department of Laboratory Medicine of the UMCG (Martijn van Faassen and Ido Kema) and PRA Health Sciences (as of July 1st: ICON). This project aims at the quantitative determination of different proteoforms of protein biomarkers. Currently, concentrations of these biomarkers are typically determined using ligand-binding assays such as ELISAs, but there is often a lack of consistency between results obtained at different laboratories, or even within a single laboratory when different lots of critical immunochemical reagents are used. Since most, if not all, protein biomarkers occur *in vivo* as a family of closely related but structurally different isoforms that may respond quite differently in a ligand-binding assay, it is increasingly realized that the generation of a single read-out may be an oversimplification. By using mass-spectrometry based methods, we expect to obtain more knowledge about this important phenomenon. An LC-MS/MS method involving immunocapture with an anti-human growth hormone (hGH) directed antibody and measurement of a specific tryptic peptide was found to be able to unambiguously quantify the major circulating hGH isoform (hGH-1, 22-kDa) in human serum and plasma without interference from three other proteoforms. By simultaneous quantification of a tryptic peptide occurring in all four forms, a semi-quantitative readout of the total hGH concentration is also obtained. The method was validated according to the most recent clinical chemistry guidelines for validation of protein biomarkers by LC-MS and a good correlation was found with the binding assay that is currently employed in the UMCG. Further comparison with assay formats in other hospitals in the Netherlands will soon be undertaken.

In a second research project, we have started setting up a method for the sex hormone binding globulin (SHBG) in human serum. Although more information will be generated in the next months, initial results seem to indicate that a straightforward tryptic digestion followed by LC-MS/MS allows quantification of the total concentration of SHBG.

3.2 Computational Mass Spectrometry

3.2.1 *Pipelines And Systems for Threshold Avoiding Quantification*

Alejandro Sánchez Brotons has developed a comprehensive bioinformatics pipeline for processing proteomics and metabolomics LC-MS/MS data obtained with data dependent acquisition (DDA). This work is the continuation of the collaboration with Frank Suits (IBM Research Australia), which started in 2008, on LC-MS/MS data pre-processing. The Pipelines And Systems for Threshold Avoiding Quantification (PASTAQ) is following the threshold avoiding philosophy, which encompasses the application of a threshold for discriminating signal from noise at the end of the pipeline using all available data from the processed dataset, and avoid to use (or apply very low) threshold in the intermediate processing step such as peak detection and quantification. PASTAQ uses a noise from signal discriminating threshold at the end of the pipeline by including peaks in the final peptide/protein quantification table, which can be detected in a large percentage of samples present in at least one of the sample groups. This threshold application is independent from the signal strength (i.e. compound quantity). Besides, it implements many novel algorithm approaches, which contribute to accurate peak quantification in the single-stage (MS1) part of the LC-MS DDA data, such as 1) use of warped mesh in m/z providing the same sampling density of peak across the whole m/z range considering peak broadening of different mass analysers, 2) detection of peak location using 2

dimensional (2D) Gaussian kernel smoothed MS1 LC-MS DDA data, 3) quantification of peak present at locations detected in point 2) by the best fitted 2D Gaussian peak, 4) relying on highly accurate automated correlation optimization warping based WARP2D retention time alignment algorithm to align chromatograms and performing peak matching using solely m/z and retention time coordinates (Figure 2.). The PASTAQ pre-processed MS1 peaks are annotated with DDA MS/MS events, in which annotation with proteomics identification has been implemented using the mzIdentML format. Annotation of MS1 peaks allows to filter identification based on uniformity of identification of matched MS1 peaks. PASTAQ performs MS1 based quantification and annotates MS1 peaks with DDA MS/MS spectra and with the concomitant identification results. PASTAQ provides accurate quantification results for non-identified peaks and collects all information such as non-identified fragment spectra and accurate mass of MS1 peaks, which can support potential identification, e.g. by manual interpretation or by applying other tools such as *de novo* sequencing algorithms. PASTAQ implements many quality control algorithms, such as showing LC-MS profile similarity based on sum of overlapping peak volume before and after time alignment, and shows total and base peak ion chromatograms of the processed files.
PASTAQ has been benchmarked with a mixture of proteome prepared with various ratios of Hela cell line, yeast and *Escherichia Coli* protein extracts, as well as artificial stable isotope labelled QCONCAT proteins spiked-in with 4 orders of concentration level magnitude. The pipeline showed improved quantification compared to MaxQuant for low and middle abundant proteins and peptides. The core of the pipeline is a highly efficient C++ library, providing access to low level functionalities and have Python bindings that allow to combine the various steps to complete the workflow of various designs. The paper has been published in Analytical Chemistry (PMID 34355890) and the code is available under the MIT Licence on the GitHub: https://pastaq.horvatovichlab.com/.

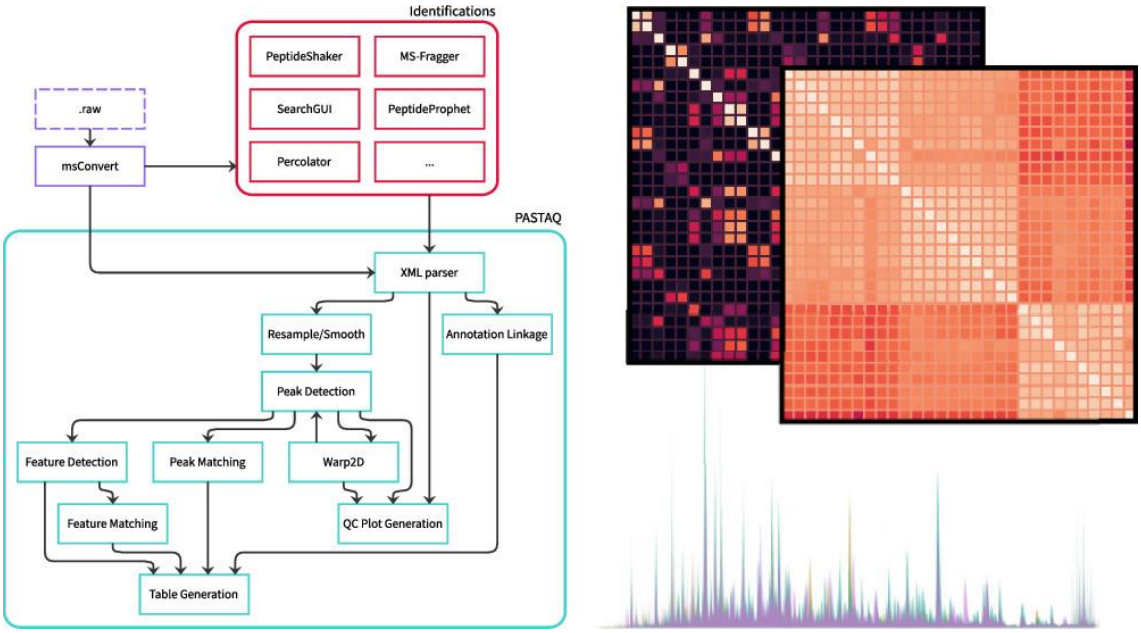

Figure 2. Scheme of the main modules of PASTAQ DDA MS1 pipeline and various options to import proteomics identification from open-source tools such as SearchGUI, MSFragger and PeptideShaker (left scheme). Right upper plot shows the pairwise similarity matrix of chromatograms using the sum of overlapping peak volumes before (background plot) and after (front plot) retention time alignment using a Warp2D algorithm and base peak chromatogram of the analysed sample after retention time alignment (bottom right plot). Figure from PMID 34355890.

3.2.2 *Dynamic thresholding for EIC reconstruction in XCMS*

The width of peaks in mass spectra are changing with different relations to m/z in data acquired with different mass analyzers. Xiaodong Feng with other colleagues have developed the theoretical framework to calculate exactly the mass range that include a peak in the m/z domain of LC-MS MS1 data and which can be used to extract an ion chromatogram suitable for accurate compound quantification (Figure 3.). This theory has been implemented in XCMS and is called "dynamic binning". The algorithm was assessed with a differential spiked human lipidomics serum sample and its performance was compared with the peak picking/quantification methods implemented in the original version of XCMS, the open source mzMine and the commercial Progenesis workflow. The dynamic binning peak picking/quantification algorithm showed better performance than compared to mzMine and the original version of XCMS and provided similar quantification performance as Progenesis. The article summarizing the results has been published open access in Analytical Chimica Acta (PMID 34172146) and the source code was made publicly available at the GitHub at https://github.com/xiaodfeng/DynamicXCMS.

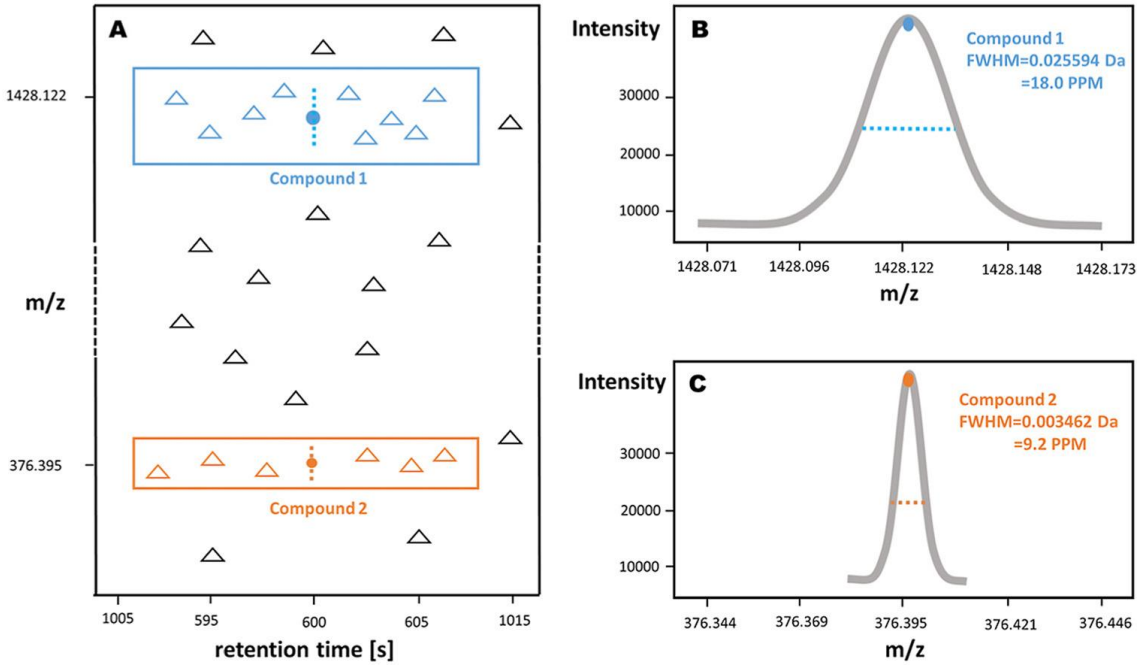

Figure 3. Piece of LC-MS map showing the isotope peak of two compounds at low and high m/z (plot A) and their corresponding peak width in data measured by the Orbitrap high resolution instrument (plots B and C). The mathematical theory to consider such peak width difference has been developed and implemented in the "dynamic binning" algorithm. Figure from PMID 34172146.

3.2.3 *Removing bias in shrunken partial correlation*

Network reconstruction applied on an omics dataset using Gaussian Graphical Models (GGM) with shrunken partial correlation is suffering from bias, due to the necessity to use a shrinkage approach. Omics data typically have much lower sample size than number of variables (i.e. measured compounds) also known as "high dimensionality problem". Victor Bernal has developed a theoretical framework showing the non-linear characteristics of the bias of partial correlation, as well as a parametric method to remove partially the bias from the learned GGM partial correlation network (Figure 4.). The efficiency of the bias removal was shown in the simulated dataset with various ratios of sample size and variables, and its effect was shown in a experimental transcriptomics dataset. This work constitutes of an important step forward, identifying one of the most key limitations of GGM-based network learning. Victor's work describes the mathematical background of bias characterisation, and proposes a method, which can

partially remove the bias in an analysed omics dataset. The work has been published open access in BMC Bioinformatics (PMID 34493207), the R code has been made public at https://github.com/V-Bernal/UnShrunk. This work has been made in close collaboration with Marco Grzegorczyk and Victor Guryev.

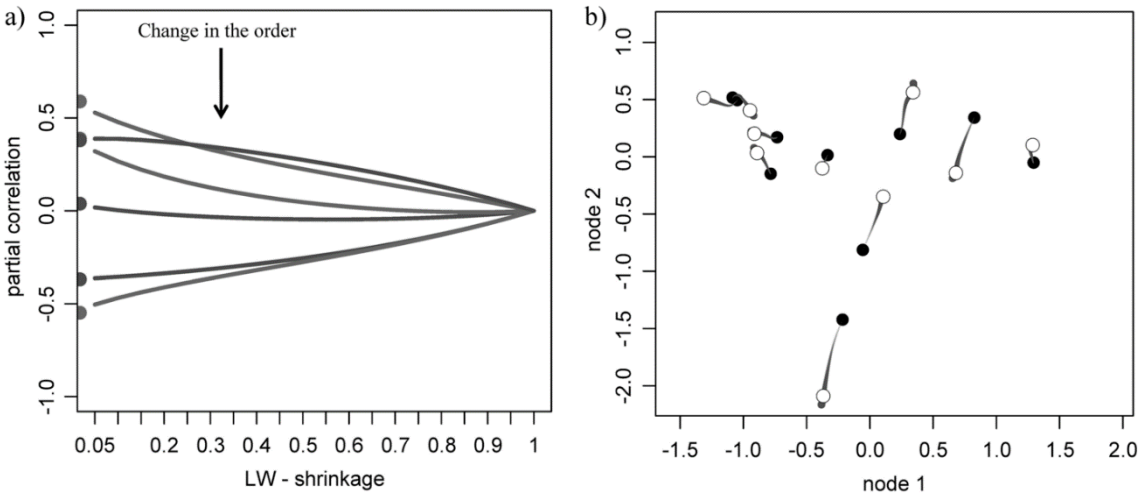

Figure 4. Non-linear bias caused by the shrinkage method used in a GGM network reconstruction of an omics dataset having much lower sample size than number of measured variables. Panel a) shows the non-linear change of partial correlation as well as the change in ordering of the strength of partial correlation using various shrinkage value. Panel b) presents a scatter plot of the original (black dots) and shrunk (white dots) data point at a shrinkage of 0.65 of two variables (nodes) as well as its path by gradually changing the shrinkage from 0 to 0.65. The data shown in this plot were made using simulation of a random network. Figure from PMID 34493207.

3.2.4 *Proteogenomics data integration*

Common proteomics identification uses a protein amino acid sequence from accurately annotated public databases such as Uniprot, Swissprot and Ensembl. These databases use so-called canonical sequences, which sequences contain a limited number of protein variants, differing between individuals (germline variants) and mutations acquired through somatic events during the life of a person. These variants play a key role in disease onset, development, and in determining which treatment will be efficient. To this end, the study of protein variants plays a crucial role in understanding molecular mechanisms of complex diseases, such as COPD and cancer, and to identify efficient personalised treatment. Performing proteomics identification of LC-MS/MS data using a protein sequence database, which includes all variants detected in a disease using genomics sequencing, would include too many variants increasing the false positive identifications. Additionally, many not yet discovered somatic and germline variants may exist, which would be missed following this identification strategy. Prediction of sample and patient specific protein variants from genomics/transcriptomics data is the optimal way to identify sample specific protein variants in proteomics LC-MS/MS data. Protein variants detection can be performed accurately from genomics and transcriptomics data obtained from the same sample from which the proteomics profile was acquired. This approach is called proteogenomics. The potential of such a proteogenomic approach has been shown in our recent paper presenting a proteogenomics study for COPD showing the identification of several novel protein variants, such as a new exon for SORBS1 gene (PMID 31937552). Yanick Paco Hagemeijer, under shared supervision with Victor Guryev and with a X-Omics funding, is developing a proteogenomics data integration pipeline using a NextFlow workflow engine with Docker/Kubernetes containers and FAIR (Findable, Accessible, Interoperable and Reusable) data and parameter management. The pipeline development is in the final stage, and we aim to publish the pipeline showing its

performance to identify sample specific protein variants in 2022. This year, Yanick has published a book chapter on proteogenomics data integration, and he contributed to the protein variant prediction of 60 melanoma samples using whole exome sequencing data collected by the Swedish Melanoma Moonshot Consortium. This work has been submitted to the bioRxiv preprint server (see Scientific output section "5.2 Articles on preprint servers" for details).

3.2.5 *Melanoma Bioinformatics in the Swedish Cancer Moonshot*

The Swedish Cancer Moonshot lead by Prof. Gyorgy Marko-Varga (University of Lund) has the aim to better understand the molecular mechanism of Melanoma and, with the earned new knowledge, find a breakthrough in Melanoma treatment. The project includes clinically well-characterised sample collections from multiple clinical centres in Sweden and Hungary, an advanced biobanking infrastructure to store blood and tissue compartments, the use of digital pathology with machine learning and artificial intelligence to identify and annotate anatomical and pathological characteristics of collected tumor tissue samples, producing deep multi-omics molecular profiles at genome, transcriptome, (phospho)proteome and metabolome levels and complex bioinformatics integration and analysis of all measured and collected data. The work is centralised at the University of Lund (Sweden) and is embedded in a large international collaboration with several laboratories in Hungary (University of Szeged and Semmelweis, Marcell Szasz and Istvan Nemeth), US (New York University, David Fenyo), and Brazil (University Federal of Rio de Janeiro, Gilberto Domont) and The Netherlands (University of Groningen, Peter Horvatovich). In this work, Peter Horvatovich is participating in the project as bioinformatic expert, which is supported by an honorary position at the University of Lund. In 2021, this research line resulted in 3 large publications presenting various aspects of Melanoma, such as discovery of prognostic and predictive biomarker to predict patient's survival using deep proteomics analysis of paraffin archived melanoma tissue (PMID 34885218) and presented the Melanoma Atlas defining molecular pathology of Melanoma (PMID 34323402) and complementing melanoma transcriptome with deep proteomics profile (Figure 5, PMID 34323403).

3.3 New chemical reagent to identify Advanced Glycosylated End-product (AGE)

3.3.1 *Detection of AGE modified proteins*

Advanced Glycosylated End-products (AGE) are formed by a non-enzymatic Maillard reaction between sugar and lysine and arginine residues of proteins. AGE modified proteins can form cross-links between proteins, as well as chemically modify the lysine and arginine residues. The AGE modified proteins may have altered the biological function and contribute to the onset and development of multiple complex disease such as diabetes type II and COPD. Saskia Sokoliova is a PhD candidate enrolled in the Molecular Life and Health programme of the Faculty of Science and Engineering. Her research project is focusing on the development of new sets of chemical reagents, which can label a potential site for AGE modifications in proteins, and allow enrichment of AGE modified proteins. Some of the developed reagents can be activated by photocleavage and permit detection of AGE modified proteins by fluorescent readout and mass spectrometry. This project is performed in collaboration with Martin D Witte (Stratingh Institute, Chemical Biology group) and resulted in a set of reagents, which are currently tested in model proteins and various human cell lines. The goal of this project is to provide a chemical tool set to study AGE modified proteins and study their effect in complex diseases such as COPD and diabetes Type II. Next year, we expect several papers from this work and the successful thesis submission of Saskia Sokoliova.

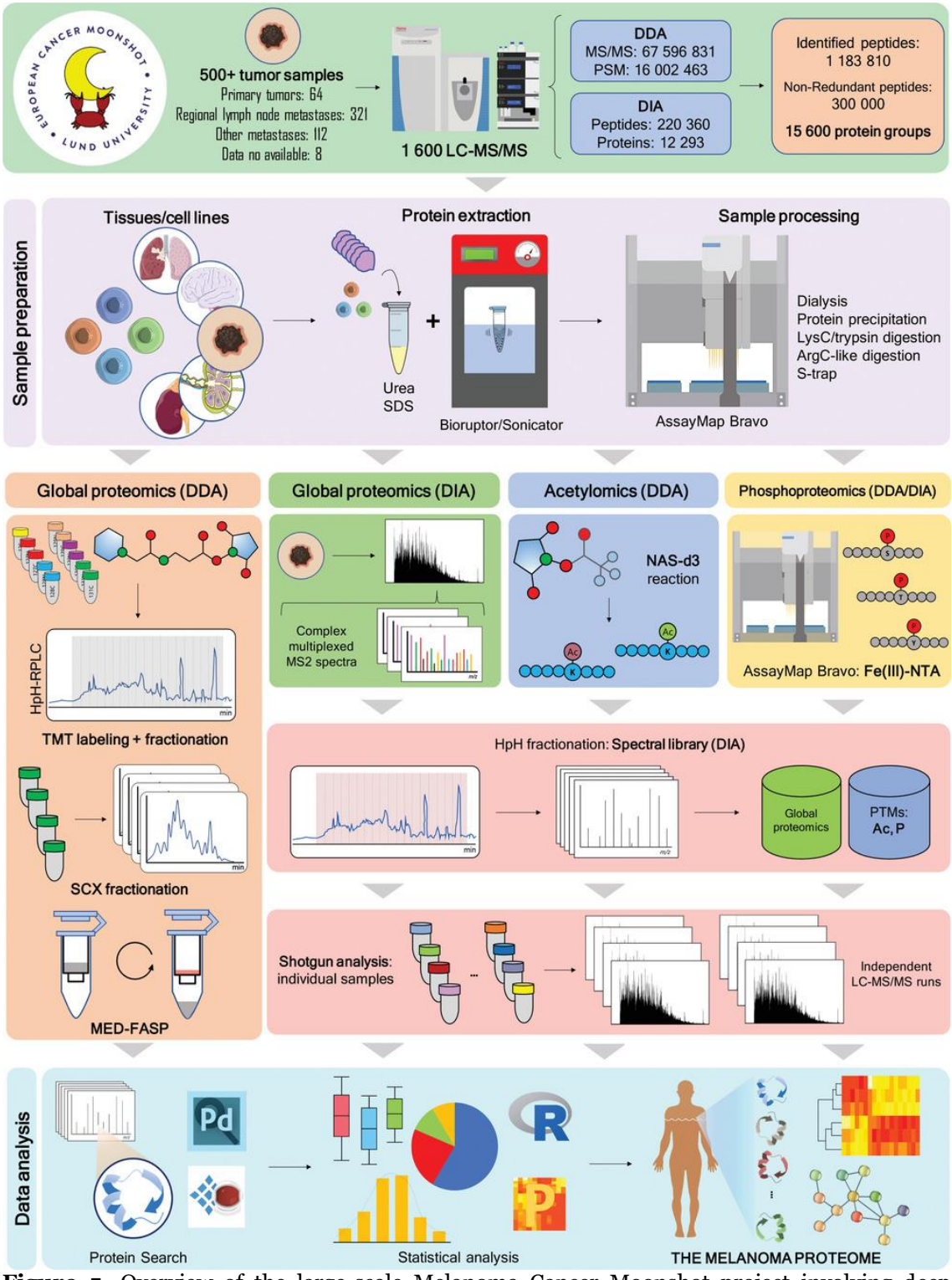

Figure 5. Overview of the large-scale Melanoma Cancer Moonshot project involving deep proteomics sequencing, other omics, digital pathology data of 500 melanoma tumor samples analysed so far in the project. Figure from PMID 34323403.

3.4 Electrochemistry-Mass Spectrometry

The different research lines of this project are run in close collaboration between the Analytical Biochemistry Group, the Interfaculty Mass Spectrometry Center (Hjalmar Permentier) and the BIOS Lab-on-a-chip Group at Twente University (Mathieu Odijk, Wouter Olthuis, Albert van den Berg). The major topics of the project are the

electrochemical conversion of drug molecules into metabolites and the electrochemically-assisted synthesis of added value pharmaceutical intermediates.

3.4.1 *Electrochemically-assisted synthesis of value-added pharmaceutical intermediates*

The synthesis of N-dealkylated metabolites and drug precursors by electrochemistry was first shown in 2020 for tropane alkaloids and was extended to opiates which are even more challenging. Ali Alipour (PhD student), in collaboration with Faizan Bhat from the group of Gerrit Poelarends (GRIP, RUG), succeeded by introducing TEMPO as a catalytic intermediate in the electrochemical reaction. This work was published in ChemElectroChem in 2021. Analogous to the tropane alkaloids, the opiates have naturally occurring precursors, including morphine and thebaine, which are used to produce semisynthetic active drug compounds such as naltrexone. The key reaction is demethylation of the tertiary amine in the structure. Direct electrochemical reaction proved to be selective, but we found that addition of TEMPO in combination with optimized reaction conditions boosted the yields of the desired N-dealkylated noropiates to 50-80%, achieving gram-scale product amounts (Figure 6.). Mechanistic studies were also carried out and suggested that the electrochemically generated oxoammonium species from TEMPO oxidizes the opiate substrate to an iminium intermediate which then hydrolyses to the noropiate.

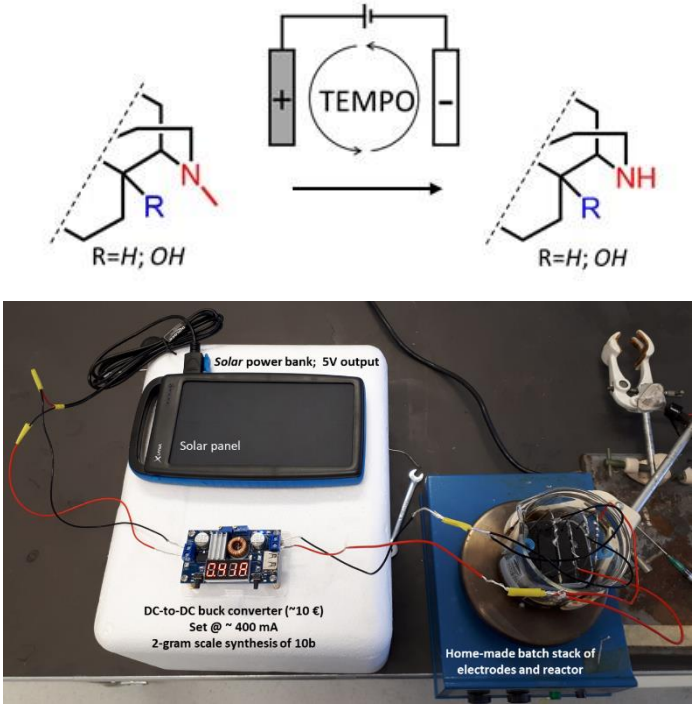

Figure 6. Top panel: Reaction scheme and conditions for the N-demethylation of opiates.
Bottom panel: Homemade system using a solar power bank connected by a DC-to-DC buck converter to an electrochemical batch cell with stacked porous graphite electrodes.

Work focussed on the N-dealkylation of drug metabolites on nanoporous gold (NPG) catalytic surfaces has finished in 2021 and the results have been submitted to peer review. This work has been done by Ali Alipour in our group in collaboration with Elchin Jafariyeh-Yazdi from the group of Jun Yue (ENTEG, RUG), Mojgan Hadian from the group of Alexander Dömling (GRIP, RUG), and Arne Wittstock from the University of Bremen (Germany). NPG, produced by Prof Wittstock by dealloying gold-silver pieces, are reactive towards organic molecules. This reaction is used for important conversions of e.g. methanol to methyl formate. We have used this compound for the first time to convert more complex drug molecules and found that the N-dealkylation reaction is prevalent under the right conditions; notably the presence of an oxidant such as air or TEMPO is required (Figure 7.). We have shown that the reaction is reproducible and

works for a variety of drugs with secondary and tertiary amine moieties, achieving yields of 30-70%.

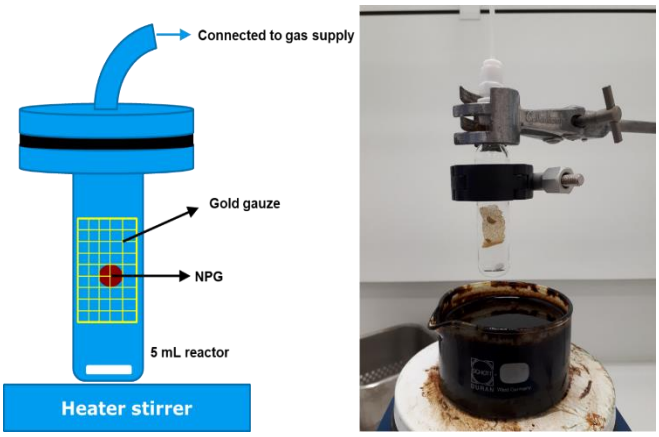

Figure 7. Left: schematic of the designed 5mL reactor including a gold gauze as NPG holder and a rubber-clamp system to make the system leak-free; Right: a photograph of the reactor in practice.

3.4.2 *N-Dealkylation of pharmaceuticals on gold particles*

This project is a 'spin-off' from the original project to synthesize drug metabolites that are due to N-dealkylation mediated by members of the Cytochrome P450 enzyme family. As reported previously, we discovered that such reactions can proceed without any electrical potential on the surface of nanoporous gold. However, obtaining reproducible results has been difficult and the physical-chemical parameters that play a role in this reaction remained partially unexplored (Figure 8.).

To shed more light onto this reaction, Jos Hermans (research technician) and Ali Alipour (PhD student) constructed a capillary column filled with gold particles that was coupled to a mass spectrometer. That way, reaction products could be analyzed 'on the fly' and different reaction conditions were tested. Jos advanced this project by setting up an intricate system that further allowed to add oxygen and various other reagents that we considered to be critical for this reaction. It is noteworthy, that dealkylated lidocaine, our test compound, occurs in good yield already in the flow-through of the gold particle column indicating that the reaction is extremely fast.

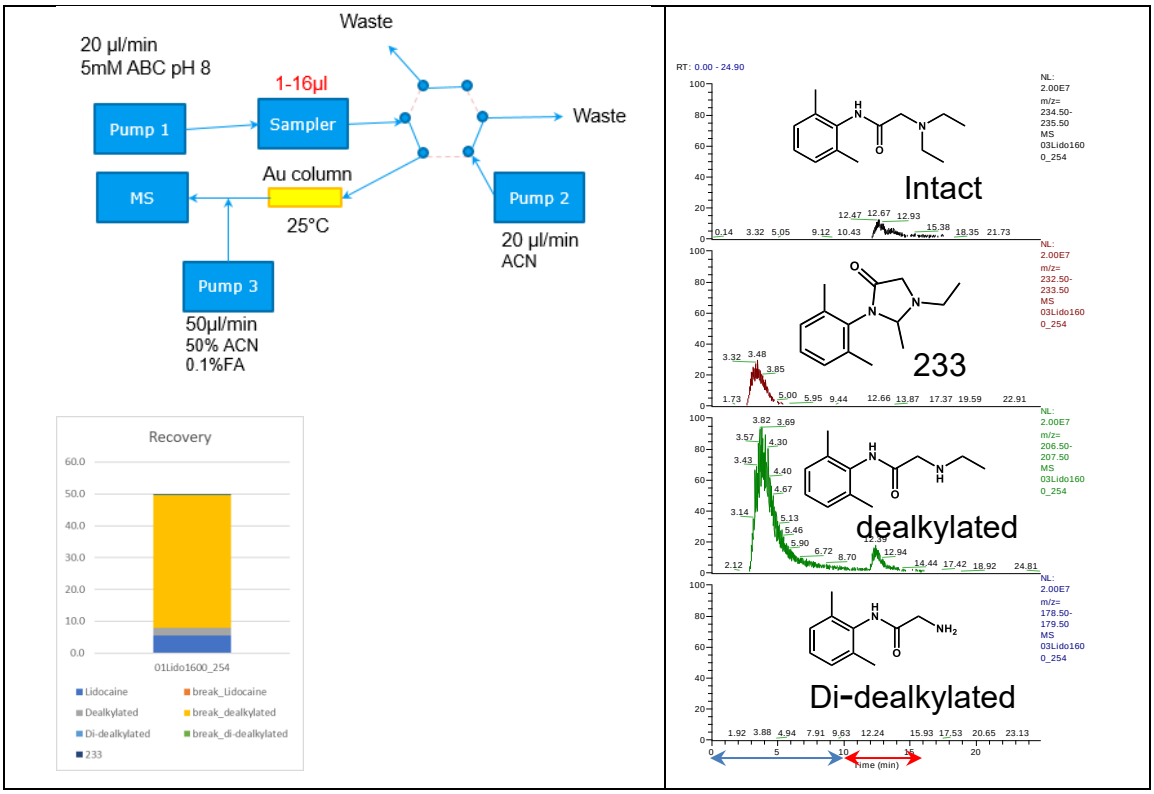

Figure 8. Basic instrumental configuration and chromatographic profiles for a 1µl, 100µM Lidocaine injection. The upper left panel shows the instrumental set-up, while the lower left panel shows a plot of the various compounds that are followed using this set-up. The right panel shows the corresponding mass spectrometric traces as extracted ion chromatograms.

3.4.3 *Electrochemical-mass spectrometric detection of neuroactive metabolites produced by gut bacteria*

Julia Aresti works as a PhD student in the Molecular Life and Health programme of the RUG on the analysis and function of neuroactive metabolites produced by gut bacteria. This MLH project is a collaboration between Analytical Biochemistry (Hjalmar Permentier) and the Microbial Physiology group (Prof. Sahar el Aidy).
The production and transformation of neuroactive compounds in the gut by microbiota can have a major effect on the host, leading to changes in neurological disease states or the effectiveness of neuroactive drugs. The first stage of the project focussed on bacterial transformation of L-DOPA. Rat fecal samples were incubated *in vitro* with L-DOPA to determine whether cecal microbiota can metabolize it. A new peak was detected in all samples by HPLC-ECD-MS/MS and ultimately identified as hydroxyphenylacetic acid.

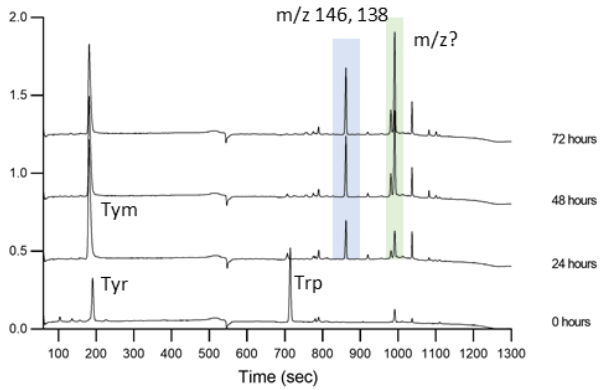

Figure 9. ADD patient fecal sample culture. Metabolite ECD profiles 0-3 days after addition of Tyr and Trp.

The modified analytical method of combining HPLC-ECD-MS helps to detect and identify gut bacterial metabolites in complex biological samples and their quantification based on both the ECD current and the MS ion signal. We have implemented the HPLC-ECD-MS method on a high-resolution Q-ToF MS to demonstrate its ability to identify additional, less abundant compounds allowing more in-depth metabolomics analyses. This proof-of-principle study is done on fecal samples of ADD patients which had been dosed with methylphenidate (MPH, ritalin). Statistical analysis of data among patients before and after medication showed a great variability of the metabolome composition between subjects, but a few compounds showed significant changes in the MS data and the ECD data separately (Figure 8.). We are currently investigating the nature and role of these compounds in the context of ADD and ritalin use. A follow-up study will focus on the ability to provide extra information on unknown metabolite functional groups by performing an on-line oxidation step using a coulometric electrochemical cell followed by MS detection of both native and oxidized metabolites.

Another study focused on the potential enzymatic conversion of MPH by gut bacteria to ritalinic acid, affecting the response to the drug in ADD patients. *In silico* analysis was used to select a range of gut bacteria with appropriate enzyme homologues for ritalin hydrolysis. Several selected bacteria appeared to be actively producing ritalinic acid, but more detailed study revealed that ritalin is in itself highly instable at pH values of 7 or higher. A clear bacterial cause for ritalin break-down in the gut could therefore not be proven, but the pH stability of ritalin deserves closer attention. Further studies into the effect of gut microbiota on other drugs are ongoing (Figure 9.).

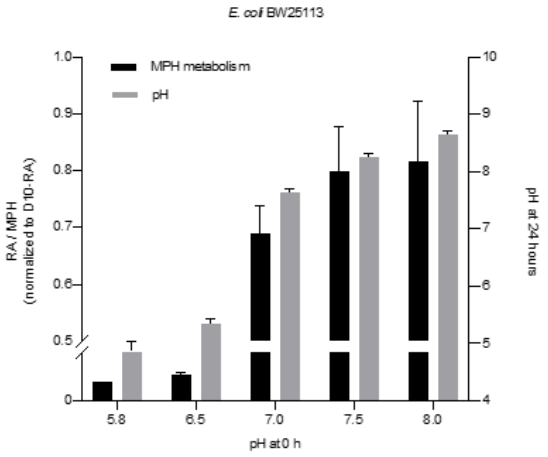

Figure 9. Apparent *E. coli* metabolism of MPH to ritalinic acid as a funtion of pH and the measured pH after 24 h growth.

Finally, the role of the neuroactive peptide substance P and its breakdown in the gut by microbiota was studied. Various bacterial strains have already been shown to be able to digest substance P into specific peptide fragments of substance P.

3.5 Proteomics

3.5.1 *Novel chemical labelling strategies in quantitative proteomics*

Advancements in liquid chromatography and mass spectrometry over the last decades have led to a significant development in mass spectrometry-based proteome quantification approaches. A widely used strategy is multiplex isotope labeling, which significantly improves the accuracy, precision and throughput of quantitative proteomics in the data-dependent acquisition (DDA) mode. In 2021, Xiaobo Tian (PhD student) developed a novel chemical labelling strategy that is adapted for the data-independent acquisition (DIA) mode.

Figure 10. below shows the concept of the Ac-IP tag, which generates identical MS2 spectra from differentially labelled precursor ions. Quantification is based on

deconvolution of the precursor ion in the MS1 spectrum. Current work focuses on improving data processing for quantitative analysis in DIA and on increasing the multiplexing capacity.

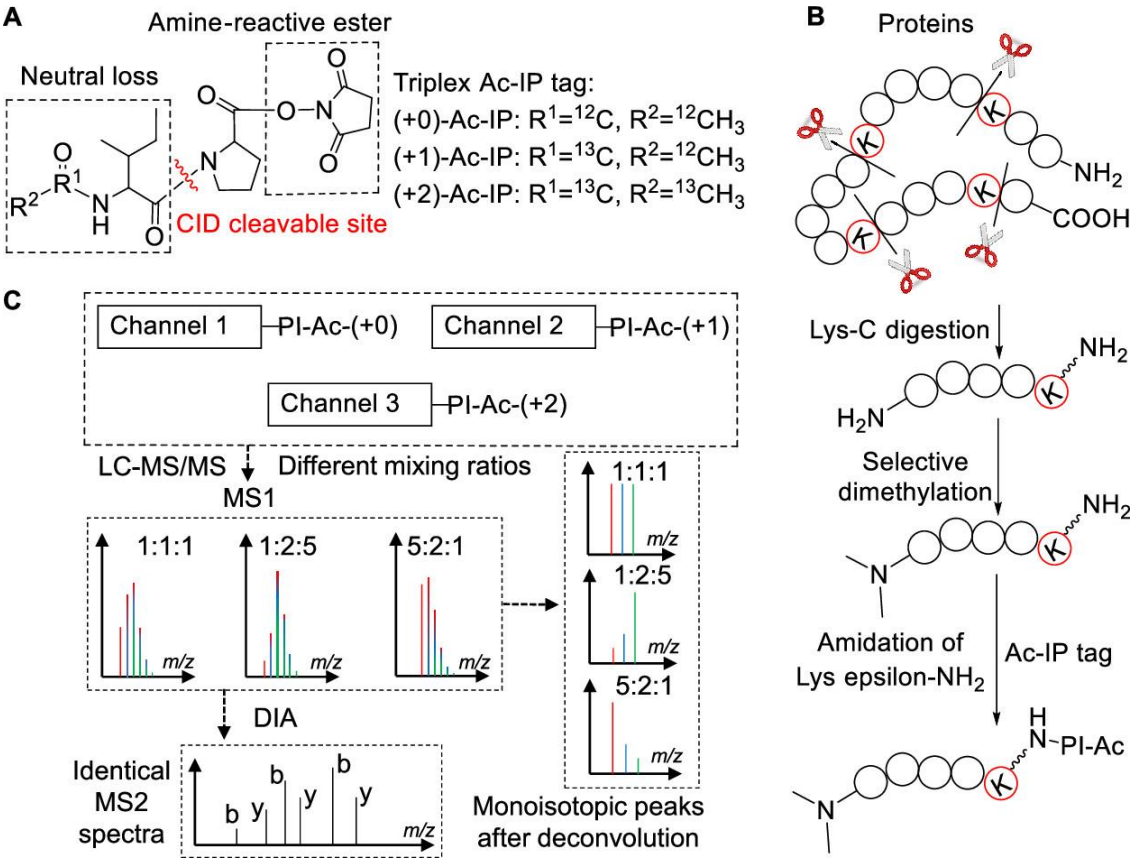

Figure 10. Design and concept of quantification based on the Ac-IP tag in DIA mode. (A) Molecular structure of the triplex Ac-IP tag. (B) Sample preparation workflow: protein digestion with LysC followed by selective N-terminal dimethylation and labelling with the Ac-IP tag. (C) Schematic representation of sample mixing and results at the MS1 and the MS2 level prior to and after deconvolution for a mixture of triplex-labelled samples. The differentially labelled samples are pooled prior to LC–MS/MS in DIA mode.

3.5.2 *Targeted protein analysis*

This research line is driven by Karin Wolters (Dept. of Pediatrics, UMCG). More and more researchers connect to Karin to develop targeted LC-MS/MS assays for their respective projects. The use of isotopically labelled internal standards in the form of synthetic concatemers created by the combination of all targeted peptides into one synthetic protein (QconCAT technology) has proven to be of great value to a range of projects, using the selected reaction monitoring (SRM) approach as the main 'workhorse'. Internally, we are currently applying these methods to protein targets related to cellular cholesterol homeostasis and metabolism, triglyceride hydrolysis and atherosclerosis (coll. Kuivenhoven), protein classes like the copper metabolism MURR1 domain (COMMD) protein family (coll. van de Sluis), protein targets related to bile acid metabolism (coll. Kuipers), proteins related to ER stress (coll. Jonker) and mitochondrial/glycolysis-related proteins (coll. Bakker). Via the established Enabling Technology Hotel (https://www.dtls.nl/technology-hotels/list/tools-for-systems-biology-applications/), we are currently developing additional panels targeting a series of proteins related to the peroxisomes in collaboration with Hans Waterham (Amsterdam Medical Center).

In a collaborative project with the group of Barbara Bakker (UMCG), we showed the potential of the targeted proteomics approach by identifying CPT1B as a key enzyme in the (loss) of lipid handling in the skeletal muscles upon the combined effects of aging and diets. Figure 11. shows a similar general upregulation of the β-oxidation proteins upon aging or increase fat diets, with the exception of CTP1B. Identifying this protein as a potential target to counteract age-dependent lipid accumulation in the skeletal muscles and potential insulin resistance.[1]

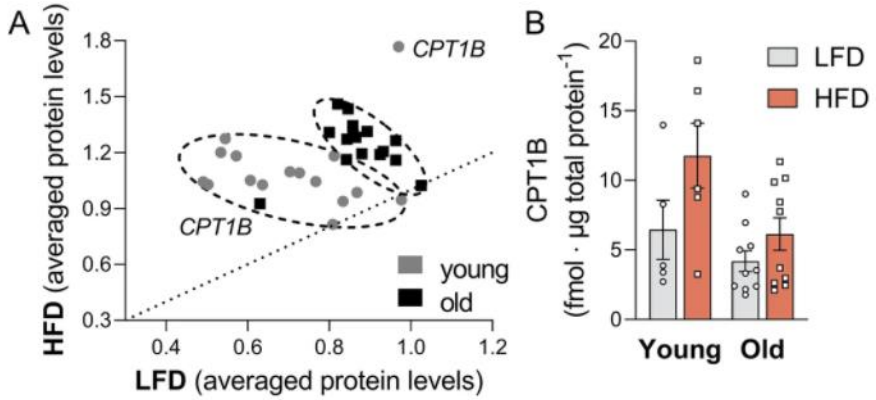

Figure 11. Targeted proteomics detection of the β-oxidation proteins in skeletal muscle tissues of young and old mice on a low (LFD) or high (HFD) fat diet (panel A) and the individual amounts of CPT1B (Panel B). This figure contains a subset of the panels from Fig4, PMID: 34330275).

3.6 Biopharmaceuticals

3.6.1 *Studying the in vivo biotransformation of the therapeutic antibodies trastuzumab and pertuzumab*

To study the *in vivo* biotransformation of the therapeutic antibodies trastuzumab and pertuzumab in breast cancer patients, we developed a powerful separation system for charge variants by cation-exchange chromatography with pH-gradient elution. More than 20 variants were partially separated at the protein level for trastuzumab (Figure 12.).

To study biotransformation *in vivo*, we developed an affinity enrichment approach using antibody-specific affimers (collaboration Avacta LifeScience). These affimers were selected by phage display to interact specifically with trastuzumab or pertuzumab, respectively, and not to interact with human IgG1 (see Figure 13.).

These methods have recently been combined to analyse charge variants in plasma of breast cancer patients that are treated with a combination of both antibodies (collaboration NKI-Dutch Cancer Institute). The main challenge was to find elution conditions from the affimer column that are compatible with the subsequent cation-exchange separation.

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[1] Age-related susceptibility to insulin resistance arises from a combination of CPT1B decline and lipid overload. Vieira-Lara MA, Dommerholt MB, Zhang W, Blankestijn M, Wolters JC, Abegaz F, Gerding A, van der Veen YT, Thomas R, van Os RP, Reijngoud DJ, Jonker JW, Kruit JK, Bakker BM. BMC Biol. 2021 Jul 30;19(1):154. doi: 10.1186/s12915-021-01082-5.PMID: 34330275

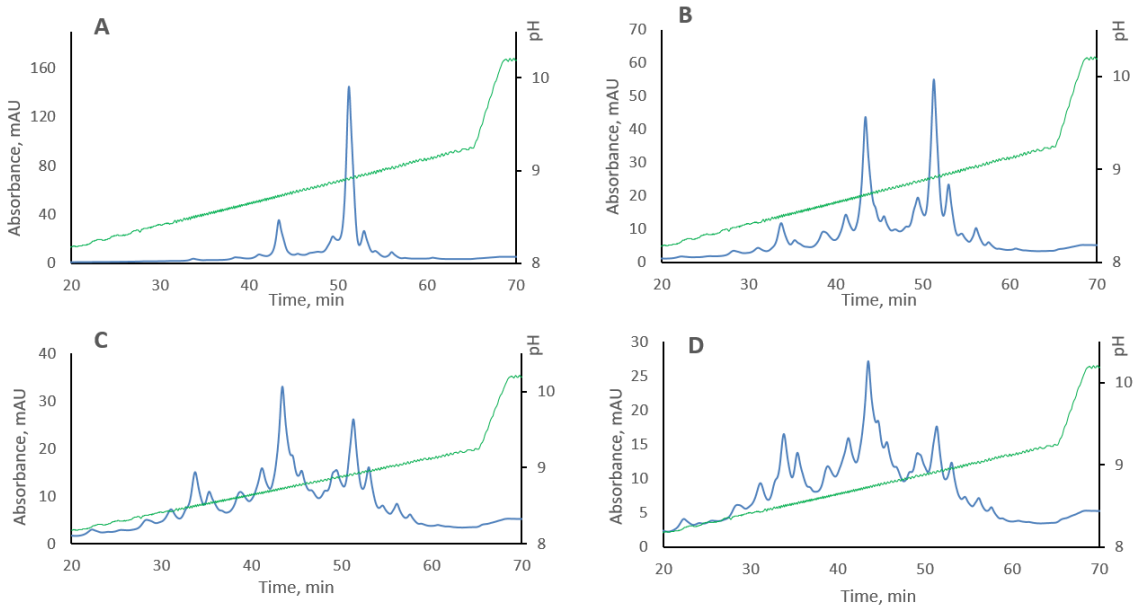

Figure 12. Charge state profile of trastuzumab upon stressing for 3 weeks at pH 7.4; 37 °C in PBS. (A) Starting material (clinical grade trastuzumab); (B) 1-week stressed trastuzumab; (C) 2 weeks stressed trastuzumab; (D) 3 weeks stressed trastuzumab. The green line indicates the measured pH gradient. Absorbance was measured at 280 nm.

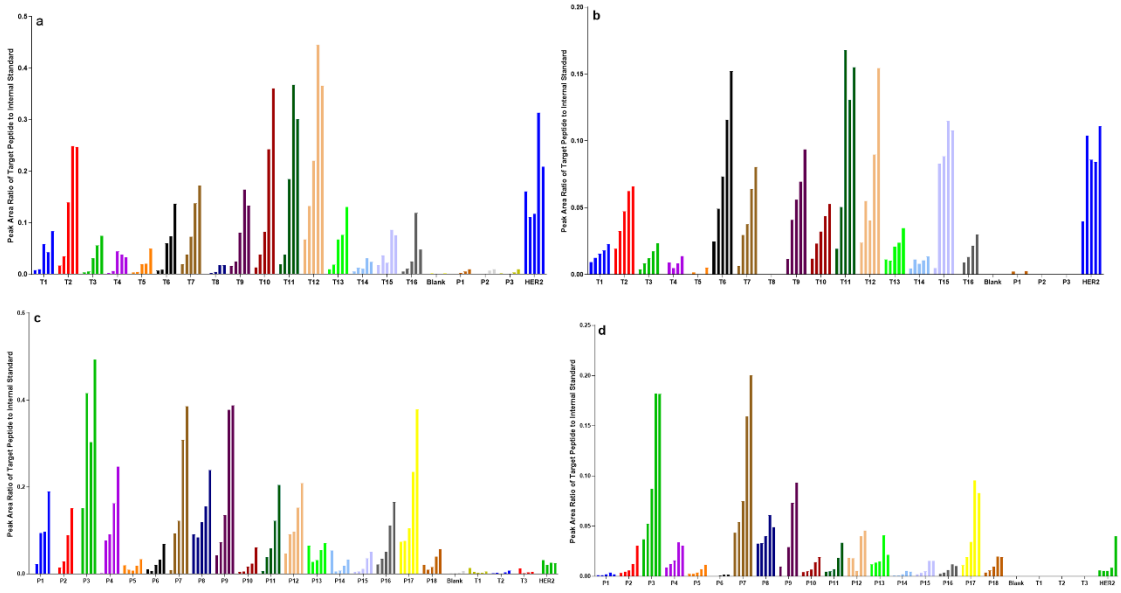

Figure 13. Prioritization of anti-trastuzumab and anti-pertuzumab Affimer® reagents using a microtiter plate-based binding assay and LC-MS in the SRM mode (System 1) as readout. Performance evaluation of the different anti-trastuzumab affimers in a) PBS and b) 50% plasma is based on the peak area ratios of the antibody-derived signature peptide (FTISADTSK) to the added stable-isotope-labelled peptide standard. Numbers T1-16 represent anti-trastuzumab affimers and P1-P3 anti-pertuzumab affimers (controls for cross-reactivity). Performance evaluation of the different anti-pertuzumab affimers c) in PBS and d) in 50% plasma is based on the peak area ratios between the antibody-derived signature peptide (FTLSVDR) and the added stable-isotope-labelled peptide standard. Numbers P1-18 represent anti-pertuzumab affimers and T1-T3 are anti-trastuzumab affimers (controls for cross-reactivity). HER2 is the target protein (positive control) in both cases. The five bars represent increasing antibody amounts (75, 150, 300, 750 and 1500ng corresponding to 0.75, 1.5, 3, 7.5 and15µg/mL from left to right) deposited on the plate in 100µL volume.

3.7 Proteome and metabolome regulation studies

3.7.1 *Macrophage polarization in inflammation – the regulatory role of the proteome, protein acetylation and energy metabolism*

Diabetes mellitus type II and obesity are characterized by low-grade chronic inflammation and metabolic dysfunction (meta-inflammation), observed in all tissues involved in energy homeostasis. Macrophages play an important role in these tissues and can activate into specialized subsets by cues from their micro-environment to handle a variety of tasks. Many different macrophages subsets have been described and they are also defined by differential metabolic reprogramming taking place to fuel their main functions. Since DMT II and obesity are characterized by metabolic alterations at the organism level, these alterations may also induce changes in macrophage metabolism resulting in unique macrophage activation patterns in diabetes and obesity.

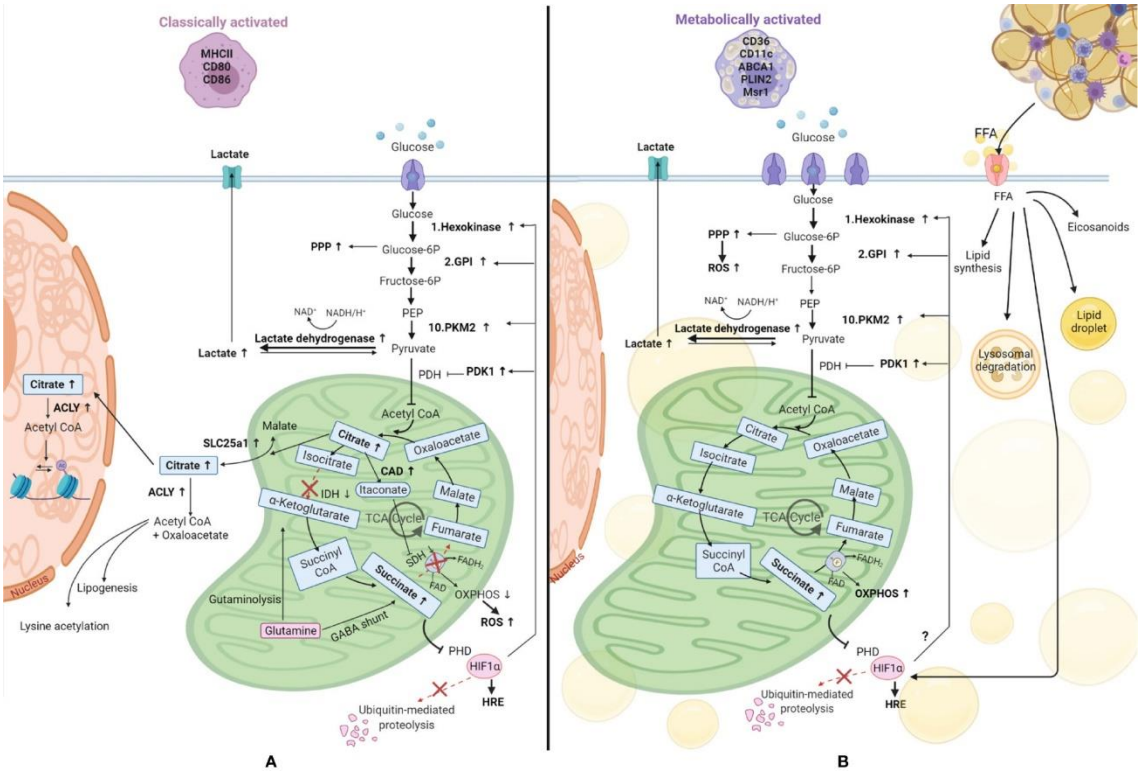

Figure 14. Comparison of metabolic reprogramming of classically and metabolically activated macrophages. (A) Classically activated macrophages and their metabolism. (B) Metabolically activated macrophages and their metabolism. Adipose tissue macrophages in obesity internalize free fatty acids (FFA) and lipids from the dying adipocytes, becoming foam cells. These FFA can be used to synthesize new lipids, can be stored in lipid droplets, can be catabolized through the lysosomal pathway or be used to produce inflammatory lipid mediators called eicosanoids. Glucose is the main source of energy also in metabolically activated macrophages, where the glucose transporter is overexpressed, and it is catabolized by glycolysis, which is upregulated, providing substrates for the pentose phosphate pathway (PPP), also upregulated. Also the metabolic pathway OXPHOS (oxidative phosphorylation) is upregulated in these cells, underlying their high energy demand. Succinate production is increased in these cells. This metabolite inhibits prolyl hydroxylase domain (PHD) proteins, resulting in less hydroxylation of hypoxia-inducible factor 1-alpha (HIF-1a), which circumvents its degradation, allowing its binding to hypoxia response elements (HRE) on target genes. HIF-1a is also promoted by FFA and we hypothesize it might promote the switch to glycolysis by inducing glycolytic enzymes as it happens in classically activated macrophages. TCA, tricarboxylic acid cycle; SLC25a1, solute carrier family 25 member 1; ACLY, ATP citrate lyase; IDH, isocitrate dehydrogenase; SDH, succinate dehydrogenase; CAD, cis-aconitate decarboxylase; GABA, g-aminobutyric acid; PHD, prolyl hydroxylase domain; ROS, reactive oxygen species; PKM2, pyruvate kinase M2; GPI, glucose-6-

phosphate isomerase; PEP, Phosphoenolpyruvate; PDH, Pyruvate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; FAO, fatty acid oxidation. Figure from PMCID: PMC8602812[2]

We notably studied the relation between changes in metabolism and polarization (Figure 14.), and we focused on different possibilities of measuring a range of metabolites intra-and extracellularly in a precise and comprehensive manner, as summarized in a recently published review article.
The work on this project has recently focussed on macrophage polarization related to chronic obstructive pulmonary disease (COPD) in close collaboration with Barbro Melgert (Department of Molecular Pharmacology).

3.7.2 *Establishment of a combined metabolic and chemical labeling approach to investigate site-specific acetylation dynamics by LC-MS*

This research line is a collaboration with Marcel Kwiatkowski, Kathrin Thedieck (University of Innsbruck, Austria) and Ines Heiland (UiT, The Arctic University of Norway, Tromsø, Norway) performed by the PhD student Alienke van Pijkeren (2+2 PhD student; currently in Innsbruck). An LC-MS/MS based method and data analysis pipeline was developed to investigate acetylation dynamics on histone proteins. We developed a novel flux methodology that allows for the first time to quantify site-specific reaction rates of acetylation and deacetylation. This approach, recently published in Analytical Chemistry (van Pijkeren et al., PMID: 34519498) combines metabolic and chemical labeling (CoMetChem) using stable isotopes. For metabolic labeling, cells were incubated with [U-^{13}C]-glucose. After extraction, nonacetylated protein lysine residues were chemically acetylated with $^{13}C_6$,D_6-acetic anhydride. In this way, chemically equivalent acetylated isotopologue species are generated. These acetylated isotopologue species can be differentiated by high-resolution mass spectrometry. We showed that CoMetChem enables the site-specific quantification of the incorporation and loss of lysine acetylation over time, allowing the determination of reaction rates for acetylation and deacetylation. Figure 16. gives an overview of the CoMetChem workflow and summarizes the results obtained by analyzing the acetylation dynamics of the histone 3 (18-26) peptide, K18QLATK23AAR, that can be acetylated on both lysine residue 18 (K18) and 23 (K23).

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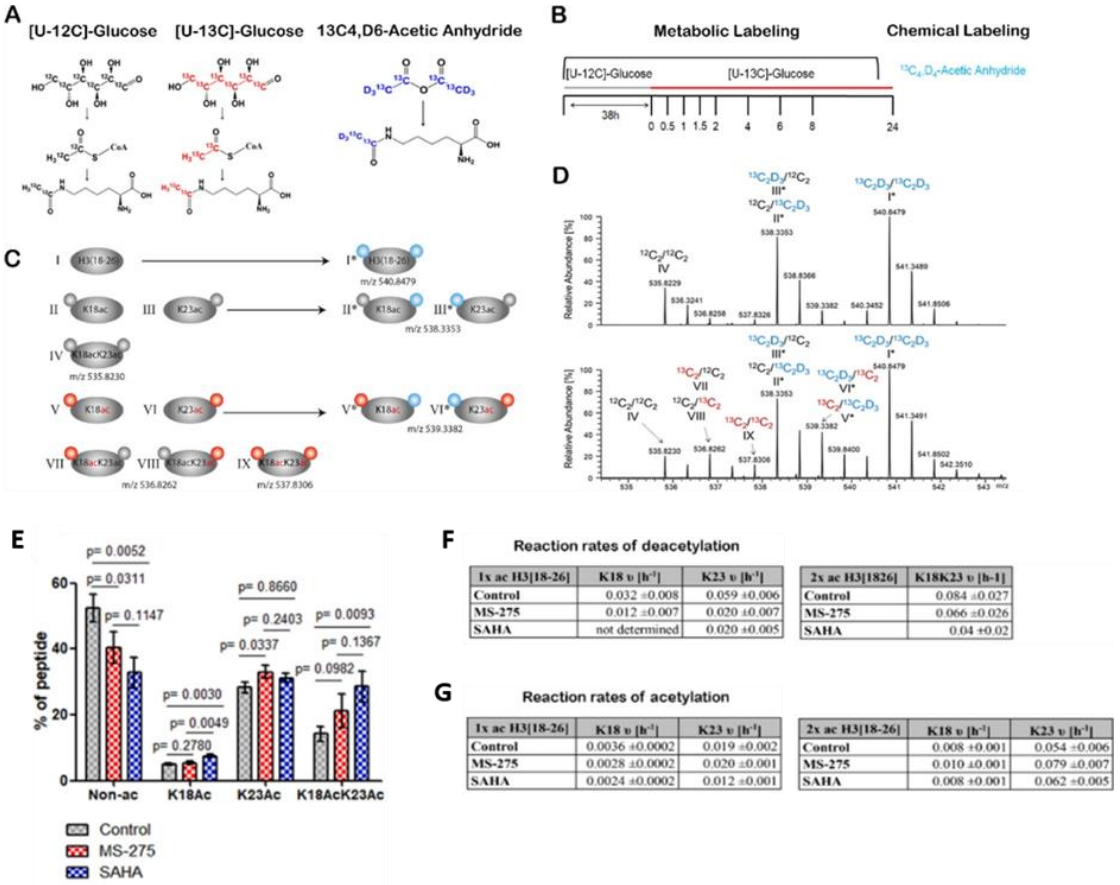
[2] Meta-Inflammation and Metabolic Reprogramming of Macrophages in Diabetes and Obesity: The Importance of Metabolites. Russo S., Kwiatkowski M., Govorukhina N., Bischoff R. and Melgert B. Front Immunol. 2021 Nov 5;12:746151. doi: 10.3389/fimmu.2021.746151. eCollection 2021.



1x ac H3[18-26]	K18 υ [h⁻¹]	K23 υ [h⁻¹]
Control	0.032 ±0.008	0.059 ±0.006
MS-275	0.012 ±0.007	0.020 ±0.007
SAHA	not determined	0.020 ±0.005

2x ac H3[1826]	K18K23 υ [h-1]
Control	0.084 ±0.027
MS-275	0.066 ±0.026
SAHA	0.04 ±0.02

1x ac H3[18-26]	K18 υ [h⁻¹]	K23 υ [h⁻¹]
Control	0.0036 ±0.0002	0.019 ±0.002
MS-275	0.0028 ±0.0002	0.020 ±0.001
SAHA	0.0024 ±0.0002	0.012 ±0.001

2x ac H3[18-26]	K18 υ [h⁻¹]	K23 υ [h⁻¹]
Control	0.008 ±0.001	0.054 ±0.006
MS-275	0.010 ±0.001	0.079 ±0.007
SAHA	0.008 ±0.001	0.062 ±0.005

Figure 16. CoMetChem workflow for the analysis of site-specific histone acetylation dynamics. (A) CoMetChem combines metabolic and chemical labeling using stable isotopes. The metabolic labeling using [U-^{12}C]-Glc or [U-^{13}C]-Glc results in $^{12}C_2H_3$ (gray)- or $^{13}C_2H_3$ (red)-containing acetyllysines, respectively. The chemical acetylation of nonacetylated lysines using $_{13}C_6$,D_6-AA results in $^{13}C_2D_3$ (blue)-containing acetyllysines. (B) Cells were first cultured in a [U-$_{12}$C]-glucose-containing medium (gray) followed by medium replacement to [U-^{13}C]-glucose-containing medium (red). The nuclei were isolated from the samples at different time points, followed by nucleus isolation and histone extraction, chemical derivatization of unmodified lysine residues at the protein level using $^{13}C_6$,D_6-AA (blue), and tryptic digestion and quantitative LC–MS analysis. (C) Schematic representation of all possible acetylated H3 species covered by the H3(18-26) peptide generated metabolically (left panel, Roman numerals without asterisk) and the corresponding isotopologues generated by combination with chemical acetylation (right panel, Roman numerals with asterisk). Acetyl groups derived from [U-^{12}C]-Glc are indicated in gray, [U-^{13}C]-Glc-derived acetyl groups are indicated in red, and $^{13}C_6$,D_6-AA-derived acetyl groups are indicated by blue circles. (D) MS1 spectra of the H3(18-26) isotopologues upon CoMetChem labeling before medium exchange (t = 0) and 4 h of incubation with [U-^{13}C]-Glc (t = 4 h). The spectra show the different H3(18-26) isotopologues, and the colors indicate whether the acetyl groups of the K18 (left) and K23 (right) residues are derived from [U-^{12}C]-Glc (black), [U-^{13}C]-Glc (red), or $^{13}C_6$,D_6-AA (blue). (E) Bar chart (mean with standard deviation) showing abundance levels of the non-acetylated (Non-Ac), single-acetylated (K18ac, K23ac) and double-acetylated (K18acK23ac) H3(18-26) species after 16 hours of incubation with the histone deacetylase inhibitors MS-275, SAHA or the carrier (control). (F) Site-specific deacetylation rates of K18ac and K23ac of the single-acetylated (1× ac) H3(18-26) species and deacetylation rate for the double-acetylated peptide. (G) Site-specific acetylation rates of K18ac and K23ac of the single-acetylated (1×ac) and double-acetylated (2×ac) H3(18-26) species. n = 3 independent experiments. Statistical analyses were performed using the two-tailed unpaired t-test.

3.7.3 *Establishment of a simultaneous extraction method to perform metabolomics and proteomics analysis from the same biological sample*

In addition, we worked on a method which enables the simultaneous extraction of polar, ionic metabolites, amino acids, proteins and lipids as well as extracellular metabolites from a single sample based on liquid-liquid extraction using $CHCl_3$-MeOH. After collection of the different fractions, they can be analyzed by optimized MS-based methods as illustrated in Figure 15. Currently, we are finalizing a manuscript evaluating the influence of different buffer systems (sodium dodecyl sufate-, sodium deoxycholate-, and urea-based) on the proteome accessibility from the interphases. We further applied this simultaneous proteo-metabolomics approach to investigate the regulatory interplay between energy metabolism and proteome in the context of tuberous sclerosis complex (TSC) disease.

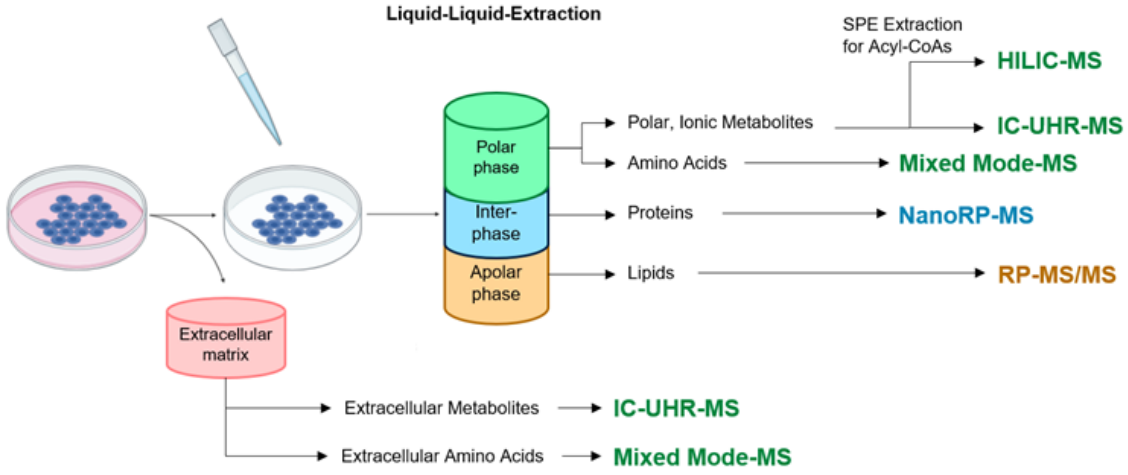


Figure 15. Workflow for the simultaneous extraction and MS-based analysis of extracellular metabolites and amino acids, polar, ionic metabolites, amino acids, proteins, and lipids. SPE: solid-phase extraction, HILIC-MS: Hydrophilic Interaction Liquid Chromatography Mass Spectrometry, IC-UHR-MS: Ion Chromatography Ultra High-Resolution Mass Spectrometry, NanoRP-MS: Nano Reversed Phase Mass Spectrometry, RP-MS/MS: Reversed Phase Tandem Mass Spectrometry.

4 Interfaculty Mass Spectrometry Center (IMSC)

For the IMSC, the year 2021 was again an eventful year with several important changes. We have seen the expected uptick in service requests in the first half of 2021, after the difficult first COVID19 year 2020. New instruments have arrived or are in the process of acquisition. Older instruments have finally been switched off after many years of service. Unfortunately, the IMSC technician employed by RUG has left us in 2021. This has caused significant delays in service, particularly in new projects on small molecule analysis and quantification and implementation of new workflows. We expect to fill the position again in the beginning of 2022. Finally, Prof. Rainer Bischoff retired in 2021, and we would like to thank him for the work and support for the IMSC as a core facility embedded within the Analytical Biochemistry research group. Prof. Peter Horvatovich has taken over the position as scientific head of the IMSC.

Preparations in the form of tender procedures for new investments in LCMS instrumentation were initiated in 2021. The acquisition of a new high-resolution LC-MS system for proteomics was completed at the end of 2021 and it is expected to be installed in the first quarter of 2022. The system will be a Thermo Orbitrap Exploris 480, the same type of instrument that was installed in 2020. Instead of a regular nanoLC, we have acquired an EvoSep One nanoLC, which uses integrated SPE tips and a preformed gradient to drastically speed up proteomics runs. This LC instrument and the extra capacity of the second Exploris 480 MS is expected to greatly increase the sample throughput for 'routine' clinical proteomics studies. This investment was funded by UMCG in the context of the core facility plan.

The acquisition of a high-resolution LC-MS system for metabolomics is in preparation for early 2022 and will be funded jointly by FSE, GRIP and IMSC. This instrument will be dedicated to untargeted metabolomics analysis and will take over the function of the older QToF and Orbitrap instruments on which we currently are developing metabolomics methods.

For targeted quantitative applications for small molecules we have acquired two used API4000 instruments kindly provided by our industrial collaborators. These instruments are now used instead of the API3000 and TSQ Quantum systems in the IMSC. They are reasonably sensitive, and importantly, quite robust for a wide variety of applications. We are also in the process of upgrading our quantitative workflow with stricter procedures to improve both the project throughput and data quality.

Figure 17*: New Exploris 480 (left), EvoSep One (middle) and Bravo (right) equipment.*

The Agilent Bravo liquid-handling system was put into service this year and is currently being tested for several proteomics sample preparation workflows, such as the SP3 protocol. It is capable of both automated, high-throughput liquid-handling and sample purification, and should benefit a variety of applications including proteomics & metabolomics sample preparation.

Overall, the IMSC has operated on the same level as previous years (with the exception of 2020), with a small positive cashflow after taking into account several costly repairs and investments in lab equipment. The scope of the analyses also remained similar,

covering both proteomics and small molecule quantitation, as well as analysis of among other lipids, intact proteins, vitamins & cofactors, amino acids, and numerous synthetic molecules. In 2021 UMCG projects for the first time accounted for more service work than RUG projects, with a small number of projects for companies. The prospects for 2022 look good with plenty of work to make the best use of the new equipment and update and streamline protocols for our standard proteomics as well as targeted quantitative workflows. Development of workflows for untargeted metabolomics and data independent proteomics are some of the focus points. We have several collaborations with RUG and UMCG research groups on these subjects and hope to host several PhD students to continue these studies in the course of 2022.

5 Ph.D. projects

Peter Bults (ICON)
Pharmacokinetics and biotransformation of biopharmaceuticals by liquid chromatography with unit-mass and high-resolution mass spectrometric detection
Promotor: Nico van de Merbel
Start: January 2015 (thesis defense April 11, 2022)

Wenxuan Zhang (UMCG)
Lipidomics in Systems Medicine
Promotors: Folkert Kuipers & Dirk-Jan Reijngoud
Start: November 2015
Thesis defense: May 10, 2021

Victor Bernal Arzola
Clinical big data for multifactorial diseases: from molecular profiles to precision medicine
Promotor: Peter Horvatovich
Start: July 2016 (thesis expected to be defended in April 2022)

Yang Zhang
Proteogenomic and targeted metabolomic analysis of ovarian cancer heterogeneity and its contribution to recurrence and therapy resistance
Promotor: Peter Horvatovich
Start: January 2017 (thesis defense February 1, 2022)

Alienke van Pijkeren (UMCG)
Protein acetylation dynamics – elucidating the connection between energy metabolism and gene expression in age-related inflammatory diseases
Promotor: Rainer Bischoff
Start: September 2017

Xiaobo Tian (CSC scholarship)
Electrochemistry for protein and peptide chemistry
Promotor: Rainer Bischoff
Start: October 2017
Thesis defense: September 24, 2021

Xiaodong Feng
Improving identification and quantification in metabolomics applying modern data analysis techniques
Co-promoter: Peter Horvatovich (First Promoter Ido Kema).
Started, October 2017, defense expected in first half of 2022

Ali Alipour Najmi Iranag
Electrochemistry – Mass Spectrometry in the synthesis of drug metabolites and precursors for pharmaceuticals
Promotor: Rainer Bischoff
Start: November 2017

Baubek Spanov
Bioanalytical methodology to study the *in vivo* biotransformation of therapeutic proteins
Promotor: Rainer Bischoff

Start: May 2018

Oladapo Olaleye
Methodology for studying protein species of therapeutic proteins
Promotor: Rainer Bischoff
Start: June 2018

Alejandro Sánchez Brotons
Development of a generic framework for pre-processing LC/GC-MS(/MS) data obtained with data-dependent and data-independent acquisition
Promotor: Peter Horvatovich
Start: June 2018

Saskia Sokoliova
A chemoproteomic approach to study advanced glycation end-products
Promotor: Peter Horvatovich
Start: July 2018

Sara Russo
Regulation of macrophage polarization and inflammation in Diabetes Mellitus Type II (DMT-II) and obesity through energy metabolism and protein acetylation
Promotor: Rainer Bischoff
Start: September 2018

Janine Stam
Determining exosomal proteins as potential biomarkers for drug-induced cholestasis
Promotor: Rainer Bischoff
Start: October 2018

Julia Aresti Sanz
Detection and characterization of novel metabolites from the gut microbiota with liquid chromatography- electrochemistry-mass spectrometry, and identification of their biological functions
Supervisor Hjalmar Permentier (promotor: Sahar el Aidy)
Start: April 2018

Bas Sleumer (ICON)
Quantification of biomarker isoforms
Promotor: Nico van de Merbel
Start: March 2019

Yanick Paco Hagemeijer
Proteogenomics data integration for the X-Omics initiative
Promotor: Peter Horvatovich
Start: February 2020

Khadija Al-Saad
High resolution liquid chromatography mass spectrometry screening in sports antidoping for the detection of sulfate Phase II metabolites of endogenous and exogenous prohibited substances
Promotor: Peter Horvatovich
Start: May 2021

6 Theses

Tian, X., Chemical approaches for quantitative proteomics. 2021, [Groningen]: University of Groningen. 217 p.

Zhang, W., Applying lipidomics strategies to study lipid metabolic diseases. 2021, [Groningen]: University of Groningen. 203 p.

7 Scientific Output

7.1 Scientific publications (peer-reviewed, published in 2021)

1. Al-Jaal, B., Latiff, A., Salama, S., Hussain, H. M., Al-Thani, N. A., Al-Naimi, N., Al-Qasmi, N., Horvatovich, P. & Jaganjac, M., Analysis of Multiple Mycotoxins in the Qatari Population and Their Relation to Markers of Oxidative Stress. 8-Apr-2021, In: Toxins. 13, 4, 10 p., 267.

2. Aresti-Sanz, J., Schwalbe, M., Pereira, R. R., Permentier, H. & El Aidy, S., Stability of methylphenidate under various ph conditions in the presence or absence of gut microbiota. Aug-2021, In: Pharmaceuticals. 14, 8, 14 p., 733.

3. Bernal, V., Bischoff, R., Horvatovich, P., Guryev, V. & Grzegorczyk, M., The 'un-shrunk' partial correlation in Gaussian graphical models. 7-Sep-2021, In: Bmc Bioinformatics. 22, 1, 15 p., 424.

4. Betancourt, L. H., Gil, J., Sanchez, A., Doma, V., Kuras, M., Murillo, J. R., Velasquez, E., Çakır, U., Kim, Y., Sugihara, Y., Parada, I. P., Szeitz, B., Appelqvist, R., Wieslander, E., Welinder, C., Almeida, N. P., Woldmar, N., Marko-varga, M., Eriksson, J., Pawłowski, K. & 37 others, The Human Melanoma Proteome Atlas—Complementing the melanoma transcriptome. 1-Jul-2021, In: Clinical and Translational Medicine. 11, 7, e451.

5. Betancourt, L. H., Gil, J., Kim, Y., Doma, V., Çakır, U., Sanchez, A., Murillo, J. R., Kuras, M., Parada, I. P., Sugihara, Y., Appelqvist, R., Wieslander, E., Welinder, C., Velasquez, E., Almeida, N. P., Woldmar, N., Marko-varga, M., Pawłowski, K., Eriksson, J., Szeitz, B. & 37 others, The human melanoma proteome atlas—Defining the molecular pathology. 1-Jul-2021, In: Clinical and Translational Medicine. 11, 7, e473.

6. Dietze, J., van Pijkeren, A., Egger, A. S., Ziegler, M., Kwiatkowski, M. & Heiland, I., Natural isotope correction improves analysis of protein modification dynamics, 27-Oct-2021, (E-pub ahead of print) In: Analytical and Bioanalytical Chemistry.

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7.2 Articles on preprint servers

1. Jeovanis Gil, Yonghyo Kim, Beáta Szeitz, Viktória Doma, Uğur Çakır, Natália Pinto de Almeida, Yanick Paco Hagemeijer, Victor Guryev, Jenny G Johansson, Yogita Sharma, Indira Pla Parada, Zsolt Horvath, Jéssica de Siqueira Guedes, Gustavo Monnerat, Gabriel Reis Alves Carneiro, Fábio CS Nogueira, Boram Lee, Henriett Oskolas, Enikő Kuroli, Judit Hársing, Yutaka Sugihara, Magdalena Kuras, Roger Appelqvist, Elisabet Wieslander, Gilberto B Domont, Bo Baldetorp, Runyu Hong, Gergely Huszty, Laura Vizkeleti, József Tímár, David Fenyö, Lazaro Hiram Betancourt, Johan Jakobsson, Johan Malm, Aniel Sanchez, A Marcell Szász, Peter Horvatovich, Melinda Rezeli, Sarolta Kárpáti, György Marko-Varga, Proteogenomics Reveals how Metastatic Melanoma Modulates the Immune System to Allow Immune Evasion, biorxiv.org, doi: https://doi.org/10.1101/2021.04.10.439245.

7.3 Proceedings

1. Bekker, N., Van Pijkeren, A., Wolters, J.C. Brotons, A.S., Guryev, V., Bischoff, R., Alkema, W., Van Den Berge, M., Horvatovich, P., Timens, W., Brandsma, C. A., Proteomics approach to identify COPD-related changes in pulmonary fibroblasts, European Respiratory Journal 58 (suppl 65), 2021.

7.4 Scientific publications (peer-reviewed, accepted)

1. Gupta, A., Burgess, J. K., Borghuis, T., de Vries, M. P., Kuipers, J., Permentier, H. P., Bischoff, R., Slebos, D. J., Pouwels, S. D., Identification of Damage Associated Molecular Patterns and Extracellular Matrix Proteins as Major Constituents of the Surface Proteome of Lung Implantable Silicone/Nitinol Devices, Acta Biomaterialia (accepted January 07, 2022).

2. van der Kamp, M. F., Halmos, G. B., Guryev, V., Horvatovich, P. L., Schuuring, E., van der Laan, B. F. A. M., van der Vegt, B., A literature review on age specific oncogenetic pathways in head and neck squamous cell carcinoma – are elderly a different subcategory? accepted in Cellular Oncology.

3. Spanov, B., Aboagye, V., Olaleye, O., Govorukhina, N., van de Merbel, N. C., Bischoff, R., Effect of trastuzumab-HER2 complex formation on stress-induced modifications in the CDRs of trastuzumab. Frontiers in Chemistry 2022 Vol. 9 Issue 1124 (DOI: 10.3389/fchem.2021.794247).

7.5 Book chapter

1. Hagemeijer, Y. P., Guryev, V., Horvatovich, P., Accurate Prediction of Protein Sequences for Proteogenomics Data Integration, Clinical Proteomics, 2022, 233-260.

7.6 Lectures

- Rainer Bischoff, Mass Spectrometry for Analysis of Critical Quality Attributes of Biologics/Biosimilars, Mumbai, India (March 05, 2021) – online workshop.
- Rainer Bischoff, Quantitative (Bio)analysis of Proteins by LC-MS, Semnan University, Iran (October 26, 2021) – online lecture.
- Rainer Bischoff, Bioanalysis of therapeutic proteins by LC-MS, 40th International Symposium on the Separation of Proteins, Peptides & Polynucleotides, Porto, Portugal (November 7 – 10, 2021).
- Alipour Najmi, A. Electrochemical N-demethylation of tropane and opiate alkaloids. CHAINS 2021, Veldhoven, The Netherlands (December 7-8, 2021) - online lecture
- Alipour Najmi, A. An electrosynthesis approach for the N-demethylation of tropane and opiate alkaloids. ElCheMS4: 6th International Workshop on Electrochemistry/Mass Spectrometry, Münster, Germany (May 27-28, 2021) - online lecture.
- Peter Horvatovich, “Processing mass spectrometry imaging data for quantitative spatial molecular profiling”, millennium nucleus of ion channel associated disease (MiniCAD) Seminar, Chile (January 18, 2021) – online lecture.
- Peter Horvatovich, “Introduction to proteomics and proteogenomics application in COPD”, Seminar, Jinan University, China (October 26, 2021) – online lecture.
- Peter Horvatovich, “The role of proteogenomics in understanding molecular mechanisms of COPD”, X-Omics Festival, The Netherlands (April 12, 2021) – online lecture.
- Peter Horvatovich, “Accurate quantification of intact drug distribution in tissue using mass spectrometry imaging Data processing perspective”, Figon, Leiden, The Netherlands (September 27, 2021).
- Xiaodong Feng, “Dynamic binning peak detection for LC-MS pre-processing”, International Conference on Non-Targeted Screening 2021 (ICNTS21), Erding, Germany (October 4-7, 2021).
- Alejandro Sanchez Brotons, International Conference on Non-Targeted Screening 2021 (ICNTS21), Erding, Germany (October 4-7, 2021).
- Alejandro Sanchez Brotons, Pipelines And Systems for Threshold Avoiding Quantification of LC-MS/MS data (PASTAQ), The Netherlands (October 6, 2021) – online lecture.
- Alejandro Sanchez Brotons, “Recalibrating Mass Spectra Improves Precision in Label-Free Proteomics Analyses”, BioSB Conference (June 15-16, 2021) – online lecture.
- Yanick Hagemeijer, Making workflows (more) FAIR pt. 3 proteogenomics edition, X-Omics, FAIR workshop, The Netherlands, (May 7, 2021) – online lecture.
- Merbel, N.C. van de, Analytical consequences of the in vivo deamidation of trastuzumab and pertuzumab - comparison of the results of three bioanalytical

platforms. European Bioanalysis Forum Focus Workshop, (June 18, 2021) – online lecture.
- Merbel, N.C. van de, Bioanalytical support of the development of deuterated drugs by LC-MS/MS - Quantification of deuterated levodopa and metabolites in plasma and urine. Webinar, (September 29, 2021) – online lecture.
- Merbel, N.C. van de, A tiered approach to method validation for the support of a bioequivalence trial with ibuprofen. European Bioanalysis Forum, 13th Annual Open Symposium (November 24, 2021) – online lecture.

7.7 Bioinformatics source code

- PASTAQ LC-MS/MS proteomics data pre-processing tool. https://github.com/PASTAQ-MS/PASTAQ.
- R project to Unshrunk partial correlation using shrinkage estimator. https://github.com/V-Bernal/UnShrunk.

7.8 Poster presentations

- Shteynberg, D. D., Midha, M. K., Hoopmann, M. R., Deutsch, E. W., Tian, X., Permentier, H. P., Bischoff, R., Moritz, R. L. Versatile Quantitative Analysis of Isobarically Tagged DIA and DDA Data by Trans-Proteomic Pipeline Tools. HUPO Reconnect 2021, Vancouver, Canada (November 15-19, 2021) - online meeting.
- Tian, X., Bischoff, R., Permentier, H.P. The Isotopic AC-IP Tag Enables Multiplexed Proteome Quantification in Data-Independent Acquisition Mode. HUPO Reconnect 2021, Vancouver, Canada (November 15-19, 2021) - online meeting.
- Wang, L., Bravo-Ruiseco, G., Liu, L., Feng, X., Permentier, H. P. , Horvatovich, P. L., He, T., van Dijl, J. M.,. Harmsen, H. J. M. The effect of calcium palmitate on bacteria associated with infant gut microbiota, 6th World Congress of Paediatric Gastroenterology, Hepatology and Nutritione, Vienna, Austria (June 2-5, 2021).
- Szeitz, B., Woldmar, N., Valkó, Z., Megyesfalvi, Z., Bárány, N., Paku, S., László, V., Bugyik, E., Lang, C., Szász, A. M., Pizzatti, L., Marko-Varga, G., Schelch, K., Horvatovich, P., Döme, B., Rezeli, M. Proteomic signatures of small cell lung cancer subtypes, HUPO Reconnect 2021 , (November 15-19, 2021) - online meeting.
- Szeitz, B., Kuras, M., Rodriguez, J., Eriksson, J., Horvath, Z., Szász, A.M., Rezeli, M., Horvatovich, P., Betancourt, L. H., Marko Varga, G., A landscape of single amino acid variants in melanoma, German Conference on Bioinformatics 2021 (September 6-8, 2021) – online meeting.
- Hagemeijer, Y., Gillett, T., Guryev, V., Horvatovich, P., Using proteogenomics to recover the non-canonical proteome, BioSB 2021 Conference, (June 15-16, 2021).
- Feng, X., Zhang, W., Kuipers, F., Kema, I., Barcaru, A. & Horvatovich, P., Dynamic binning peak detection for LC-MS pre-processing, BioSB 2021 Conference, (June 15-16, 2021) – online meeting.
- Khelifi, S., Saad, K., Vonaparti, A., Mahieddine, S., Salama, S., Saleh, A., Al-Mohannadi, M., Al-Thaiban, H., Lommen, A., Horvatovich, P., Beotra, A., Abushareeda, W., M Al Maadheed, M., Georgakopoulos, C. Ultra-Fast Retroactive Processing by MetAlign of Liquid-Chromatography High-Resolution Full-Scan Orbitrap Mass Spectrometry Data in WADA Human Urine Sample Monitoring Program, 39th Manfred Donike workshop, Cologne, Germany (March 22-23, 2021) – online workshop.
- Bekker, N., Van Pijkeren, A., Wolters, J. C., Brotons, A. S., Guryev, V., Bischoff, R., . Alkema, W., Van Den Berge, M., Horvatovich, P., Timens, W., . Brandsma, C. A. Proteomics approach to identify COPD-related changes in pulmonary fibroblasts

COPD - mechanism, Chronic diseases, Inflammation, European Respiratory Society (ERS) International Congress, (September 5–8, 2021) – online meeting.
- Sokoliova, S., Horvatovich, P., Witte, D. M., A photocaged chemical probe to study protein glycation, NWO CHAINS 2021, (December 7-8, 2021) – online meeting.

7.9 Editorships/board memberships

- Horvatovich, P., Board: Dutch Proteomics Platform
- Horvatovich, P., Secretary general, author of HUPOST and PI of Chromosome 5 for Chromosome Centric Human Proteome Project
- Horvatovich, P., Member of HUPO and German and Dutch Mass Spectrometry Societies
- Horvatovich, P., Member of EuPA Funding Committee.
- Merbel, N.C. van de, Editorial Board member Bioanalysis (Future Science Group).
- Merbel, N.C van de, Harmonization team leader of the Global Bioanalysis Consortium (GBC)
- Merbel, N.C. van de, Editorial Board member Bioanalysis (Future Science Group).
- Merbel, N.C. van de, Topic Team member: European Bioanalysis Forum
- Merbel, N.C. van de, Board: Section Analytical Chemistry (KNCV)
- Merbel, N.C. van de, Board: Working Group Pharmaceutical and Biomedical Analysis (KNCV)

7.10 Research Grants

National Roadmap for Large-Scale Research Infrastructure (NWO 184.034.019)
Netherlands X-omics Initiative.
Principal Investigator: Alain van Gool (UMCRadboud, Nijmegen).
Funding Period: 2018-2028.

GRIP PhD Scholarship
Recipient: Janine Stam.
Determining exosomal proteins as potential biomarkers for drug-induced cholestasis.
Principal Investigator: Rainer Bischoff.
Funding Period: 2018-2022.

Dutch Heart Foundation
High throughput Screening to identify novel molecules enhancing the activity of the CArdio-Protective Enzyme 5-oxoprolinase (OPLAH) for the treatment of Heart Failure. – eSCAPE-HF.
Principal Investigator: Peter van der Meer (UMC Groningen).
Funding Period: 2018-2021.

Molecular Life Sciences and Health (University of Groningen)
A chemoproteomic approach to study advanced glycation end-products.
Principal Investigators: Peter Horvatovich and Martin Witte (Stratingh Institute, University of Groningen).
Funding Period: 2017-2021.

Molecular Life Sciences and Health (University of Groningen)
Combining liquid chromatography-electrochemical detection with mass spectroscopy for powerful characterization of novel neuroactive gut bacterial metabolites with potential antimicrobial activity.
Principal Investigators: Hjalmar Permentier and Sahar El Aidy (Groningen Biomolecular and Biotechnology Institute (GBB), University of Groningen).
Funding Period: 2017-2021.

H2020-MSCA-ITN-2017; Marie Skłodowska-Curie Innovative Training Network (ITN) - European Training Network (ETN)
Analytics for Biologics (A4B).
Principal Investigator: Hartmut Schlüter (University Medicine Hamburg, Germany).
Funding Period: 2017-2020.

H2020-MSCA-COFUND-2016; Marie Skłodowska-Curie Action
'PROMINENT' Personalised Medicine in Diabetic Chronic Disease Management.
Principal Investigator: Dick de Zeeuw (UMC Groningen).
Funding Period: 2017-2020.

NWO-TTW 15230
Nano-patterned Electrochemical Surfaces for Protein Analysis and Drug Synthesis
Principal Investigator: Mathieu Odijk (Twente University, Enschede, The Netherlands).
Funding Period: 2017-2021.

EU-COST CA16113
CliniMARK: 'good biomarker practice' to increase the number of clinically validated biomarkers.
Principal Investigator: Theo Luider (Erasmus Medical Center, Rotterdam).
Funding Period: 2017-2021.

8 Teaching

8.1 Main courses

Academic Research & Communication Skills 1, WPFA18001, essay & poster mentoring	February – June 2021
Academic Research and Communication Skills 2, WBFA003-04, presentations & mini thesis mentoring, lecture	September – October 2021
Academic Research & Communication Skills 1, WBFA001-05, scientific paper analysis, mentoring	November 2021
Bioanalytical Omics Techniques/Advance Bioanalysis electives (WBBY073-05)	February 1-19, 2021
BMS from Big Data to Personalized Medicine (WMBM008-05), Proteomics applications for personalized medicine	October 1, 2021
Bachelor thesis & projects	September – November 2021
Biostatistics (WBFA011-05)	November 15, 2021 – December 7, 2021.
Bioanalysis (WBFA19004)	May 31 – July 09, 2021
Drug Development (masters)	September 13, 2021
MMIT Systems biology and Medicine (Top class 2)	September 28, 2021
Mass Spectrometry (open course)	January 13 – 14, 2022deep

Bischoff, R., member of the Board of Examiners (Examen Commissie) Pharmacy (till July 2021).
Bischoff, R., tutor for the master Medical and Pharmaceutical Sciences (MPS) (till July 2021).
Bischoff, R., tutor for the master Medical and Pharmaceutical Sciences (MPS) and masters of Biomedical Sciences (BMS).
Horvatovich, P., member of the Admission Committee (Toelating Commissie), MPS.

8.2 Special teaching activities

March 12, 2021, Teaching in “Master of medicament” at University of Strasbourg, Faculté de Pharmacy (online) course “Introduction à la protéomique”.

8.3 Student projects

- Victoria Aboagye, finished May 2021, MPS master’s project: Deamidation of asparagine in the Complementary Determining Region of (CDR) Trastuzumab in complex with Her-2. Supervisor: Baubek Spanov.
- Dominique ter Maat, start September 07, 2020, bachelor project Hanze University: Extracellulaire vesikels als potentiële biomarkers voor, door medicijnen ontwikkelde, cholestase. Supervisor: Janine Stam.
- Mats Nitert, start September 2021. Bachelor project Hanze: Development of targeted proteomics assays for potential cancer biomarkers. Supervisor: Karin Wolters.
- Marjolein Korenhof, start September 2021. Bachelor project Hanze: Determination of the anti-toxic effect of collagenase and benzonase on chlorpromazine-induced cholestasis in precision-cut liver slices. Supervisor: Janine Stam.

- Rob Frederiks, start May 2021, Pharmacy master research project on “Resolving chimeric spectra using independent component analysis”. Supervisor: Alejandro Sánchez Brotons.
- Jordan Zwerwer, start October 4, 2021, bachelor project Hanze University on “Development of an LC-MS method for the quantification of total sex hormone binding globulin in human serum”. Supervisor: Bas Sleumer.

8.4 Individual teaching

- Bachelor Pharmacy Assay: Enzymatic Digestion of Proteins in Biological Samples for Quantification with LC MS/MS - An Overview & Evaluation (Suzanne Willems). Supervision: Bas Sleumer and Nico van de Merbel.
- BMS Assay (Jing Zheng).
- Bachelor Pharmacy assay and projects (Emma Dorenbros, Aaricia Koen) on the topics of “Proteogenomic data integration”.

9 Outlook

We hope for an attenuation of the pandemic limitations in the year of 2022, which will allow us to return regularly to the laboratory, classrooms, and university workspaces, to meet each other and perform work more productively and in a better social environment.

Another key focus point for this year is to find a talented scientist having a research line focusing on the development of novel advanced proteomics/metabolomics methods or other fields of mass spectrometry. The new colleague will allow Analytical Biochemistry to expand its research and educational activities at GRIP, FSE and UMCG level, and will take over the very successful and organised teaching activities of Prof. Rainer Bischoff.

The next challenge is to get funding for scientific projects in clinical research, proteomics and metabolomics to replace the group of PhD candidates that finished their research projects. We are currently working on this by trying to acquire funding from NWO, EU, the Dutch Large Scale Research Infrastructure Initiative, as well as other organisations. And we are continuously looking for talented students with their own governmental funding. For these new projects, we now have an excellent infrastructure, including new recently acquired high-resolution mass spectrometers, automated liquid handling systems and high-performance computational clusters. We have also a rich repertoire of projects and expertise on which we can build. To give examples these are the new reagent sets for AGE modified protein identification and for DIA multiplexing, the electrochemistry-mass spectrometry integrated system allowing to perform specific transformation and labelling of drugs and metabolites, the PASTAQ codebase processing with high accuracy DDA LC-MS/MS dataset being currently extended to process DIA data and to process isotopically labelled data, the proteogenomics pipeline allowing to identify sample and patient specific protein variants, our lipidomics and metabolomics wet and dry lab workflows and pipelines, the methodology developed to study proteoforms of biologicals and the divers collection of ongoing clinical projects. Our other strength is the large collaboration network and joint projects with other academic research groups within GRIP, FSE and UMCG as well as other groups at a national and international level, such as the intensive joint work with the Swedish Melanoma Cancer Moonshot Consortium.

We are also working together with Prof. Daan Touw (UMCG) to establish a new research line and trying to acquire a MALDI mass spectrometry imaging platform with a high-resolution mass spectrometer. If it is successful, the platform will allow us to study spatial distribution of drugs, small molecules, peptides, and proteins. For this platform, we already possess of a state-of-the-art data pre-processing method and experience in studying distribution of integrins using photocleavable Ru-complex. This platform will open collaborations with the UMCG Microscopy & Imaging Center (UMIC, Ben Giepmans) for developing multi-modal imaging methods and will be the starting point for an integrated pathology-multi-omics profiling platform.

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