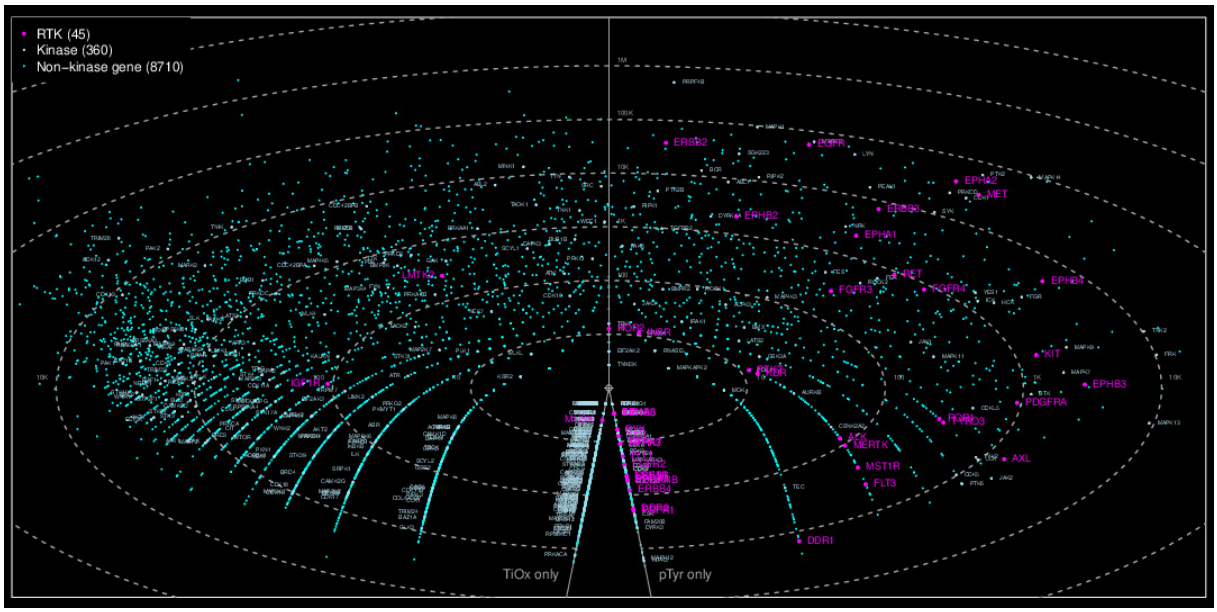
OncoProteomics Laboratory

Core Facility Mass Spectrometry-based Proteomics of Amsterdam UMC

Progress Report
2018-2021



Amsterdam UMC
Universitair Medische Centra

OncoProteomics Laboratory

Core Facility Mass Spectrometry-based Proteomics of Amsterdam UMC

Progress Report
2018-2021

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Cover image Phosphoproteome library consisting of 807110 unique high-quality kinase and susbtrate phosphopeptides, courtesy Dr. Alex A. Henneman.

Contents

Foreword

This progress report 2018-2021 covers proteomics research of the OncoProteomics Laboratory (OPL) and collaborators, as well as proteomics activities performed in the context of our function as Proteomics Core Facility. It harbors information on our mass spectrometry infrastructure and proteomics expertise, output, academic activities and includes summaries of running core and collaborative projects (detailed in appendix 2).

Here follows a brief overview of the highlights of the past 3,5 years:

- In total 58 peer-reviewed research articles were published including papers in Molecular Systems Biology, Nature Communications, Molecular and Cellular Proteomics, Cell Report, EBioMedicine, EMBO Journal, Bioinformatics and Journal of Extracellular Vesicles.
- Seven grants were obtained as PI/coPI: 1. KWF consortium grant on colorectal cancer multi-omics including phosphoproteomics (with Wessels, NKI; Verheul, Radboud MC and Trusolino, Cancer Candiolo Institute); 2. Health Holland project "Omics-Predict" on response prediction in esophageal cancer (with Van Laarhoven, Cuppen of Hartwig Medical Foundation and Merck); 3. two PhD students in the context of a Marie Curie European International Training Network "Secret" on the cancer secretory pathway; 4. Weston Brain Institute on CSF biomarkers for early Alzheimer's Disease (with Scheltens and Teunissen); 5. coPI on KWF high risk project of Pegtel and Westerman; 6. coPI on a KWF grant of Dr. De Lange and Wolthuis to investigate STAG2-deficient tumors; 7. A Netherlands eScience Center grant written bij Dr. Thang Pham on innovative AI-driven phosphoproteomics data analysis.
- To be able to support more projects and users with our small team, we acquired a liquid handling station and automated the global phosphoproteomics workflow.
- Our bioinformatics pipeline for integrative inferred kinase activity scoring in single samples (Molecular Systems Biology 2019) has now been successfully applied in multiple projects for drug response prediction, resulting in 6 published studies. Moreover, the webserver inkascore.org currently has 217 registered users world-wide who ran 495 analyses at the time of this writing.
- Important for multi-omics projects with limited sample such as needle biopsies, we showed that high quality phosphoproteomics is possible on the left-over fraction after acid guanidinium thiocyanate-phenol-chloroform extraction used for DNA and RNA isolation. See further for this and other method development, pages 58 - 66 of this report.
- Important for clinical proteomics is highthroughput reproducible measurements. In a multi-center study conducted in nine countries, we showed that harmonized MS based on data independent acquisition can yield highly reproducible and robust data (Nature Communications 2020).
- As co-chair of the Cancer-Human Proteome Project, I initiated TCPA (a la TCGA). In the past years we managed to compile a pan-cancer proteome landscape of > 1000 cancers based on data-independent acquisition mass spectrometry in collaboration with a a few laboratories. Our most notable partner is Tiannan Guo at Westlake University in China, whom we also visited with the core group in the fall of 2019. Currently we jointly analyze the data that we aim to make available via a queriable database.
- Last december, I was elected Vice-President of the European Proteomics Association so in the coming 3 years I will also be engaged with the European proteomics community.
- Finally, despite the restrictions of 2020-21, we were quite productive because luckily for all running projects we had lots of data to mine.

I hope you will enjoy reading on our OncoProteomics and other biomedical proteomics research and that this report triggers new ideas and collaborative projects.

Connie Jimenez
Head OPL, Professor of Translational OncoProteomics, Dept. Medical Oncology, Amsterdam UMC

Members of the OncoProteomics Laboratory:

Head: Prof. dr. Connie R Jimenez

Mass Spectrometry:

Dr. Sander Piersma (OPL-core: nanoLC-MS/MS)

Dry lab analysis:

Dr. Thang V. Pham (OPL core: (bio)informatics)

Dr. Jaco C. Knol (OPL core: down-stream data mining)

Wet lab research technicians

Ing. Richard Goeij-de Haas (OPL core)

Post-doctoral fellows:

Dr. Franziska Böttger (CRC and NSCLC predictive biomarkers)

Dr. Frank Rolfs (HR deficiency predictive biomarkers in breast cancer)

Dr. Alex Henneman ((bio)informatics, ICT)

PhD students:

Andrea Valles (pancreatic cancer phosphoproteomics)

Madalena Nunes Monteiro (exosomal pathway as source of biomarkers and drug targets)

Catarina de Almeida Marques (cancer secretory pathway for biomarkers target disocvery)

Iris Glykofridis (Role folliculin in renal cancer)

Giulia Mantini (Integrative analysis PDAC omics)

Ayse Erozenci (Urine exosomes PrCa)

Valentina Cordo (t-ALL phosphoproteomics)

Translational scientists:

Mariette Labots MD

Dr. Irene Bijnsdorp

Visiting PhD students:

Madiha Mumtaz (Pakistan), Mohan Shankar (India), Laura Lorenzo Sanz (Spain), Merih Ozverin (Germany), Elena Gutierrez Galindo (Germany), Alexia Gali (Greece), Elisa Suarez (Spain)

Internship students 2018-2020:

Eight master internship students (Oncology, Biomedical Sciences and Bioinformatics), four HLO internship students and three students writing their literature thesis.

OncoProteomics Laboratory Progress Report 2018-2021

Core Facility Mass Spectrometry-based Proteomics of Amsterdam UMC

Introduction

To better understand cancer in all its facets and work towards improved diagnostics and treatment of cancer, the OncoProteomics Laboratory of the Amsterdam UMC focusses on the direct global analysis of the functional building blocks of life, *i.e.*, the proteins which activity and functions are highly deranged in cancer cells. Unbiased (phospho)protein profiling by mass spectrometry-based proteomics offers a means to measure the biochemical impact of cancer-related genomic abnormalities, and thereby can bridge the gap between cancer genome information and observed cancer phenotype (Figure 1). **Therefore, OncoProteomics is of high interest for all programs/ themes of the Cancer Center Amsterdam of the Amsterdam UMC.**

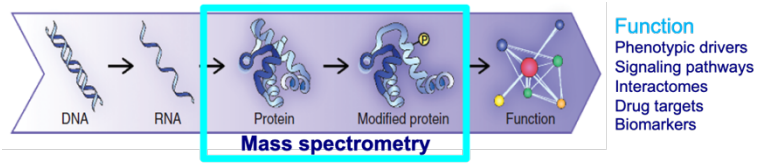

Figure 1. Proteomics, global analysis of the functionally relevant proteins

In collaboration with cancer researchers and clinicians of the CCA and external collaborators, our studies encompass the full spectrum of analyses in cancer cell lines, organoids, mouse models (both genetic and patient-derived xenografts) and clinical samples. The latter is enabled by our embedding in a clinical department (Medical Oncology) at Amsterdam UMC and our ample contacts with clinicians. **This setting ensures working on clinical needs, which is key to the mission of the Cancer Center Amsterdam.**

The multi-disciplinary OPL team includes core members with expertise in mass spectrometry, biology, biochemistry, and bioinformatics, and post-docs and PhD students with life science and clinical background (for more information, see www.oncoproteomics.nl). Since its foundation in **2006** with a **start-up grant of the Cancer Center Amsterdam**, the OPL has acquired a strong reputation as cancer proteomics center. To ensure streamlined profiling and analysis, robust label-free quantitation strategies and dedicated statistics have been developed for protein expression profiling and biomarker and drug target discovery (J. Prot. Res. 2010; J. Prot. 2014; Mol. Syst. Biol. 2019; Bioinformatics 2010, 2012, 2016, 2020).

Over the years, we have shared our know-how and provided high quality mass spectrometry data to CCA and Amsterdam UMC researchers and beyond. Our proven ability to collaborate can be evidenced from the multiple CCA and KWF projects with Jimenez as (co)PI or collaborator and investigators at CCA and multiple national institutes (see below for an overview of the ongoing cancer proteomics research collaborations).

We strongly believe that multi-disciplinary collaboration is key to achieve higher impact science with the ultimate goal to have clinical impact.

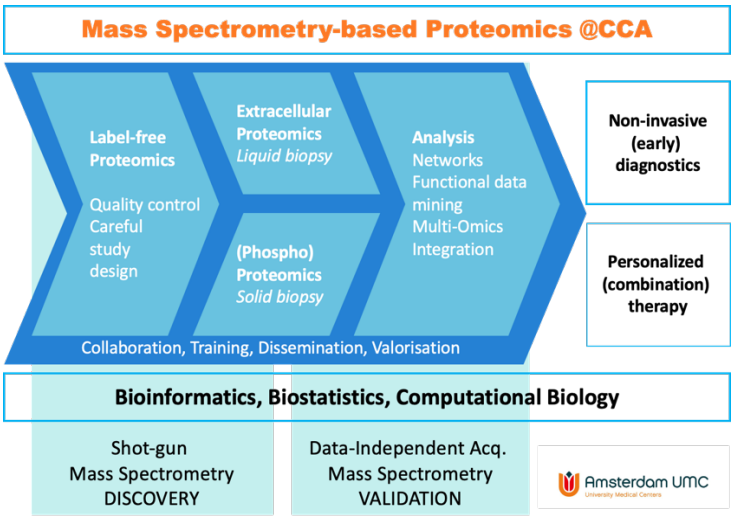

Fig. 2. Overview OncoProteomics

Cancer proteomics research @OPL

Our OncoProteomics research can be divided into **methodological research** and two broad cancer research lines (figure 2):
1. Analysis of tumor microenvironment, via secretome, exosome and proximal fluid proteomics to develop **non-invasive biomarker applications**
2. Analysis of cancer signalling pathways via intracellular (phospho)proteomics to enable **target discovery, patient stratification and response prediction.**

These research lines have been successfully applied in many **collaborative projects** in various tumor types, including colorectal cancer, breast cancer, lung cancer, pancreatic cancer, prostate cancer and leukemias (see overview in appendix). The obtained (phospho)proteome data provide a valuable addition to the existing large collections of DNA and RNA datasets, thereby contributing to the multi-omics perspective of cancer and its precursors and have revealed novel candidate biomarkers and drug targets that are in different phases of validation.
Highlights of research line 1 include the discovery and validation of novel stool markers for colorectal cancer screening using stool proteomics (Ann. Int. Med. 2017). Antibody assays for the top 10 protein stool markers are currently being tested in prospective validation cohorts with so far positive results (Ann. Int. Med. 2021). Another highlight is the urine exosome proteomics work that reveals the potential for not only non-invasive prostate cancer detection but also for non-invasive pan-cancer detection. Non-cancer highlights include our CSF proteomics efforts to discover and validate biomarkers for Alzheimer's Disease and other dementias.
Highlights of research line 2 include the application of our downscaled phosphotyrosine workflow in a clinical trial setting (Cancers 2020), underscoring the feasibility of clinical phosphoproteomics. In the coming years, we aim to perform phosphoproteomics on tumor biopsies collected in multiple clinical trials to develop improved patient selection for targeted therapy. Our newly developed computer algorithms that can pinpoint highly active protein kinases in single biological samples on the basis of label-free phosphoproteomic data (Integrative Inferred Kinase Activity (INKA) analysis, Mol. Syst. Biol. 2019) will be a key component of a future multi-omics personalized medicine pipeline. Its value was recently shown in the preclinical setting in several published studies.
We anticipate that a combined multi-OMICs view of the tumor of a patient, that includes not only the genome but also the functionally relevant proteome and phosphoproteome, will be essential to revolutionize molecular cancer therapy and deliver on the promise of personalized medicine.

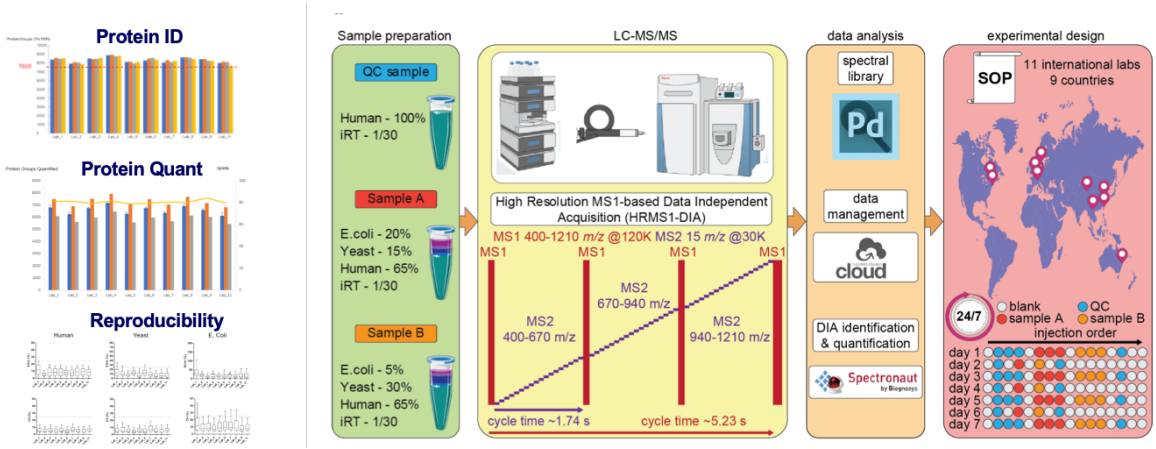

Fig. 3. Robust, sensitive, and reproducible data generation across eleven sites in nine countries on seven consecutive days in a 24/7 operation mode.

New developments in clinical cancer proteomics

One exciting new development is next generation quantitative proteomics based on data-independent acquisition (DIA) mass spectrometry. This novel approach was the focus of the NWO-Middelgroot grant that Jimenez acquired in 2016. DIA-MS uses parallel peptide fragmentation and less complex biochemical workflows, together reducing missing values, processing time and costs, thereby enabling large scale clinical proteomics. DIA-MS will be key for phosphoproteomics for precision oncology. After implementing and bench-marking DIA-MS at the OPL, we participated in a multi-center study, conducted in nine countries. We showed that harmonized MS with standardized data acquisition can yield highly reproducible data (Fig. 3. Xuan et al., Nature Communications, 2020). Currently DIA-MS is the preferred method for highthroughput (clinical) proteomics.

To capitalize on these developments, Jimenez initiated a **multi-laboratory collaborative cancer proteome profiling effort**, a la TCGA, that represents **T**he mass spectrometry-based **C**ancer **P**roteome **A**tlas (**TCPA**) project (pilot atlas with 325 cancer samples shown in Figure 4). Many CCA PIs supplied tumor tissue for this exciting effort. **The global aims of the analysis will be: 1. to better understand the molecular underpinnings of cancer; 2. to identify core and cancer type enriched molecular therapeutic targets and biomarkers.**

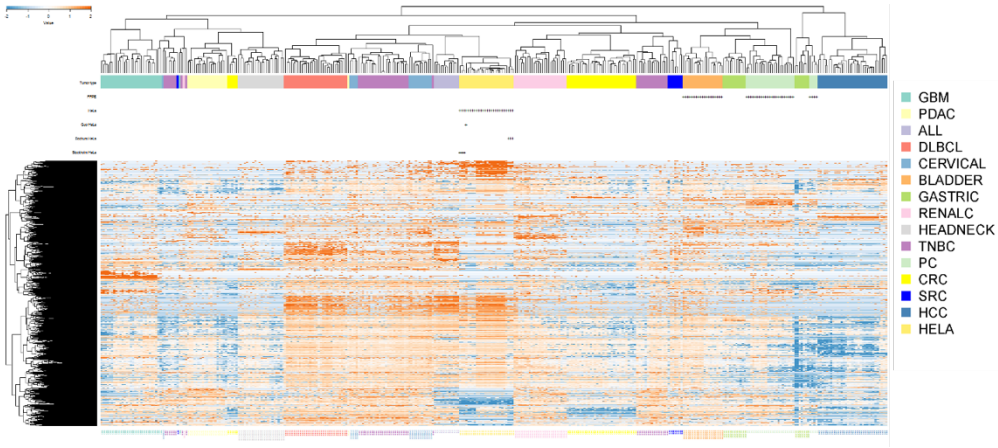

Figure 4. DIA-MS to generate pan-cancer atlas. Unsupervised cluster analysis using the first set of 325 Amsterdam tumor proteomes and Hela reference proteomes from all participating laboratories. Please note that all Hela samples cluster together, underscoring the feasibility of TCPA based on highthroughput DIA-MS.

Currently 6 clinical proteomics laboratories across Europe and Asia participate and have submitted data for integration into the atlas. A first collaborative manuscript reporting a deep mass spectral library based on deep cancer proteomes for > 15 tumor types has been published in 2020. We recently completed data generation for a draft 1.0 atlas, consisting of 1000 cancer proteomes for ~20 tumor types. This unique dataset -after tackling some ICT challenges- is being subjected to advanced data mining.

Proteomics support to users

As proteomics core facility, OPL provides support in all the steps of a proteomics experiment: study design (discussed at project intake with head OPL) and sample preparation step (research technician Dr. Richard de Haas), mass spectrometry (Dr. Sander Piersma) and dedicated analysis (Dr. Thang Pham). The analysis result is submitted in a user-friendly excel file to the end-users by Dr. Pham. Miscellaneous logistics support and optional support in functional data mining is given by Dr. Jaco Knol. For more information, see our website www.oncoproteomics.nl.

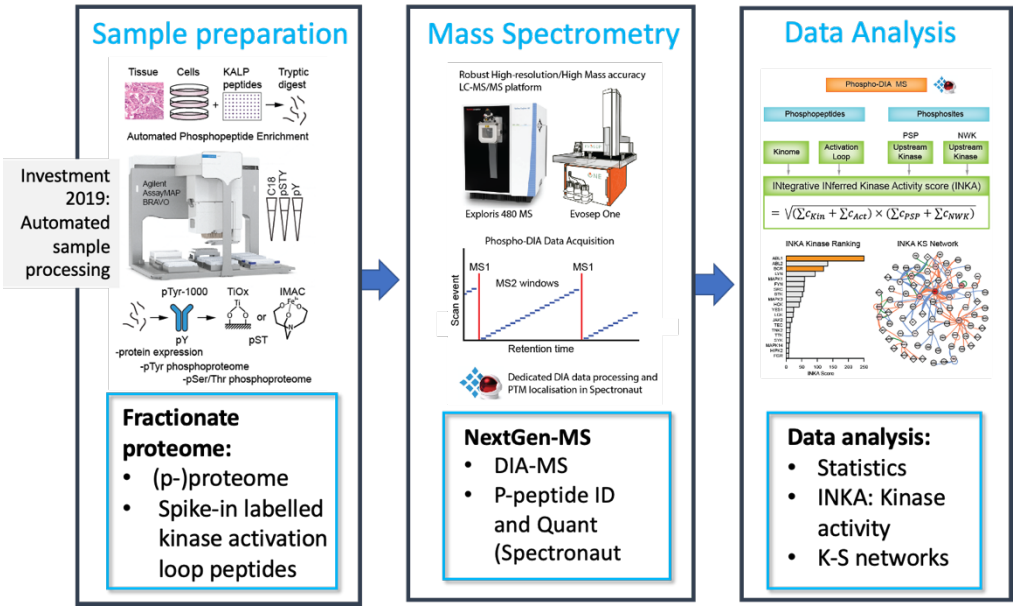

Figure 5. Steps proteomics experiment. To enable "full service" support to users, we acquired the Bravo station and recently automated the global phosphoproteomics workflow.

To showcase what we achieved in the past 3,5 years (2018-2021), the next sections of this report provide:

- An overview of the major collaborative research projects 2018-2021 (**pages 11-15**)
- A listing of publications related to OncoProteomics research lines and methodology development (pages **16-20**)
- Publications based on smaller collaborations/ core facility functions (**pages 20-22**)
- Collaborator quotes (**pages 23-27**)
- An appendix with OncoProteomics Facts and Figures (**page 27**)
- An appendix with abstracts of ongoing and completed projects employing proteomics in the past 3,5 years (**pages 28-91**)

Major OncoProteomics Projects past 3,5 years (2018-2021)

research line 1

Extracellular / biofluid proteomics for development of non-invasive biomarker applications

Urinary extracellular vesicles and their content as novel markers for minimally invasive diagnosis and prognosis of prostate cancer.
Collaborators: Dr. Irene Bijnsdorp (Dept. Urology) Prof.dr. Guido Jenster (ErasmusMC), Prof.dr. Jack Schalken (RadboudMC)

- Proteomics of urinary vesicles of patients with prostate cancer as part of a multi-OMICs effort to develop novel biomarkers
- **KWF/Alpe d'Huzes EMCR 2015-8022**, PI Jenster, coPIs Jimenez, Schalken (ends in 2021). Funding for 1 PhD student at AmsterdamUMC. This project has yielded 6 publications and 2 manuscripts.

Proteomics of the cancer secretome
Collaborators: Dr. Irene Bijnsdorp (Dept. Urology), Dr. Meike de Wit (NKI), Dr. Angelika Hauser (University of Stuttgart)

- Proteomics of cancer cell and tumor tissue intracellular proteins and secreted fractions (soluble proteins and extracellular vesicles) to identify drug targets in the secretory pathway and biomarkers for non-invasive screening
- This research started with internships of various master students. Since 2020 this research line is an EU funded project with 2 PhD students (Marie Curie ITN SECRET, coordinator Angelika Hauser).

Platelet proteomics for non-invasive cancer detection
Collaborators (various projects): Prof.dr. Henk Verheul, Prof.dr. Arjan Griffioen (Medical Oncology), Prof.dr. MGA Oude Egbrink (Maastricht UMC), Dr. E. Giovanettti, Dr. Tom Wurdinger

- In 3 projects. we established that the cancer platelet proteome harbors diagnostic information (Sabrkhany et al., 2018; Mantini et al. 2020, Walraven et al. 2021).

Novel cerebrospinal fluid protein biomarkers for progression of Mild Cognitive Impairment stages to Alzheimer's Disease
Collaborators: Prof.dr. Charlotte Teunissen, Prof.dr. Philip Scheltens

- This project is based on a long-standing CSF proteomics effort with the aim to identify biomarkers discriminating patients with mild cognitive impairment progressing or not progressing to AD. To this end, we analyzed two independent well-characterized CSF dementia cohorts, using next generation high-throughput mass spectrometry based on DIA-MS.
- Funding **Weston Brain Institute NR180096** (PI Jimenez, coPI Teunissen) for one post-doc (ended in 2021). Manuscript in preparation.

research line 2

(Phospho)proteomics of cancer cells and tissues for target discovery and precision medicine

Phosphoproteomics for precision medicine

Collaborators: Dr. Mariette Labots, Dept. Medical Oncology, Prof.dr. Henk Verheul (RadboudUMC).

- Mass spectrometry-based phosphoproteomics analyses of cancer cell lines and tumor needle biopsies collected in clinical trials
- This work was initiated with funding by Vitromics Health Services and the department and includes multiple projects (a phase I clinical study, applications in GBM, renal cancer) and several clinical trial side studies planned.

Interrogating the (phospho)proteome for drug targets in pancreatic cancer

Collaborators: Dr. Elisa Giovannetti (Medical Oncology), Dr. Maarten Bijlsma (Lexor), Prof.dr. Geert Kazemier (Surgery), Prof.dr. Hanneke van Laarhoven (Lexor/Medical Oncology)

- Phosphoproteomics analyses of patient-derived xenograft models developed by my collaborators and clinical samples as well as functional testing of potential drug targets.
- Shared PhD student in the context of a VUmc-AMC alliance project. The data of the alliantie project were used to obtain funding from KWF to continue and expand the project with currently 2 PhD students working on the project.
- **KWF VU2016-1012** project (PI Jimenez, coPIs Bijlsma and Giovannetti) (ends in 2021)
- Sofar this work has yielded 9 publications by Le Large et al. and Mantini et al. and three manuscripts are in preparation (Valles et al).

Phosphoproteomics analysis to enable precision medicine for anti-EGFR therapy in colorectal cancer

Collaborators: Prof.dr. Lodewyk Wessels (NKI), Prof.dr. Livio Trusolino (Italy) and Prof.dr. Henk Verheul (Medical Oncology, now RadboudUMC)

- Large-scale mass spectrometry-based proteomics and phosphoproteomics analyses of a unique collection of patient-derived xenograft models of colorectal cancer (n=150) that were developed and genomically characterized by Trusolino.
- Orals by PhD student Robin Beekhof at AACR2016 and HUPO2017 and publication in Mol.Systems Bio 2019.
- Based on preliminary data on 29 PDX models, a new **KWF consortium grant** was acquired (1.1 MEuro) for 2 post-docs and technician. Project start in 2020.

Phosphoproteomics for target discovery and response prediction in acute myeloid leukemia

Collaborators: Prof.dr. Jacqueline Cloos, Dr. Jeroen Janssen, Dept. Hematology

- Mass spectrometry-based phosphoproteomics analyses of cancer cell lines and patient samples from the biobank of my hematology collaborators
- Joint project funded by **CCA** with PhD student Caroline van Alphen. Work was continued by second PhD student (David Cucchi) funded by the Dept. Hematology. This effort has resulted in 3 publications and orals at several international meetings

- Grant proposals will be submitted to Pharma to further clinical validate our results.

Phosphoproteomics, genomics and integrative analysis to enable precision medicine for bifunctional immunotherapy in esophageal cancer
Collaborators: Prof.dr. Hanneke van Laarhoven, Prof.dr. Edwin Cuppen (Hartwig Medical Foundation), Dr. Gerard Stege (Merck)

- This project that started last year aims to identify biomarkers that can predict treatment response in the individual patient. To this end, we will perform genome, proteome and phosphoproteome profiling of needle biopsies collected in the Tapestry clinical trial (PI Van Laarhoven).
- Funding: Health Holland (**LSHM19083**; PI Jimenez; coPIs Van Laarhoven, Cuppen, Stege). Post-doc for proteomics/data analysis will start in 2022/23.

Discovery and clinical validation of novel protein biomarkers for homologous recombination deficient breast cancer
Collaborators: Prof.dr. Jos Jonkers (NKI), Prof.dr. Sven Rottenberg (NKI), Prof.dr. Paul van Diest (UMCU)

- Mass spectrometry-based analyses of genetic mouse models of breast cancer and currently of a large collection of patient-derived xenograft models of breast cancer by proteomics and phosphoproteomics
- The collaboration was first supported by a **CCA** grant and later supported by **KWF project: VU2013-6020** (PI Jimenez, coPIs Jonkers, Van Diest). This project ended after an extension year in 2020 and has resulted in a methodolical publication. Multiple manuscripts are being prepared.

Identification of biomarkers by whole-genome sequencing and phospho-proteomics to predict responses to high-precision cancer medicines in T-cell acute lymphoblastic leukemia
Collaborator: Dr. Jules Meijerink (Prinses Maxima)

- Phosphoproteomics analyses of genomically characterized T-ALL samples for discovery of hyper-activated kinases as novel drug targets
- Joint PhD student Valentina Cordo on a **KWF project 2016-10355** (PI Meijerink, coPI Jimenez). The first manuscript is in revision for Nature Communications.

Response prediction for cisplatin-based treatment regimens in non-small cell lung cancer using a protein-based assay
Collaborators: Dr. Idris Bahce (Dept. Pulmonology), Dr. Teodora Radonic (Dept. Pathology) Prof.dr. Anton Berns (NKI), Dr. Sjoerd Burgers (NKI), Prof.dr. Egbert Smit (NKI)

- Mass spectrometry-based proteomics analyses of patient FFPE material to identify and validate protein signatures that can predict response to platina-based treatment regimens. (Bottger et al., 2019 and manuscript in preparation).
- The collaboration was supported by **KWF project VU2014-6816** (title as above; PI Jimenez, coPIs Burgers and Grunberg). This project ended after an extension year in 2020
- Side-project: Characterization of tumor heterogeneity and cisplatin sensitivity in mouse models of small cell lung cancer (Bottger et al., 2019).

Optimized high-order low-dose drug mixtures boost selectivity and efficacy of anti-cancer targeted combination treatments
Collaborators: Prof.dr. Arjan Griffioen (dept. Medical Oncology), Dr. Patrycja Nowak-Sliwinska (Austria)

- Phoshoproteomics and bioinformatics analyses of cancer cell lines and correlation of baseline data to response to drug combinations. This work resulted in 3 publications that underscore the value of INKA analysis for prediction of kinase combination therapy.

Unravelling signaling pathways involved in colorectal adenoma-to-carcinoma progression
Collaborators: **Dr. Beatrix Carvalho (NKI), Prof.dr. Gerrit A Meijer (NKI)**

- (Phospho)proteomics analyses of patient material that was selected from the biobank by my collaborators based on pathological and genomic selection criteria
- Joint post-doc on project written jointly by Carvalho and Jimenez and that was funded by the KWF: **KWF VU2014-6813** (PI Carvalho (NKI), coPIs Jimenez, Meijer). Completed in 2020, manuscript in preparation.

Tumor-specific protein biomarkers for early detection of colorectal cancer
Collaborators: **Dr. Remond JA Fijneman, Prof.dr. Gerrit A Meijer (NKI)**

- Proteogenomics analyses of patient material that was selected from several biobanks by my collaborators, based on pathological and genomic selection criteria by joint PhD student
- **KWF VU2014-6025** (PI Fijneman, coPIs Jimenez, Meijer). This project resulted in 4 publications and a PhD thesis. Completed in 2019.

Inhibiting Extracellular Vesicle release from breast cancer cells to combat drug resistance
Collaborators: Dr. Michiel Pegtel (Dept. Pathology), Dr. Bart Westerman (Dept. Neurosurgery)

- Phosphoproteomics analyses of preclinical models to unravel signaling pathways involved in extracellular vesicle release
- **KWF High Risk Project 2018-11308**, PI Pegtel, coPIs Westerman and Jimenez

The Molecular Signalling Pathways of Folliculin (FLCN): a Tumor Suppressor in Birt-Hogg Dubé Hereditary Kidney Cancer
Collaborator: **Dr. Rob Wolthuis (Clinical Genetics)**

- Proteomics and bioinformatics analyses of isogenic cell model made folliculin knock-out with CRISPR-Cas
- Joint PhD student supported by **CCA/VUmc** funding to the Dept. Clinical Genetics (1 publication, 1 submitted and 1 in preparation). Project ended in 2021.

Improving clinical management of colon cancer through CONNECTION, a nation-wide Colon Cancer Registry and Stratification effort
Collaborators: Prof.dr. Jan Paul Medema (AMC), Prof.dr. Jan Ijzermans (ErasmusMC), Prof.dr. Koopmans (UMCU), Prof.dr. Van Krieken (Radboud MC)

- Contribution: Identification of protein biomarkers for colorectal cancer consensus subtypes that have different prognosis and potentially also different treatment response
- **KWF/Alpe d'Huzes consortium UvA2013-6331** (PI JP Medema, coPIs Ijzermans, Koopmans, Jimenez, Van Krieken). Proteomics part has ended in 2019.

Glycans to reprogram the immune response
Collaborators: Dr. Sandra. Van Vliet (MCBI), Prof.dr. Yvette van Kooyk (MCBI)

- Phosphoproteomics, proteomics and bioinformatics analyses of sugar-stimulated immune cells
- Contribution to one study of PhD student Eveline Li of the Dept. MCBI (two thesis chapters and one publication).

Mechanisms in the cellular defense against oxidative stress
Collaborators: Dr. Josephine Dorsman and Prof.dr. H. Joenje (Clinical Genetics)

- Proteomics and bioinformatics analyses of hyperoxia-resistant and sensitive cell lines to identify novel proteins and cellular mechanism(s) involved in the defense against oxidative stress
- Contribution to 2 thesis chapters of PhD student Monique Corbin of the Dept. Clinical Genetics and role as second promotor.

OncoProteomics methodology projects last 3,5 years

To ensure high quality research and optimize cancer proteomics applications and translational research, we spent considerable time on method explorations. This work was focused on benchmarking label-free proteomics, developing dedicated statistics for label-free proteomics data, exploration of phosphoproteomics capture methods, methods for exosome isolation and biofluid profiling and more and has resulted in 13 publications in the past 5 years (one of them in Nature Communications) out of total of 20 methods papers since 2010.

Transformer-based deep learning for next generation mass spectrometry-based phosphoproteomics

- Thang Pham (OPL)
- Project funded by Netherlands EScience Center for one post-doc at OPL and one AI expert at eScience.
- In this newly funded project, we will apply state-of-the-art AI technologies to our unique collection of phosphoproteomics data. When successful, this project will change current practice in DIA-MS analysis, and catalyze both cancer signaling research and biomarker and target discovery enterprises to ultimately improve cancer diagnosis and treatment.

Smaller scale experiments performed/ planned/ in progress with:

Linda Smit (Hematology); Richard Groen (Hematology); Jacqueline Cloos (Hematology); Ruud Brakenhoff (Otolaryngology); Josephine Dorsman (Clinical Genetics); Job de Lange (Clinicsl Genetics); Marjolein van Egmond (MCBI); Michiel Pegtel (Pathology); Jolanda van den Velden (Physiology); Peter Hordijk (Physiology); Robert Szulcek (Physiology); Tanja Gruijl, Victor van Beusechem (Medical Oncology); Charlotte Teunissen (Clinical Chemistry); Esther Hulleman (Pediatric Oncology); Wilbert Bitter (Medical Microbiology); Oudeelferink

(AMC); Versteeg group (AMC); Jan Paul Medema (AMC); Hanneke van Laarhoven (AMC); Maurice Aalders (AMC); Marthe Minderman (AMC); Connie Bezzina (AMC); J. deGroot (E Meulendijks) (AMC); Erik Reits (AMC); Martine Smit (VU); Ronald van Kesteren (VU); Edith Houben (VU); Winan van Houdt (NKI); Katrien Keune (Rijksmuseum); Purificacion Munoz (Spain); Madiha Mumtaz (Pakistan); Mohan Shankar (India); Elena Gutierrez Galindo (Germany); Merih Ozverin (Germany); Alexia Gali (Greece)

OncoProteomics publications

Research line 1 Secretome/ Extracellular Vesicle/ Platelet/ Biofluid Proteomics for non-invasive applications

Manuscripts in preparation:

- Erozenci LA, Pham TV, Piersma SR, Dits NFJ, van Royen ME, Moorselaar RJA, Jimenez CR, Bijnsdorp IV. **Urine storage protocol that is feasible with extracellular vesicle research and proteomics**
- Erozenci LA, Piersma SR, Pham TV, Bijnsdorp IV, Jimenez CR **Longitudinal landscape of urinary EV proteome reveals stable protein expression patterns within and between individuals**
- Erozenci LA, Feenstra, F, Piersma SR, PhamTV, Wortel, J, Jenster, G, Bijnsdorp, IV, Jimenez, CR. **Identification of the pan-cancer extracellular vesicle surface proteome and its application to detect prostate cancer in urine.**
- Erozenci LA, Piersma SR, Pham TV, Van Moorselaar J, Vis A, Jenster G, Schalken J, Verhaegh G, Bijnsdorp IV, Jimenez CR. **The urinary extracellular vesicle proteome of prostate cancer reveals distinct expression patterns and promising biomarkers**
- Monteiro M, Marques C, de Wit M, Bishop-Currey L, Dusseldorp V, Piersma S, Pham T, Knol J, Sadeghi H, Meijer G, Fijneman R, Bijnsdorp I, Jimenez C **Proteomics profiling of colorectal cancer tissue-derived exosomes and secretome reveals non-invasive candidate markers involved in tumor proliferation and progression.**
- L Vermunt, Pham, T.V., Chiasserini, Schelforst T, M Delcampo, SR Piersma, TV Pham, JC Knol, P Scheltens, CE Teunissen, CR Jimenez. **Clinical proteomics of cerebrospinal fluid identifies and validates novel biomarkers for progression of Mild Cognitive Impairment stages to Alzheimer's Disease**
- Glykofridis IE, et al. **Cancer cell secretome and exosome linked to loss of FLCN in renal cells**

Publications:

- de Klaver W, Wisse PHA, van Wifferen F, Bosch LJW, Jimenez CR, van der Hulst RWM, Fijneman RJA, Kuipers EJ, Greuter MJE, Carvalho B, Spaander MCW, Dekker E, Coupé VMH, de Wit M, Meijer GA. **Clinical Validation of a Multitarget Fecal Immunochemical Test for Colorectal Cancer Screening : A Diagnostic Test Accuracy Study**. Ann Intern Med. 2021 Sep;174(9):1224-1231.
- Komor MA, Bosch LJ, Coupé VM, Rausch C, Pham TV, Piersma SR, Mongera S, Mulder CJ, Dekker E, Kuipers EJ, van de Wiel MA, Carvalho B, Fijneman RJ, Jimenez CR, Meijer GA, de Wit M. **Proteins in stool as biomarkers for non-invasive detection of colorectal adenomas with high risk of progression**. J Pathol. 2020 Mar;250(3):288-298.
- Sabrkhany S, Kuijpers MJE, Knol JC, Olde Damink SWM, Dingemans AC, Verheul HM, Piersma SR, Pham TV, Griffioen AW, Oude Egbrink MGA, Jimenez CR. **Exploration of the platelet proteome in patients with early-stage cancer.** J Proteomics. 2018 Apr 15;177:65-74.
- Walraven M, Sabrkhany S, Knol JC, Dekker H, de Reus I, Piersma SR, Pham TV, Griffioen AW, Broxterman HJ, Oude Egbrink M, Verheul HMW, Jimenez CR. **Effects of Cancer Presence and Therapy on the Platelet Proteome.** Int J Mol Sci. 2021 Jul 30;22(15):8236.
- Le Large TYS, Meijer LL, Paleckyte R, et al. **Combined Expression of Plasma Thrombospondin-2 and CA19-9 for Diagnosis of Pancreatic Cancer and Distal Cholangiocarcinoma: A Proteome Approach.** Oncologist. 2020;25(4):e634,Äêe643.
- Mantini G, Meijer LL, Glogovitis I, In 't Veld SGJG, Paleckyte R, Capula M, Le Large TYS, Morelli L, Pham TV, Piersma SR, Frampton AE, Jimenez CR, Kazemier G, Koppers-Lalic D,

Wurdinger T, Giovannetti E. **Omics Analysis of Educated Platelets in Cancer and Benign Disease of the Pancreas.** Cancers (Basel). 2020 Dec 29;13(1):66.

- Erozenci LA, Böttger F, Bijnsdorp IV, Jimenez CR. **Urinary exosomal proteins as (pan-)cancer biomarkers: insights from the proteome.** FEBS Lett. 2019 Jul;593(13):1580-1597.
- Bijnsdorp IV, Jimenez CR. **Large-Scale Urinary Proteome Dataset Across Tumor Types Reveals Candidate Biomarkers for Lung Cancer**. EBioMedicine. 2018 Apr;30:5-6.
- Erozenci LA, Piersma SR, Pham TV, Bijnsdorp IV, Jimenez CR. **Longitudinal stability of urinary extracellular vesicle protein patterns within and between individuals**. Sci Rep. 2021 Aug 2;11(1):15629.
- Bijnsdorp IV, Schelfhorst T, Luinenburg M, Rolfs F, Piersma SR, de Haas RR, Pham TV, Jimenez CR. **Feasibility of phosphoproteomics to uncover oncogenic signalling in secreted extracellular vesicles using glioblastoma-EGFRVIII cells as a model**. J Proteomics. 2021 Feb 10;232:104076.
- Erdbrügger U, Blijdorp CJ, Bijnsdorp IV, Borràs FE, Burger D, Bussolati B, Byrd JB, Clayton A, Dear JW, Falcón-Pérez JM, Grange C, Hill AF, Holthöfer H, Hoorn EJ, Jenster G, Jimenez CR, Junker K, Klein J, Knepper MA, Koritzinsky EH, Luther JM, Lenassi M, Leivo J, Mertens I, Musante L, Oeyen E, Puhka M, van Royen ME, Sánchez C, Soekmadji C, Thongboonkerd V, van Steijn V, Verhaegh G, Webber JP, Witwer K, Yuen PST, Zheng L, Llorente A, Martens-Uzunova ES. **Urinary extracellular vesicles: A position paper by the Urine Task Force of the International Society for Extracellular Vesicles**. J Extracell Vesicles. 2021 May;10(7):e12093.
- Garcia de Durango CR, Monteiro MN, Bijnsdorp IV, Pham TV, De Wit M, Piersma SR, Knol JC, Pérez-Gordo M, Fijneman RJA, Vidal-Vanaclocha F, Jimenez CR. **Lipopolysaccharide-regulated secretion of soluble and vesicle-based proteins from a panel of colorectal cancer cell lines.** Proteomics Clin Appl. 2021 May;15(2-3):e1900119.
- Böttger F, Schaaij-Visser TB, de Reus I, Piersma SR, Pham TV, Nagel R, Brakenhoff RH, Thunnissen E, Smit EF, Jimenez CR. **Proteome analysis of non-small cell lung cancer cell line secretomes and patient sputum reveals biofluid biomarker candidates for cisplatin response prediction.** J Proteomics. 2019 Mar 30;196:106-119.

Research line 2 Proteomics and phosphoproteomics of tissues for target discovery and diagnostic, prognostic and predictive biomarkers

Manuscripts in preparation:

- H. van der Wijngaart, R. Beekhof, JC. Knol, A. Henneman, R. de Goeij, S. Piersma, TV. Pham, C.R. Jimenez, HMW. Verheul, M. Labots. **Predictive biomarkers for response to sunitinib in patients with Renal Cell Carcinoma**.
- ME van Linde, M Labots, CG Brahm, KE Hovinga, PC de Witt Hamer, RJ Honeywell, R de Goei-Haas, H Dekker, SR Piersma, T Pham, WP Vandertop, CR Jiménez, HMW Verheul. **Tumor drug concentration and phosphoproteomic profiles after two weeks of treatment with sunitinib in patients with newly diagnosed glioblastoma.**
- Tineke E. Buffart, Rosanne A.H.M. van den Oord, Adriënne van den Berg, Riet Hilhorst, Niek Bastiaensen, Hans F.M. Pruijt, Adriaan van den Brule, Peet Nooijen, Mariette Labots, Richard R de Goeij-de Haas, Henk Dekker, Sander R. Piersma, Thang V. Pham, Theo van der Leij, Rik de Wijn, Rob Ruijtenbeek, Connie R., Jiménez, Henk M.W. Verheul. **Time dependent effect of cold ischemia on the phosphoproteome and protein kinase activity in colorectal cancer tissue freshly obtained from patients.**
- Tineke E. Buffart et al. **Phosphoproteomics of right and left-sided colon cancers reveals differentially activated drug targets.**
- Vallés-Martí A, Mantini G, Le Large T.S., Pham T.V., Piersma S.R., Knol J.C., Kazemier G, Laarhoven, H, Bijlsma M.F., Giovannetti E., Jiménez C.R. **Individualized low-drug combinations in PDAC: a phosphoproteomic approach.**
- Vallés-Martí A, Mantini G, et al. **Phosphoproteome landscape of PDAC.**
- Robin Beekhof, Andrea Bertotti, Valentina Vurchio, Francesca Cottino, Eugenia R. Zanella, Giorgia Migliardi, Alex A. Henneman, Jaco C. Knol, Richard R. de Haas, Sander R. Piersma, Mariette Labots, Henk M.W. Verheul, Livio Trusolino, Connie R. Jimenez. **Phosphoproteomic profiles associated with cetuximab sensitivity and resistance in colorectal cancer xenograft tumors.**
- Bottger et al, **The phosphoproteomic landscape associated with sensitivity and**

resistance to EGFR blockade in colorectal cancer.

- Cordo et al. **Phosphoproteomics of a panel tALL cell lines reveals oncogenic signaling and hyperactive tyrosine kinases as targets for treatment**
- Cordo et al. **Phosphoproteomics of tALL patient samples**
- F. Rolfs, S. Piersma, T. Pham, J. Knol, P. ter Brugge, R. de Bruijn, J. Wesseling, E. Marangoni, V. Serra, J. Jonkers* & C. R. Jimenez*. **Identification and validation of protein biomarkers for homologous recombination deficiency in breast cancer using patient-derived xenograft models and clinical samples**
- Glykofridis IE, et al. **Signaling pathway linked to loss of FLCN in renal cells**

Publications:

- Beekhof R, van Alphen C, Henneman AA, Knol JC, Pham TV, Rolfs F, Labots M, Henneberry E, Le Large TY, de Haas RR, Piersma SR, Vurchio V, Bertotti A, Trusolino L, Verheul HM, Jimenez CR. **INKA, an integrative data analysis pipeline for phosphoproteomic inference of active kinases.** *Mol Syst Biol*. 2019 Apr 12;15(4):e8250.
- van Beijnum JR, Weiss A, Berndsen RH, Wong TJ, Reckman LC, Piersma SR, Zoetemelk M, de Haas R, Dormond O, Bex A, Henneman AA, Jimenez CR, Griffioen AW, Nowak-Sliwinska P. **Integrating Phenotypic Search and Phosphoproteomic Profiling of Active Kinases for Optimization of Drug Mixtures for RCC Treatment**. Cancers (Basel). 2020 Sep 21;12(9):2697.
- Zoetemelk M, Ramzy GM, Rausch M, Koessler T, van Beijnum JR, Weiss A, Mieville V, Piersma SR, de Haas RR, Delucinge-Vivier C, Andres A, Toso C, Henneman AA, Ragusa S, Petrova TV, Docquier M, McKee TA, Jimenez CR, Daali Y, Griffioen AW, Rubbia-Brandt L, Dietrich PY, Nowak-Sliwinska P. **Optimized low-dose combinatorial drug treatment boosts selectivity and efficacy of colorectal carcinoma treatment**. Mol Oncol. 2020 Oct 5;14(11):2894–919.
- Rausch M, Weiss A, Zoetemelk M, Piersma SR, Jimenez CR, van Beijnum JR, Nowak-Sliwinska P. **Optimized Combination of HDACI and TKI Efficiently Inhibits Metabolic Activity in Renal Cell Carcinoma and Overcomes Sunitinib Resistance**. Cancers (Basel). 2020 Oct 28;12(11):3172.
- Franziska Böttger*, Ekaterina A. Semenova*, Ji-Ying Song, Jan van der Vliet, Miranda Cozijnsen, Giustina Ferone, Rajith Bhaskaran, Lorenzo Bombardelli, Sander R. Piersma, Thang V. Pham, Connie R. Jimenez*, Anton Berns*. **Tumor heterogeneity underlies differential cisplatin sensitivity in mouse models of small cell lung cancer**. Cell Rep. 2019 Jun 11;27(11):3345-3358.e4.
- Labots M, Pham TV, Honeywell RJ, Knol JC, Beekhof R, de Goeij-de Haas R, Dekker H, Neerincx M, Piersma SR, van der Mijn JC, van der Peet DL, Meijerink MR, Peters GJ, van Grieken NCT, Jiménez CR, Verheul HMW. **Kinase Inhibitor Treatment of Patients with Advanced Cancer Results in High Tumor Drug Concentrations and in Specific Alterations of the tumor phosphoproteome.** Cancers 2020 Feb 1;12(2) pii: E330.
- Mantini G, Vallés AM, Le Large TYS, Capula M, Funel N, Pham TV, Piersma SR, Kazemier G, Bijlsma MF, Giovannetti E, Jimenez CR. **Co-expression analysis of pancreatic cancer proteome reveals biology and prognostic biomarkers.** Cell Oncol (Dordr). 2020 Dec;43(6):1147-1159.
- Le Large TY, Mantini G, Meijer LL, Pham TV, Funel N, van Grieken NC, Kok B, Knol J, van Laarhoven HW, Piersma SR, Jimenez CR, Kazemier G, Giovannetti E, Bijlsma MF. **Microdissected pancreatic cancer proteomes reveal tumor heterogeneity and therapeutic targets.** JCI Insight. 2020 Aug 6;5(15):e138290.
- Le Large TYS, El Hassouni B, Funel N, Kok B, Piersma SR, Pham TV, Olive KP, Kazemier G, van Laarhoven HWM, Jimenez CR, Bijlsma MF, Giovannetti E. **Proteomic analysis of gemcitabine-resistant pancreatic cancer cells reveals that microtubule-associated protein 2 upregulation associates with taxane treatment.** Ther Adv Med Oncol. 2019 Ma 10;11:1758835919841233.
- Le Large TYS, El Hassouni B, Kazemier G, Piersma SR, van Laarhoven HWM, Bijlsma MF, Jimenez CR, Giovannetti E. **Multidrug-resistant transporter expression does not always result in drug resistance.** Cancer Sci. 2018 Oct;109(10):3360-3362.
- Le Large TY, Bijlsma MF, Kazemier G, van Laarhoven HM, Giovannetti E, Jimenez CR. **Key biological processes driving metastatic spread of pancreatic cancer as identified by multi-omics studies.** Semin. Cancer Biol. 2017 Mar30.

- Le Large TYS, Bijlsma MF, El Hassouni B, Mantini G, Lagerweij T, Henneman AA, Funel N, Kok B, Pham TV, de Haas R, Morelli L, Knol JC, Piersma SR, Kazemier G, van Laarhoven HWM, Giovannetti E, Jimenez CR. **Focal adhesion kinase inhibition synergizes with nab-paclitaxel to target pancreatic ductal adenocarcinoma.** J Exp Clin Cancer Res. 2021 Mar 9;40(1):91.
- Komor MA, de Wit M, van den Berg J, Martens de Kemp SR, Delis-van Diemen PM, Bolijn AS, Tijssen M, Schelfhorst T, Piersma SR, Chiasserini D, Sanders J, Rausch C, Hoogstrate Y, Stubbs AP, de Jong M, Jenster G, Carvalho B, Meijer GA, Jimenez CR, Fijneman RJA; NGS-ProToCol Consortium. **Molecular characterization of colorectal adenomas reveals POFUT1 as a candidate driver of tumor progression**. Int J Cancer. 2020 Apr 1;146(7):1979-1992.
- Komor MA, Bosch LJ, Bounova G, Bolijn AS, van-Diemen PD, Rausch C, Hoogstrate Y, Stubbs AP, de Jong M, Jenster G, van Grieken NC, Carvalho B, Wessels LF, Jimenez CR, Fijneman RJ, Meijer GA; NGS-ProToCol Consortium. **Consensus molecular subtypes classification of colorectal adenomas.** J Pathol. 2018 Jul 3.
- van den Berg I, van de Weerd S, Roodhart JML, Vink GR, van den Braak RRJC, Jimenez CR, Elias SG, van Vliet D, Koelink M, Hong E, van Grevenstein WMU, van Oijen MGH, Beets-Tan RGH, van Krieken JHJM, IJzermans JNM, Medema JP, Koopman M; **CONNECTION-study group. Improving clinical management of colon cancer through CONNECTION, a nation-wide colon cancer registry and stratification effort (CONNECTION II trial): rationale and protocol of a single arm intervention study.** BMC Cancer. 2020 Aug 18;20(1):776.
- Rolfs F, Piersma SR, Jimenez CR. **Re-assessment of the high-grade serous ovarian cancer phosphoproteome - Identification of kinase candidates in TCGA tumors for future clinical intervention.** EBioMedicine. 2019 Mar;41:34-35.
- Jimenez CR, Zhang H, Kinsinger CR, Nice EC. **The cancer proteomic landscape and the HUPO Cancer Proteome Project.** Clin Proteomics. 2018 Jan 25;15:4.
- Cucchi DGJ, Van Alphen C, Zweegman S, Van Kuijk B, Kwidama ZJ, Al Hinai A, Henneman AA, Knol JC, Piersma SR, Pham TV, Jimenez CR, Cloos J, Janssen JJWM. **Phosphoproteomic Characterization of Primary AML Samples and Relevance for Response Toward FLT3-inhibitors**. Hemasphere. 2021 Jun 12;5(7):e606.
- Carolien van Alphen, Jacqueline Cloos, Sander R. Piersma, Jaco C. Knol, Thang V. Pham, Johan van Meerloo, Gert J. Ossenkoppele, Henk MW. Verheul, Jeroen JWM. Janssen* and Connie R. Jimenez* **Phosphotyrosine-based phosphoproteomics of a panel AML cell lines reveals oncogenic signaling and hyperactive tyrosine kinases as targets for treatment**. Mol. Cell.Prot. 2020, Feb. 26.
- Glykofridis IE, Knol JC, Balk JA, Westland D, Pham TV, Piersma SR, Lougheed SM, Derakhshan S, Veen P, Rooimans MA, van Mil SE, Böttger F, Poddighe PJ, van de Beek I, Drost J, Zwartkruis FJ, de Menezes RX, Meijers-Heijboer HE, Houweling AC, Jimenez CR, Wolthuis RM**. Loss of FLCN-FNIP1/2 induces a non-canonical interferon response in human renal tubular epithelial cells**. Elife. 2021 Jan 18;10:e61630.

Methodological proteomics research

Manuscripts in preparation:

- Thang V Pham, Frank Rolfs, Jim Termeulen, Alex A Henneman, Sander R Piersma, Connie R Jimenez **Assessment of quantitation and statistical methods for DIA mass spectrometry-based proteomics data**

Publications:

- Frank Rolfs, Sander R. Piersma, Mariana Paes Dias, Jos Jonkers,* Connie R. Jimenez. **Feasibility of phosphoproteomics on leftover samples after RNA extraction with guanidinium thiocyanate** Mol. Cell. Prot. 2021.
- Zhu T, Zhu Y, Xuan Y, Gao H, Cai X, Piersma SR, Pham TV, Schelfhorst T, Haas RRGD, Bijnsdorp IV, Sun R, Yue L, Ruan G, Zhang Q, Hu M, Zhou Y, Van Houdt WJ, Le Large TYS, Cloos J, Wojtuszkiewicz A, Koppers-Lalic D, Böttger F, Scheepbouwer C, Brakenhoff RH, van Leenders GJLH, Ijzermans JNM, Martens JWM, Steenbergen RDM, Grieken NC, Selvarajan S, Mantoo S, Lee SS, Yeow SJY, Alkaff SMF, Xiang N, Sun Y, Yi X, Dai S, Liu W, Lu T, Wu Z, Liang X, Wang M, Shao Y, Zheng X, Xu K, Yang Q, Meng Y, Lu C, Zhu J, Zheng J, Wang B, Lou S, Dai Y, Xu C, Yu C, Ying H, Lim TK, Wu J, Gao X, Luan Z, Teng X, Wu P, Huang S,

Tao Z, Iyer NG, Zhou S, Shao W, Lam H, Ma D, Ji J, Kon OL, Zheng S, Aebersold R, Jimenez CR, Guo T. **DPHL: A DIA Pan-human Protein Mass Spectrometry Library for Robust Biomarker Discovery.** Genomics Proteomics Bioinformatics. 2020 Apr;18(2):104-119.

- Xuan Y, Bateman NW, Gallien S, Goetze S, Zhou Y, Navarro P, Hu M, Parikh N, Hood BL, Conrads KA, Loosse C, Kitata RB, Piersma SR, Chiasserini D, Zhu H, Hou G, Tahir M, Macklin A, Khoo A, Sun X, Crossett B, Sickmann A, Chen YJ, Jimenez CR, Zhou H, Liu S, Larsen MR, Kislinger T, Chen Z, Parker BL, Cordwell SJ, Wollscheid B, Conrads TP. **Standardization and harmonization of distributed multi-center proteotype analysis supporting precision medicine studies.** Nat Commun. 2020 Oct 16;11(1):5248.
- Pham TV, Henneman AA, Jimenez CR. **iq: an R package to estimate relative protein abundances from ion quantification in DIA-MS-based proteomics**. Bioinformatics. 2020 Jan 7. pii: btz961.
- Beekhof R, van Alphen C, Henneman AA, Knol JC, Pham TV, Rolfs F, Labots M, Henneberry E, Le Large TY, de Haas RR, Piersma SR, Vurchio V, Bertotti A, Trusolino L, Verheul HM, Jimenez CR. **INKA, an integrative data analysis pipeline for phosphoproteomic inference of active kinases.** Mol Syst Biol. 2019 Apr 12;15(4):e8250.
- Mantini G, Pham TV, Piersma SR, Jimenez CR. **Computational Analysis of Phosphoproteomics Data in Multi-Omics Cancer Studies**. Proteomics. 2021 Feb;21(3-4):e1900312.
- Buffart TE, van den Oord RAHM, van den Berg A, Hilhorst R, Bastiaensen N, Pruijt HFM, van den Brule A, Nooijen P, Labots M, de Goeij-de Haas RR, Dekker H, Piersma SR, Pham TV, van der Leij T, de Wijn R, Ruijtenbeek R, Jiménez CR, Verheul HMW. **Time dependent effect of cold ischemia on the phosphoproteome and protein kinase activity in fresh-frozen colorectal cancer tissue obtained from patients.** Clin Proteomics. 2021 Feb 18;18(1):8.
- van Alphen C, Cucchi DGJ, Cloos J, Schelfhorst T, Henneman AA, Piersma SR, Pham TV, Knol JC, Jimenez CR, Janssen JJWM. **The influence of delay in mononuclear cell isolation on acute myeloid leukemia phosphorylation profiles.** J Proteomics. 2021 Apr 30;238:104134.

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Miscellaneous publications

Smaller collaborations/ core facility last 3,5 years

AMSTERDAM UMC

Dept. Medical Oncology

- Poel D, Boyd LNC, Beekhof R, Schelfhorst T, Pham TV, Piersma SR, Knol JC, Jimenez CR, Verheul HMW, Buffart TE. **Proteomic Analysis of miR-195 and miR-497 Replacement Reveals Potential Candidates that Increase Sensitivity to Oxaliplatin in MSI/P53wt Colorectal Cancer Cells**. Cells. 2019 Sep 19;8(9). pii: E1111.
- López González M, Oosterhoff D, Lindenberg JJ, Milenova I, Lougheed SM, Martiáñez T, Dekker H, Quixabeira DCA, Hangalapura B, Joore J, Piersma SR, Cervera-Carrascon V, Santos JM, Scheper RJ, Verheul HMW, Jiménez CR, Van De Ven R, Hemminki A, Van Beusechem VW, De Gruijl TD. **Constitutively active GSK3β as a means to bolster dendritic cell functionality in the face of tumour-mediated immune suppression.** Oncoimmunology. 2019 Jul 19;8(10):e1631119.
- El Hassouni B, Granchi C, Vallés-Martí A, Supadmanaba IGP, Bononi G, Tuccinardi T, Funel N, Jimenez CR, Peters GJ, Giovannetti E, Minutolo F. **The dichotomous role of the glycolytic metabolism pathway in cancer metastasis: Interplay with the complex tumor microenvironment and novel therapeutic strategies.** Semin Cancer Biol. 2020 Feb;60:238-248.

Dept. Pathology

- Verweij FJ, Bebelman MP, Jimenez CR, Garcia-Vallejo JJ, Janssen H, Neefjes J, Knol JC, de Goeij-de Haas R, Piersma SR, Baglio SR, Verhage M, Middeldorp JM, Zomer A, van Rheenen J, Coppolino MG, Hurbain I, Raposo G, Smit MJ, Toonen RFG, van Niel G, Pegtel DM. Quantifying exosome secretion from single cells reveals a modulatory role for GPCR signaling. J Cell Biol. 2018 Mar 5;217(3):1129-1142.

Dept. Clinical Genetics

- van Dijk FS, Semler O, Etich J, Köhler A, Jimenez-Estrada JA, Bravenboer N, Claeys L, Riesebos E, Gegic S, Piersma SR, Jimenez CR, Waisfisz Q, Flores CL, Nevado J, Harsevoort AJ, Janus GJM, Franken AAM, van der Sar AM, Meijers-Heijboer H, Heath KE, Lapunzina P, Nikkels PGJ, Santen GWE, Nüchel J, Plomann M, Wagener R, Rehberg M, Hoyer-Kuhn H, Eekhoff EMW, Pals G, Mörgelin M, Newstead S, Wilson BT, Ruiz-Perez VL, Maugeri A, Netzer C, Zaucke F, Micha D. **Interaction between KDELR2 and HSP47 as a Key Determinant in Osteogenesis Imperfecta Caused by Bi-allelic Variants in KDELR2.** Am J Hum Genet. 2020 Nov 5;107(5):989-999.

In prep:

- M.V Corbin[1], D.A.P Rockx[1], T.V. Pham[2], S.R. Piersma[2], J.C. Knol[2], H. Joenje[1], C.R. Jimenez[2*], J.C.Dorsman[1*] **Loss of NARFL function deregulates iron regulatory and cohesin complex cleavage proteins under hyperoxia-induced oxidative stress**
- M.V Corbin[1]; D.A.P Rockx[1]; T.V. Pham[2]; S.R. Piersma[2]; J.C. Knol[2]; H. Joenje[1]; C.R. Jimenez[2*]; J.C.Dorsman[1*] **Protein biomarkers with potential relevenace for cellular resistance against oxidative stress**

Dept. Microbiology

- Phan TH, van Leeuwen LM, Kuijl C, Ummels R, van Stempvoort G, Rubio-Canalejas A, Piersma SR, Jiménez CR, van der Sar AM, Houben ENG, Bitter W. **EspH is a hypervirulence factor for Mycobacterium marinum and essential for the secretion of the ESX-1 substrates EspE and EspF.** PLoS Pathog. 2018 Aug 13;14(8):e1007247.
- Ates LS, Dippenaar A, Ummels R, Piersma SR, van der Woude AD, van der Kuij K, Le Chevalier F, Mata-Espinosa D, Barrios-Payán J, Marquina-Castillo B, Guapillo C, Jiménez CR, Pain A, Houben ENG, Warren RM, Brosch R, Hernández-Pando R, Bitter W. **Mutations in ppe38 block PE_PGRS secretion and increase virulence of Mycobacterium tuberculosis**. Nat Microbiol. 2018 Feb;3(2):181-188.

Dept. Physiology

- Schuldt M, Pei J, Harakalova M, Dorsch LM, Schlossarek S, Mokry M, Knol JC, Pham TV, Schelfhorst T, Piersma SR, Dos Remedios C, Dalinghaus M, Michels M, Asselbergs FW, Moutin MJ, Carrier L, Jimenez CR, van der Velden J, Kuster DWD. **Proteomic and Functional Studies Reveal Detyrosinated Tubulin as Treatment Target in Sarcomere Mutation-Induced Hypertrophic Cardiomyopathy**. Circ Heart Fail. 2021 Jan;14(1):e007022.
- Schuldt M, Dorsch LM, Knol JC, Pham TV, Schelfhorst T, Piersma SR, Dos Remedios C, Michels M, Jimenez CR, Kuster DWD, van der Velden J. **Sex-Related Differences in Protein Expression in Sarcomere Mutation-Positive Hypertrophic Cardiomyopathy**. Front Cardiovasc Med. 2021 Mar 1;8:612215.
- Kawasaki M, Meulendijks ER, van den Berg NWE, Nariswari FA, Neefs J, Wesselink R, Baalman SWE, Jongejan A, Schelfhorst T, Piersma SR, Pham TV, van Boven WJP, Driessen AHG, Jimenez CR, de Groot JR. **Neutrophil degranulation interconnects over-represented biological processes in atrial fibrillation.** Sci Rep. 2021 Feb 3;11(1):2972.
- Turaihi AH, Serné EH, Molthoff CF, Koning JJ, Knol J, Niessen HW, Jose Th Goumans M, van Poelgeest EM, Yudkin JS, Smulders YM, Jimenez CR, van Hinsbergh VW, Eringa EC. **Perivascular Adipose Tissue Controls Insulin-Stimulated Perfusion, Mitochondrial Protein Expression and Glucose Uptake in Muscle Through Adipomuscular Arterioles.** Diabetes. 2020 Jan 31. pii: db181066.

Dept. Clinical Chemistry

- van Steenoven I, Koel-Simmelink MJA, Vergouw LJM, Tijms BM, Piersma SR, Pham TV, Bridel C, Ferri GL, Cocco C, Noli B, Worley PF, Xiao MF, Xu D, Oeckl P, Otto M, van der Flier WM, de Jong FJ, Jimenez CR, Lemstra AW, Teunissen CE. **Identification of novel cerebrospinal fluid biomarker candidates for dementia with Lewy bodies: a proteomic approach**. Mol Neurodegener. 2020 Jun 18;15(1):36. doi: 10.1186/s13024-020-00388-2. PMID: 32552841; PMCID: PMC7301448.
- Bridel C, Koel-Simmelink MJA, Peferoen L, Derada Troletti C, Durieux S, Gorter R, Nutma E, Gami P, Iacobaeus E, Brundin L, Kuhle J, Vrenken H, Killestein J, Piersma SR, Pham TV, De Vries HE, Amor S, Jimenez CR, Teunissen CE. **Brain endothelial cell expression of**

SPARCL-1 is specific to chronic multiple sclerosis lesions and is regulated by inflammatory mediators in vitro. Neuropathol Appl Neurobiol. 2018 Jun;44(4):404-416.

Dept. MCBI

- Li RE, de Haas A, Rodríguez E, Kalay H, Zaal A, Jimenez CR, Piersma SR, Pham TV, Henneman AA, de Goeij-de Haas RR, van Vliet SJ, van Kooyk Y. **Quantitative Phosphoproteomic Analysis Reveals Dendritic Cell- Specific STAT Signaling After α2-3-Linked Sialic Acid Ligand Binding.** Front Immunol. 2021 Apr 22;12:673454.

AMC

- Castillo J, Knol JC, Korver CM, Piersma SR, Pham TV, de Goeij-de Haas RR, van Pelt AMM, Jimenez CR, Jansen BJH. Human Testis **Phosphoproteome Reveals Kinases as Potential Targets in Spermatogenesis and Testicular Cancer**. Mol Cell Proteomics. 2019 Mar 15;18(Suppl 1):S132-S144..

EXTERNAL

VU: De Groof TWM, Bergkamp ND, Heukers R, Giap T, Bebelman MP, Goeij-de Haas R, Piersma SR, Jimenez CR, Garcia KC, Ploegh HL, Siderius M, Smit MJ. **Selective targeting of ligand-dependent and -independent signaling by GPCR conformation-specific anti-US28 intrabodies.** Nat Commun. 2021 Jul 16;12(1):4357.

UMCU Stokman MF, Bijnsdorp IV, Schelfhorst T, Pham TV, Piersma SR, Knol JC, Giles RH, Bongers EMHF, Knoers NVAM, Lilien MR, Jiménez CR, Renkema KY. **Changes in the urinary extracellular vesicle proteome are associated with nephronophthisis-related ciliopathies.** J Proteomics. 2019 Feb 10;192:27-36.

LUMC Bijen HM, Hassan C, Kester MGD, Janssen GMC, Hombrink P, de Ru AH, Drijfhout JW, Meiring HD, de Jong AP, Falkenburg JHF, Jimenez CR, Heemskerk MHM, van Veelen PA. **Specific T Cell Responses against Minor Histocompatibility Antigens Cannot Generally Be Explained by Absence of Their Allelic Counterparts on the Cell Surface.** Proteomics. 2018 Jun;18(12):e1700250.

NKI den Uil SH, de Wit M, Slebos RJC, Delis-van Diemen PM, Sanders J, Piersma SR, Pham TV, Coupé VMH, Bril H, Stockmann HBAC, Jimenez CR, Meijer GA, Fijneman RJA. **Quantitative analysis of CDX2 protein expression improves its clinical utility as a prognostic biomarker in stage II and III colon cancer.** Eur J Cancer. 2021 Feb;144:91-100.

NKI Steenbeek SC, Pham TV, de Ligt J, Zomer A, Knol JC, Piersma SR, Schelfhorst T, Huisjes R, Schiffelers RM, Cuppen E, Jimenez CR*, van Rheenen J*. **Cancer cells copy migratory behavior and exchange signaling networks via extracellular vesicles**. EMBO J. 2018 Jun 14. pii: e8357. [Epub ahead of print. PMID: 29907695 Shared senior authors.

MUMC: Vaes N, Schonkeren SL, Rademakers G, Holland AM, Koch A, Gijbels MJ, Keulers TG, de Wit M, Moonen L, Van der Meer JRM, van den Boezem E, Wolfs TGAM, Threadgill DW, Demmers J, Fijneman RJA, Jimenez CR, Vanden Berghe P, Smits KM, Rouschop KMA, Boesmans W, Hofstra RMW, Melotte V. **Loss of enteric neuronal Ndrg4 promotes colorectal cancer via increased release of Nid1 and Fbln2.** EMBO Rep. 2021 Jun 4;22(6):e51913.

Spain: González-Alonso P, Zazo S, Martín-Aparicio E, Luque M, Chamizo C, Sanz-Álvarez M, Minguez P, Gómez-López G, Cristóbal I, Caramés C, García-Foncillas J, Eroles P, Lluch A, Arpí O, Rovira A, Albanell J, Piersma SR, Jimenez CR, Madoz-Gúrpide J, Rojo F. **The Hippo Pathway Transducers YAP1/TEAD Induce Acquired Resistance to Trastuzumab in HER2-Positive Breast Cancer**. Cancers (Basel). 2020 Apr 29;12(5):1108.

Lead Pharma Castillo J, Knol JC, Korver CM, Piersma SR, Pham TV, de Goeij-de Haas RR, van Pelt AMM, Jimenez CR, Jansen BJH. **Human Testis Phosphoproteome Reveals Kinases as Potential Targets in Spermatogenesis and Testicular Cancer.** Mol Cell Proteomics. 2019 Mar 15;18(Suppl 1):S132-S144.

Italy Loredana Ruggeri, Elena Urbani, Davide Chiasserini, Federica Susta, Pier Luigi Orvietani, Emanuela Burchielli, Sara Ciardelli, Maria Speranza Massei, Stefano Bruscoli, Sander R. Piersma, Dunia Ramarli, Luciano Binaglia, Connie R. Jimenez, Georg A. Hollander and Andrea Velardi. **Natural killer cells trigger beta-2-microglobulin production to enhance immune reconstitution** (submitted)

What do collaborators say about the OPL?

Dr. Jacqueline Cloos, Dept. Hematology, collaborator since 2012:

"De afdeling Hematologie heeft samen met het OPL een aantal mooie studies lopen waar al heel interessante data uit zijn gekomen. Kroon op het werk is een artikel in Molecular and Cellular Proteomics waarin we laten zien dat leukemiecellen blaasjes uitscheiden met daarin met name eiwitten betrokken bij splicing. In een ander succesvol project karakteriseren we specifiek het tyrosine fosfoproteoom van verschillende leukemiecellen om aan de hand van deze profielen de juiste kinase remmers te selecteren voor het behandelen van de patiënt. Naast de technische support bij de experimenten krijgen we gelukkig ook veel support voor de data analyses om de biologische significantie te vinden in de grote datasets. **Omdat de (gefosforyleerde) eiwitten veel zeggen over de functionele processen in de cel is proteomics een mooie aanvulling op de genomics en transcriptomics en zullen we ook in de toekomst nog veel gebruik maken van deze faciliteit.**"

Dr. Linda Smit, Dept. Hematology, collaborator since 2014:

Mijn onderzoeksgroep is op zoek naar eiwitten die gebruikt kunnen worden als therapie doelwitten en de terugkeer van leukemie kunnen voorkomen. De proteomics faciliteit geleid door Connie Jimenez heeft al een **fantastische bijdrage** geleverd aan dit onderzoek door proteomics te doen op de cellen die verantwoordelijk zijn voor de leukemie terugkeer. **We hebben op deze manier al verschillende eiwitten geïdentificeerd die mogelijk gaan leiden tot een nieuwe therapie die de overleving van leukemie patiënten gaat verbeteren**.

Dr. Michiel Pegtel, Dept. Pathology, collaborator since 2007:

De Exosomens research group (ERG), is een multidisciplinaire onderzoeksgroep van het VUmc die internationaal bekend staat om baanbrekend onderzoek naar exosomen en de rol die deze nanoscopische vetblaasjes spelen in kanker en autoimmuniteit. **Sind de start van dit laboratoriumonderzoek zijn met behulp van massaspectometrie en innovatieve data-analyse methodes met behulp van en ontwikkeld door het OPL verschillende nieuwe fundamentele inzichten verkregen**. Recent heeft dit geleid tot een publicatie in **PNAS** waarin werd aangetoond met massaspectometrie dat RNA-bindende eiwitten een inhiberende rol spelen in ontstekingen veroorzaakt door uitgescheiden RNA moleculen. Tevens is met behulp van de kwantitatieve analyse methode van het OPL ontdekt dat druggable 'membraan fusie' eiwitten oncogenese bevorderen omdate deze ongecontroleerde exosomen productie in kankercellen 'aan' zetten. Het is de verwachting dat door middel van **nieuwe isolatietechnieken mede ontwikkeld door het ERG in combinatie met gevoelige eiwit detectie technieken van het OPL nieuwe diagnostische testen** kunnen worden ontwikkeld voor kanker en auto-immuniteit met een superieure sensitiviteit en specificiteit.

Dr. Irene Bijnsdorp, Dept. Urology, collaborator since 2012:

Research of the Dept. of Urology focusses on protein marker identification for detection and stratification of prostate cancer patients into risks groups. To this end we analyze small extracellular vesicles (exosomes) that have emerged as biomarker-rich treasure troves. Together with dr. C. Jimenez of the OPL, we profiled the proteome of urinary exosomes leading to the identification of over 3000 proteins. **This is much more than has been identified before by others** (usually not exceeding 1500 proteins). Initial proof-of-concept proteomics profiling led to the improvement of exosome isolation of urine EVs. Furthermore, after data mapping and **key (bio)statistical support provided by the OPL-group, led to the identification of potential PCa biomarkers**. This work was the basis for the financial support (KWF, Alpe d'Huzes) of a collaborative project together with Prof. Jenster (Erasmus MC) and Prof. Schalken (Radboud UMC). This project will take advantage of the **excellent infrastructure and expertise in exosome-proteomics** (Dr. Jimenez) and it is expected that this project will provide a strong basis for developing a liquid biopsy-based diagnostic/prognostic test for PCa.

Prof. Ruud Brakenhoff, Dept.Otolaryngology-Head&Neck Surgery, collaborator since 2010:

The Tumor Biology lab focuses its research on the diagnosis and treatment of patients with tumors in the upper aerodigestive tract. Using genome-wide siRNA screens we identified a variety of novel molecular targets that might be exploited to improve future therapy protocols, combined with standard first line treatments. One of the major research efforts is to elucidate the molecular pathways these drug targets act in, and using (phospho)proteomics approaches both primary and secondary substrate proteins can be identified, and the effects of inhibitors analyzed. This was recently published for the MASTL protein, a kinase that sensitizes lung cancer cells for radiotherapy (Nagel et al. Mol Cancer Ther 2015). **This work will certainly be followed up for targets and inhibitors being studied, and proteomics and phosphoproteomics are the ideal pipelines for such studies.**

Dr. Josephine Dorsman, Dept. Clinical Genetics, collaborator since 2010:

“Het is goed om in-huis een kOPLoper in protemics te hebben, om nieuwe inzichten & OPLossingen te vinden voor basale en translationale vragen. Ook daadwerkelijk gebeurd in samenwerking: Fanconi & Zuurstof tolerantie ! Omdat het juist de eiwitten in de cel zijn die het werk doen, verwachten wij dat het OPL onderzoek nog veel gaat **OPLeveren** in de toekomst.”

Dr. Rob Wolthuis, Dept. Clinical Genetics, collaborator since 2015:

“In the era of cancer -omics, we see two critical developments: the first one is directed at resolving biological and clinical implications of cancer mutations. Already at an early stage, Connie Jimenez started to address these by effectively exploiting a combination of cancer genomics and proteomics. Secondly, there is an enormous need for new combination therapies that could overcome drug resistance associated with targeted mono-therapies. This absolutely requires advanced molecular pathway analyses and functional kinase studies, which are technically very challenging. The Jimenez lab has generated powerful new assays for rapid phosphoproteomics and links them to state-of-the art bioinformatics, a great combination. **With these expertises in full operation, the lab functions at a state-of-the-art international level now, and has tremenduous value for cancer research at the CCA.** We look forward to continuing our various research lines with the OPL"

Dr. Juan Vallejo, Dept. Molecular Cell Biology & Immunology, collaborator since 2015:

“At the group of Dendritic Cell Immunobiology we focus on the role of glycan-binding receptors on the modulation of immune responses and their potential use as targeting receptors for anti-cancer vaccination. One of the most interestign features of these broad family of receptors is that, besides mediating efficient antigen uptake, they also trigger intracellular signaling that modulatest dendritic cell activation. However, the nature of the signaling events involved in this pathway remains only partly uncovered, and next generation phosphoproteomics will be extremely useful in shedding light on this processes.”

Prof. Wilbert Bitter, Dept. Medical Microbiology, collaborator since 2006:

“The proteomics facility of the OPL has been crucial for our work on the tubercle bacillus. We have generated various secretion mutants and the detailed proteomic analysis of these mutants has shown important new insights in the working of this major pathogen. The high-end equipment and expertise of the OPL researchers in the data processing helped us to place ourselves in the forefront of tuberculosis research. Especially because some of the most important proteins turned out to be extremely challenging to capture by proteomics. This work has resulted in 8 publications including papers in PloS Pathogens and PloS Genetics, as well as in new strategies for vaccine development.”

Prof. Arjan Griffioen, Dept. Medical Oncology, collaborator since 2014:

'The Oncoproteomics Laboratory very successfully assisted several projects that are currently running in the Angiogenesis Laboratory. Proteomics approaches were applied in

the search for alternative isoforms of the tumor vascular marker vimentin and for the discovery of diagnostic biomarkers present in cancer patient thrombocytes. The expert collaboration has been efficient, fast and successful. A future collaborative project will focus on the mechanisms of targeted combination therapy by phophoproteomics.'

Dr. Mariette Labots M.D., Dept. Medical Oncology (clinical staff), collaborator since 2012:

Als medisch oncoloog leg ik patiënten met uitgezaaide kanker dagelijks uit hoe targeted therapies werken. En ook dat we op voorhand niet weten of de behandeling wel zal aanslaan, of, dat als deze blijkt te werken, op termijn meestal toch ongevoeligheid zal ontstaan. Vaak vraag ik om toestemming voor het nemen van een tumorbiopt voor onderzoek: omdat we samen met het OPL hard werken aan een (fosfo)eiwittest om in de toekomst te kunnen bepalen welke signaleringsroutes actief zijn in de tumor, om hiermee de meest geschikte behandeling te kunnen selecteren voor een individuele patient.
Zo hebben we in de afgelopen jaren de voor personalized medicine veelbelovende fosfoproteomics-technologie toepasbaar weten te maken op maar een heel klein stukje tumorweefsel van patienten. Dit maakt verdere ontwikkeling van deze technologie voor de klinische praktijk mogelijk. Ik ben er van overtuigd dat (fosfo)proteomics een belangrijke bijdrage zal leveren aan het realiseren van therapieselectie voor individuele patienten.
De samenwerking met het OncoProteomicsLab is inspirerend, snel en biedt mede door de inbedding binnen de afdeling medische oncologie veel mogelijkheden voor translationeel onderzoek. De onderzoeksfocus binnen het OPL-lab naar het toepassen van fosfoproteomics voor personalized medicine is uniek in Nederland. Connie Jimenez weet mede dankzij state-of-the-art apparatuur de snelle ontwikkelingen binnen dit onderzoeksveld bij te houden en neemt hierin ook internationaal een voortrekkende positie in.

Prof. Tanja de Gruijl, Dept. Medical Oncology, collaborator since 2016:

“The immunotherapy lab, in a VUmc/AMC Alliance collaborative project with the lab of Prof. Theo Geijtenbeek, is studying ways in which to optimally leverage autophagy in melanoma, in order to boost T cell immunity in vivo. Together with the Proteomics lab we hope to asess the protein content of differentially generated autophagosomes for their immunogenic potential, both in terms of (neo-)epitopes and immune stimulatory signals.
In addition, in our search for predictive immune biomarker profiles in patients treated with immunotherapies, targeted therapies and even more conventional chemo- or radiotherapies, we hope in future to translate systemic phenotypic immune effector cell subset signatures to (phospho)proteomic signatures, building on the technical know-how and unique expertise of Dr Jiménez and her lab."

Prof. Jan Paul Medema, Lexor, collaborator since 2014:

“One of LEXOR’s research lines focusses on colorectal cancer prognosis and response to therapy. We identified biologically subgroups with highly distinctive gene expression patterns and clinical features, which includes a subtype with dismal prognosis. In collaboration with the OPL we are currently unravelling the proteome complement of these CRC transcriptome-based subtypes. We obtained promising, in-depth proteome data of the AMC colon tumors that may pave the way to novel immunohistochemical test. Together with also ErasmusMC, UMCU and RadboudMC, we obtained funding from KWF/ Alpe d’Huzes that will enable us to expand the CRC proteome dataset and to translate these findings into a clinically applicable test.”

Dr. Charlotte Teunissen, Dept. Clinical Chemistry, collaborator since 2005:

“Het OPL heeft een grondige en zeer betrouwbare workflow opgezet voor liquor proteomics. Hiermee hebben we verschillende studies kunnen doen, waardoor we inzicht gekregen hebben in het liquor proteome, en nieuwe kandidaat biomarkers ontdekt hebben. Hierdoor hebben we zowel diagnostische en mechanistisch/pathologische vervolgstudies kunnen uitvoeren.”

Prof. dr. Jolanda van der Velden, Dept. Physiology, collaborator since 2015:

Prof. Jolanda van der Velden, Dr. Diederik Kuster and Maike Schuldt study determinants of disease progression in genetic cardiac disease within a reserach consortium, which is funded by the Netherlnads Heart Foundation (CVON-DOSIS):
The proteomics facility of the OPL is key in identifying cellular pathways that are centrally involved in disease progression in hypertrophic cardiomyopathy. Proteomics analysis has been performed in cardiac tissue samples from 60 genotyped and clinically well-characterized patients. We expect to report our results in a joint publication. The identified disease modifiers will be tested in functional assays to establish their exact role in cardiac disease.

Prof. Gerrit Meijer, Dr. Remond Fijneman, NKI, collaborator since 2006:

“10 years of OPL = 10 years of collaborative and successful CRC research: congratulations! The OPL turned in-depth protein profiling of colorectal tumors into a reality, which added a new dimension to our research. Major achievements: The collaborative research with OPL has yielded many protein biomarkers, and has boosted research for early detection of CRC. Near future work: The OPL enables antibody-independent validation of protein biomarkers, thereby bridging the gap between biomarker discovery and assay development for clinical applications. The pleasant atmosphere around the OPL offers an inviting environment for Master students, PhD students, and postdocs to learn what proteomics is about.”

Prof. Onno Kranenburg, UMCU, collaborator since 2007:

“The Kranenburg research group aims to devise novel therapeutic strategies aiming to prevent and effectively treat metastasis in CRC, in part by targeting cancer stem cells. The OPL has been instrumental in elucidating drug resistance mechanisms in CRC stem cells, which has resulted in the publication of 6 co-authored papers in high-impact journals in the cancer and proteomics domains. We have recently further identified novel targets for therapy that drive tumor growth and metastasis in the most aggressive (mesenchymal/stem-like) CRC subtype. In addition, we have developed a diagnostic tool allowing us to select such patients for targeted therapy. We hope to renew our collaborations with the OPL in ‘proof-of-concept’ clinical and organoid-based studies as (phospho-)proteomics-based evaluation of drug response will be an essential part of future trial design.”

Prof. Jos Jonkers, NKI, collaborator since 2007:

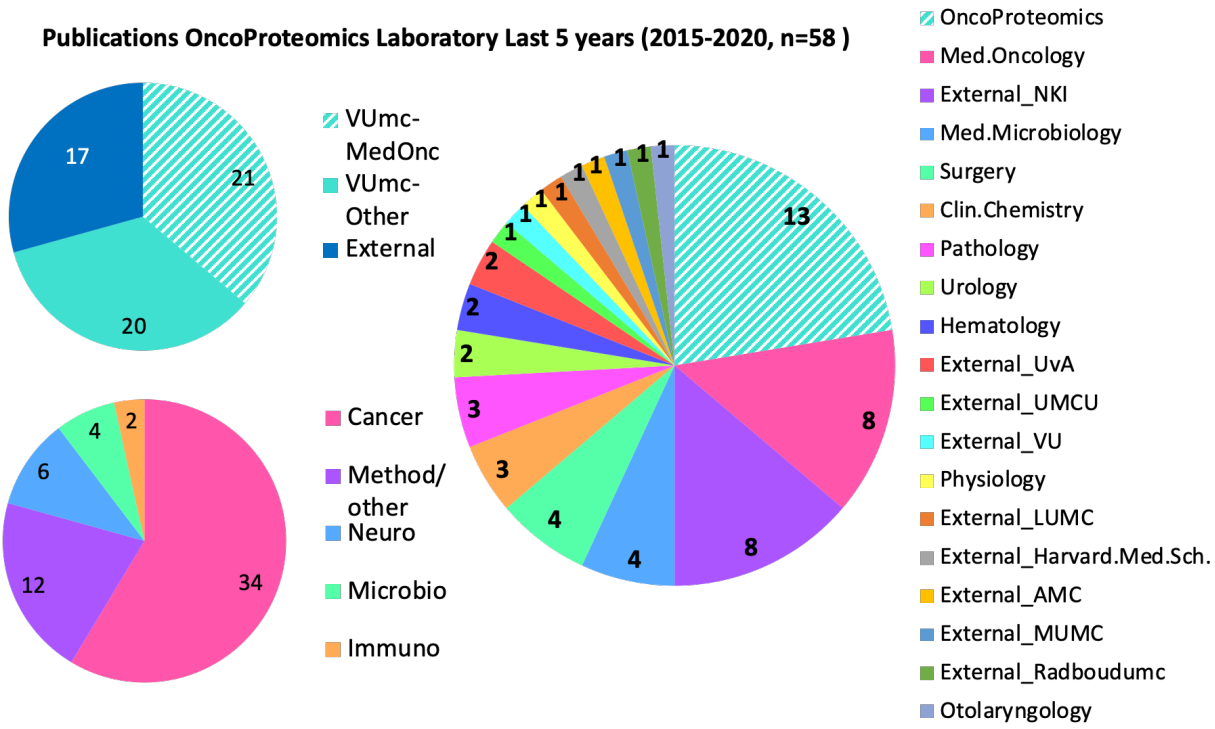
“The focus of our group is on the genetic dissection of human breast cancer through the use of advanced mouse models. In the past 9 years, our collaboration with the OPL has given us insight into the proteome of our genetically engineered mouse model for BRCA1- and BRCA2- associated hereditary breast cancer. The results of this fruitful collaboration have been published in 2 papers in the nr 1 proteomics paper in the field, Molecular and Cellular Proteomics and a joint KWF project. Currently we are using phosphoproteomics on our patient-derived xenograft models for homologous recombination repair deficient breast cancer. First pilot results have been very promising and I look forward to the results of this continuing joint discovery.”

Prof. Jacco van Rheenen, NKI, collaborator since 2015:

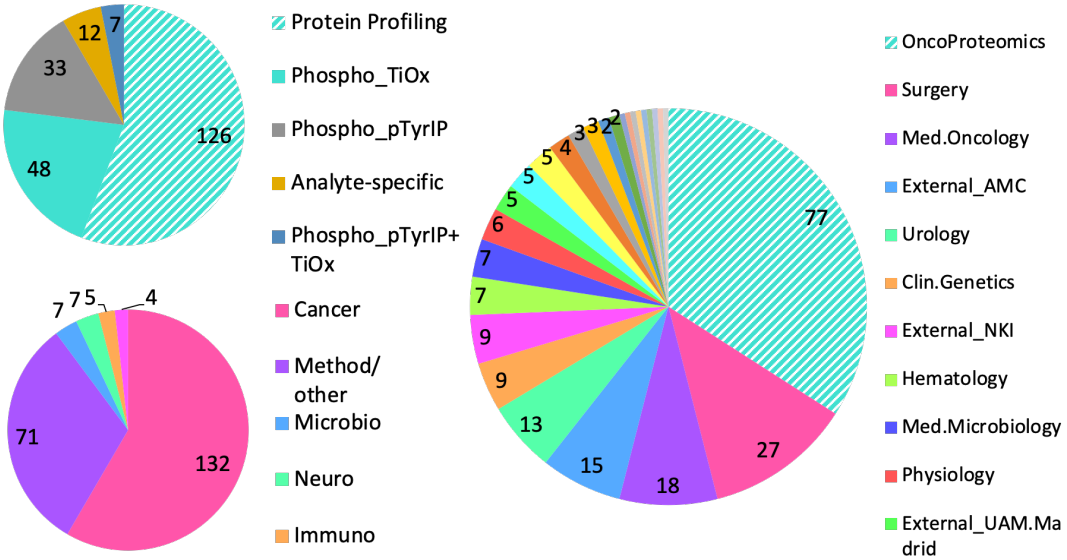
“Our lab is a world-leader in the high resolution in vivo imaging of the behavior of cells in living mice. Last year, using in vivo imaging, we have shown how malignant tumor cells can phenocopy their behavior to more benign cells through extracellular vesicles (Zomer et al, Cell 2015). In collaboration with the OPL we are now tackling the molecular mechanisms behind this phenomenon. Together with the OPL, we are now identifying the protein content that is responsible for the metastatic behavior that is phenocopied. This may lead to the identification of new drug targets for tumor growth and metastasis.
We prepared our own samples at the OPL and we received excellent help with hands-on lab work and protocols optimized by the OPL. With the experience in data analysis of the OPL, we are sure we can get the best out of our data.”

Appendix 1.
OncoProteomics Laboratory Facts and Figures

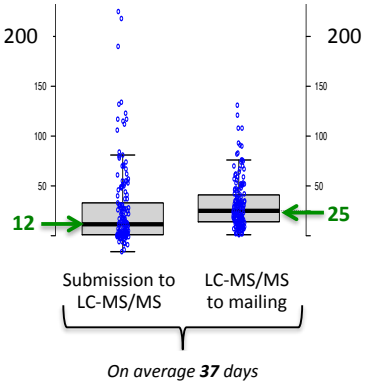
Publications OncoProteomics Laboratory Last 5 years (2015-2020, n=58)



Mass Spectrometry Runs OncoProteomics Laboratory Last 5 years (2015-2020, n=226 exps)



Turn-around time between sample submission and data return is very reasonable, typically ~4 weeks.
Larger datasets may take longer.



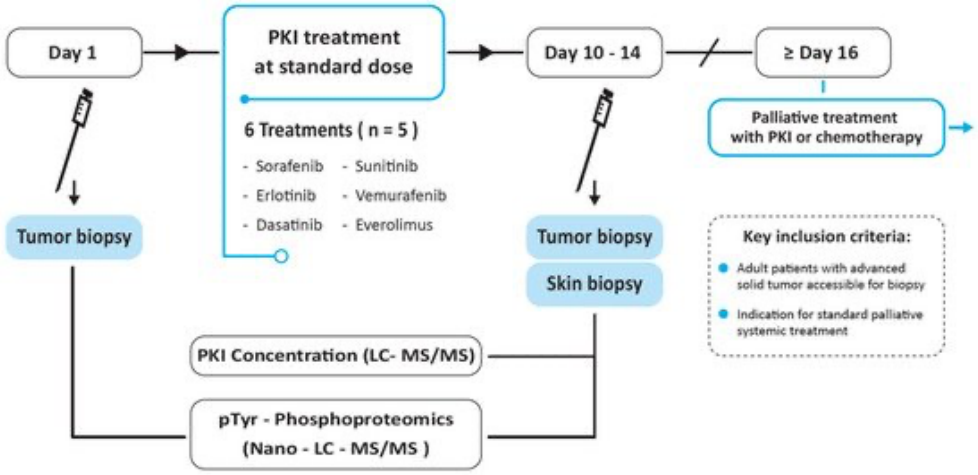
Appendix 2.
Abstracts of ongoing and completed projects employing proteomics in the past 3,5 years

Pan-cancer

Kinase Inhibitor Treatment of Patients with Advanced Cancer Results in High Tumor Drug Concentrations and in Specific Alterations of the Tumor Phosphoproteome

Labots M, Pham TV, Honeywell RJ, Knol JC, Beekhof R, de Goeij-de Haas R, Dekker H, Neerincx M, Piersma SR, van der Mijn JC, van der Peet DL, Meijerink MR, Peters GJ, van Grieken NCT, Jiménez CR, Verheul HMW

Cancers (Basel) 2020 Feb 1;12(2):330. doi: 10.3390/cancers12020330. PubMed PMID: 32024067.



Identification of predictive biomarkers for targeted therapies requires information on drug exposure at the target site as well as its effect on the signaling context of a tumor. To obtain more insight in the clinical mechanism of action of protein kinase inhibitors (PKIs), we studied tumor drug concentrations of protein kinase inhibitors (PKIs) and their effect on the tyrosine-(pTyr)-phosphoproteome in patients with advanced cancer. Tumor biopsies were obtained from 31 patients with advanced cancer before and after 2 weeks of treatment with sorafenib (SOR), erlotinib (ERL), dasatinib (DAS), vemurafenib (VEM), sunitinib (SUN) or everolimus (EVE). Tumor concentrations were determined by LC-MS/MS. pTyr-phosphoproteomics was performed by pTyr-immunoprecipitation followed by LC-MS/MS. Median tumor concentrations were 2-10 µM for SOR, ERL, DAS, SUN, EVE and >1 mM for VEM. These were 2-178 × higher than median plasma concentrations. Unsupervised hierarchical clustering of pTyr-phosphopeptide intensities revealed patient-specific clustering of pre- and on-treatment profiles. Drug-specific alterations of peptide phosphorylation was demonstrated by marginal overlap of robustly up- and downregulated phosphopeptides. These findings demonstrate that tumor drug concentrations are higher than anticipated and result in drug specific alterations of the phosphoproteome. Further development of phosphoproteomics-based personalized medicine is warranted.

Leukemia

The influence of delay in mononuclear cell isolation on acute myeloid leukemia phosphorylation profiles *Accepted for publication in Journal of Proteomics*

Carolien van Alphen[1,2], David G.J. Cucchi[1], Jacqueline Cloos[1], Tim R.A. Schelfhorst[2], Sander R. Piersma[2], Thang V. Pham[2], Jaco C. Knol[2], Connie R. Jimenez[2*], Jeroen J.W.M. Janssen[1*]

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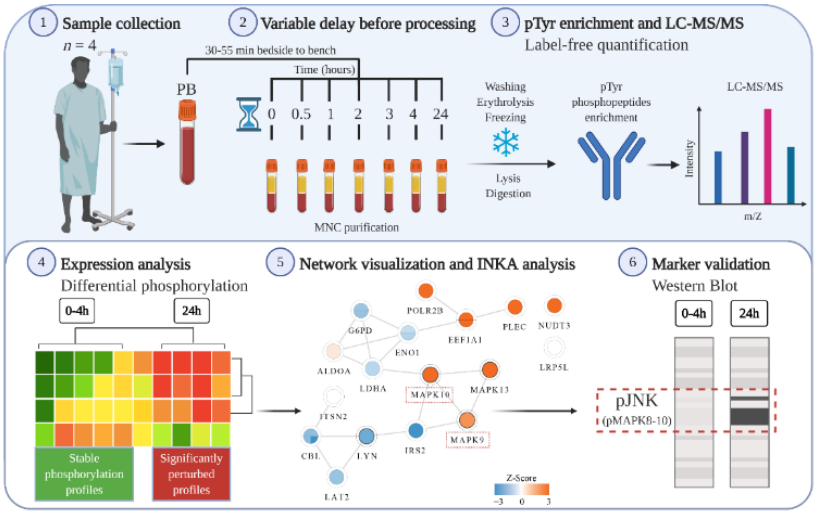
[2]*Department of Medical Oncology, OncoProteomics Laboratory, Cancer Center Amsterdam, Amsterdam UMC, Vrije Universiteit, Amsterdam, The Netherlands*



INTRODUCTION - Mass-spectrometry (MS) based phosphoproteomics is increasingly used to explore aberrant cellular signaling and kinase driver activity, aiming to improve kinase inhibitor (KI) treatment selection in malignancies. Phosphorylation is a dynamic, highly regulated post-translational modification that may be affected by variation in pre-analytical sample handling, hampering the translational value of phosphoproteomics-based analyses.

AIM - To investigate the effect of delay in mononuclear cell isolation on acute myeloid leukemia (AML) phosphorylation profiles.

APPROACH - We performed MS on immuno-precipitated phosphotyrosine (pY)-containing peptides isolated from AML samples after seven pre-defined delays before sample processing (direct processing, thirty minutes, one hour, two hours, three hours, four hours and 24 hours delay). We confirmed candidate phosphorylation markers using western blot.

RESULTS - Up to four hours, pY phosphoproteomics profiles show limited variation. However, in samples processed with a delay of 24 hours, we observed significant change in these phosphorylation profiles, with differential phosphorylation of 22 pY phosphopeptides ($p < 0.01$). This includes increased phosphorylation of pY phosphopeptides of JNK and p38 kinases indicative of stress response activation.

CONCLUSIONS - Based on these results, we conclude that processing of AML samples should be standardized at all times and should occur within four hours after sample collection.

(Phospho)proteomic characterization of primary AML samples and relevance for response towards FLT3 inhibitors

David G.J. Cucchi[1], Carolien Van Alphen[1,2], Sonja Zweegman[1], Bo Van Kuijk[1], Zinia Kwidama[1], Adil al Hinai[3], Sander R. Piersma[2], Jaco C. Knol[2],Thang V. Pham[2], Alex Henneman[2], Connie R. Jimenez[2]*, Jeroen J.W.M. Janssen[1], Jacqueline Cloos[1]*

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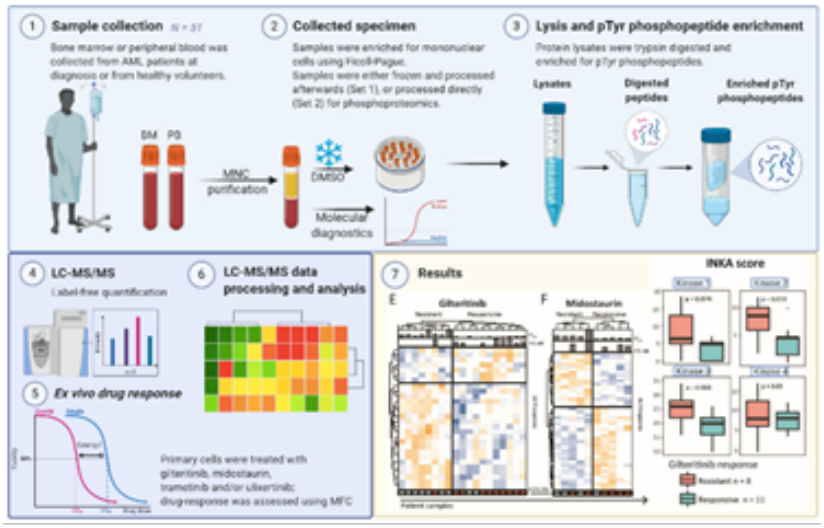
[3]*Department of Hematology, Erasmus MC Cancer Institute, University Medical Center, Rotterdam, The Netherlands*

INTRODUCTION - Internal Tandem Duplications (ITDs) of the FMS-tyrosine-like Kinase 3-gene (*FLT3*) are the most common genetic aberrations in acute myeloid leukemia (AML). The general consensus stresses the function of *FLT3*-ITD in ligand-independent activation of FLT3 and downstream pathways. However, also *FLT3*-WT patients respond to FLT3 TKIs, indicating alternative possibilities of FLT3 pathway activation or off target effects leading to unexpected treatment response. Therefore, we used phosphoproteomics to unravel mechanisms of FLT3-TKI response in primary AML samples.

AIM - A fundamental understanding on phosphoproteome-wide profile changes in *FLT3*-ITD AML is lacking, as well as phosphorylation markers that are predictive for response to FLT3-inhibitor therapy. Thus, we aimed to (1) assess differentially phosphorylated phosphoproteins in *FLT3*-WT AML and *FLT3*-ITD AML and (2) to identify differentially phosphorylated kinases and/or specific phosphorylation profiles that are predictive for response to FLT3 inhibitors in primary AML samples.

APPROACH - We performed label-free phosphotyrosine phosphoproteomics on a set of 51 molecularly characterized primary AML samples (27 *FLT3*-WT, 24 *FLT3*-ITD), divided over two cohorts. We compared phosphoproteomic profiles in *FLT3*-WT and *FLT3*-ITD AML. Kinase activity scores were calculated using INKA. We assessed *ex vivo* response to midostaurin and gilteritinib using liquid culture and cell viability testing using flow cytometry in 33 primary AMLs, and additionally assessed response towards trametinib and ulixertinib in 11 samples. Drug response towards combinations with FLT3-TKIs and ERK-Is was assessed in nine samples. *Ex vivo* FLT3-TKI response was related to kinase phosphorylation status, INKA score and *FLT3*-ITD status.



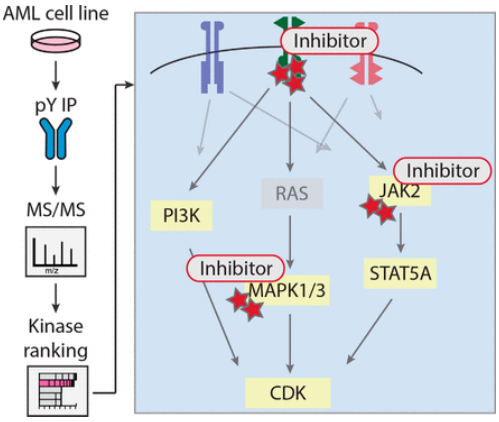
RESULTS - In the first cohort, 61 phosphoproteins were differentially phosphorylated between *FLT3*-WT and *FLT3*-ITD AML. *FLT3*-ITD AML was characterized by high STAT5, SPTLC2, CYB5R1 and LYN phosphorylation, while LCK, KIT and PIK3R1 phosphorylation was low in *FLT3*-ITD samples, compared to *FLT3*-WT. Surprisingly, phosphorylation of FLT3 did not seem dependent on the presence of in-sample *FLT3*-ITD. In liquid culture, *FLT3*-ITD samples were, as expected, more responsive towards gilteritinib, and midostaurin, compared to *FLT3*-WT samples. However, drug response could not be fully explained by the presence of *FLT3*-ITD, with responses observed in *FLT3*-WT patients and resistance, exemplified by relatively high LC50 values, in *FLT3*-ITD samples. In order to unravel this, we compared phosphoproteomic profiles, independent from the presence of the mutation, in responsive and resistant samples based on the median LC50. We observed 28 phosphosites with differential phosphorylation ($p < 0.05$), possibly explaining response in *FLT3*-WT samples and vice-versa. Gilteritinib-resistant samples were characterized by higher INKA scores of several kinases, indicating that activation of FLT3-independent pathways may overcome FLT3 inhibition and rely on alternative pathways for survival.

CONCLUSIONS - We present novel phosphotyrosine-based proteomics data characterizing AML with a focus on *FLT3*-ITD and *FLT3*-TKI response. These robust data, exemplified by the observation of key proteins and phosphorylation events in (*FLT3*-ITD) AML, serve as a reference for further identification of (phospho)proteomic markers associated with *FLT3*-ITD AML and FLT3-TKI response and the development of strategies to improve responses.

Phosphotyrosine-based phosphoproteomics for target identification and drug response prediction in AML cell lines

Van Alphen C, Cloos J, Beekhof R, Cucchi DGJ, Piersma SR, Knol JC, Henneman AA, Pham TV, van Meerloo J, Ossenkoppele GJ, Verheul HM, Janssen JJ, Jimenez CR

Mol Cell Proteomics 2020 May;19(5):884-899. doi: 10.1074/mcp.RA119.001504. PubMed PMID: 32102969.



Acute myeloid leukemia (AML) is a clonal disorder arising from hematopoietic myeloid progenitors. Aberrantly activated tyrosine kinases (TK) are involved in leukemogenesis and are associated with poor treatment outcome. Kinase inhibitor (KI) treatment has shown promise in improving patient outcome in AML. However, inhibitor selection for patients is suboptimal.In a preclinical effort to address KI selection, we analyzed a panel of 16 AML cell lines using phosphotyrosine (pY) enrichment-based, label-free phosphoproteomics. The Integrative Inferred Kinase Activity (INKA) algorithm was used to identify hyperphosphorylated, active kinases as candidates for KI treatment, and efficacy of selected KIs was tested.Heterogeneous signaling was observed with between 241 and 2764 phosphopeptides detected per cell line. Of 4853 identified phosphopeptides with 4229 phosphosites, 4459 phosphopeptides (4430 pY) were linked to 3605 class I sites (3525 pY). INKA analysis in single cell lines successfully pinpointed driver kinases (PDGFRA, JAK2, KIT and FLT3) corresponding with activating mutations present in these cell lines. Furthermore, potential receptor tyrosine kinase (RTK) drivers, undetected by standard molecular analyses, were identified in four cell lines (FGFR1 in KG-1 and KG-1a, PDGFRA in Kasumi-3, and FLT3 in MM6). These cell lines proved highly sensitive to specific KIs. Six AML cell lines without a clear RTK driver showed evidence of MAPK1/3 activation, indicative of the presence of activating upstream RAS mutations. Importantly, FLT3 phosphorylation was demonstrated in two clinical AML samples with a *FLT3* internal tandem duplication (ITD) mutation.Our data show the potential of pY-phosphoproteomics and INKA analysis to provide insight in AML TK signaling and identify hyperactive kinases as potential targets for treatment in AML cell lines. These results warrant future investigation of clinical samples to further our understanding of TK phosphorylation in relation to clinical response in the individual patient.

Phospho-Proteomic Profiling of T-Cell Acute Lymphoblastic Leukemia Identifies Targetable Kinase Activities and Novel Treatment Combination Strategies

Valentina Cordo'[1], Rico Hagelaar[1], Vera M. Poort[1], Richard Goeij-de Haas[2], Alex A. Henneman[2], Sander R. Piersma[2], Thang V. Pham[2], Connie R. Jimenez[2#], Jules P.P. Meijerink[1#]

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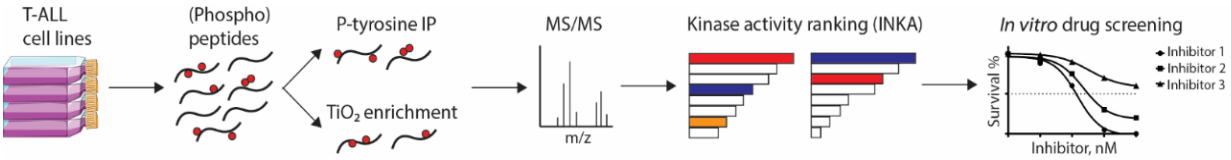
[#]shared senior authorship



INTRODUCTION - Intensive multi-agent treatment has boosted survival up to 80% of pediatric T-cell acute lymphoblastic leukemia (T-ALL) patients. Nevertheless, relapsed patients have a poor prognosis due to acquired therapy resistance and detrimental chemotherapy-induced side effects. Therefore, novel targeted therapies are urgently needed since further intensification of the current high-risk treatment regimen is not feasible for refractory/relapsed cases.

Protein kinase inhibitors (PKIs) are successful cancer treatments. However, targetable kinases activated by gene fusions are rare in T-ALL. The most common *NUP214-ABL1* fusion is detected in less than 6% of T-ALL patients at diagnosis, mostly in minor subclones. Nevertheless, protein kinases can be hyper-activated even in the absence of genomic defects and these signaling activities can drive survival and proliferation of leukemic blasts. Thus, together with the identification of genomic aberrations, phospho-proteomics can provide additional information on aberrant kinases and pathways activation and signaling networks that can offer important opportunities for targeted therapies.

AIM - We aimed to identify relevant kinase activities in T-ALL cell lines and validate selected kinases as possible therapeutic targets to pinpoint targetable leukemia vulnerabilities and design effective (combination) treatment strategies.

APPROACH - Protein extracts from 11 T-ALL cell lines were enriched for phospho-peptides by titanium dioxide enrichment and anti-phosphorylated tyrosine immunoprecipitation followed by liquid chromatography – tandem mass spectrometry (MS). Subsequently, the Integrative Inferred Kinase Activity (INKA) pipeline was used to rank active kinases in each sample (Beekhof et al., 2019). Based on these data, selected kinase inhibitors were tested in vitro as single treatment or in combinations.

RESULTS - MS-based phospho-proteome profiling of 11 T-ALL cell lines identified about 3700 tyrosine phospho-sites and more than 13300 serine/threonine phospho-sites. Various SRC-family members are broadly activated in T-ALL including LCK, SRC, FYN, and YES1 while ABL1, LYN, and FGR were detected only in specific lines. Additionally, CDK1/2 were found to be highly activated in all cell lines. We then tested cellular response to clinically relevant PKIs based on predicted kinase activities. The *in vitro* drug screening showed a cytostatic effect with G1-arrest upon treatment with the CDK1/2 inhibitor milciclib in all the cell lines tested (50nM < IC50 < 1uM).

Despite a general SRC-family kinases activation profile, dual SRC/ABL inhibitors like dasatinib reduced cellular viability only in the 2 cell lines with *ABL1* or *LCK* aberrations (IC50 < 10nM) while other lines that lack *ABL* or *LCK* rearrangements were affected at much higher drug concentration (IC50 > 3uM, which is beyond the clinical achievable plasma concentration in patients). Thus, PKIs were tested in combination based on additional kinase activities detected. A subset of T-ALL cell lines showed activation of the INSR and IGF1R kinases. Since the activation of the INSR/IGF1R axis can promote resistance to tyrosine kinase inhibitors, we tested the effect of combined inhibition of SRC/LCK and INSR/IGF1R kinases in T-ALL. Interestingly, simultaneous inhibition of the SRC-family kinases by dasatinib and the INSR/IGF-1R axis by BMS-754807 led to a drastic reduction of cell survival at nanomolar concentrations, even in dasatinib-resistant cells. These results suggest that INSR/IGF1R inhibition can sensitize cells to dasatinib treatment and highlight a novel possible combination strategy for T-ALL.

CONCLUSIONS - Ranking kinase activities from phospho-proteomic data can guide the use of PKIs as treatment option for T-ALL patients. Moreover, kinase activity profiling can provide insights to design efficient combination treatments for personalized medicine.

REPORTS

- Poster presentation: December 2019: American Society of Hematology annual congress 2019 (ASH 2019, Orlando, Florida, USA)
- Oral Presentations: January 2020, Dutch Hematology Congress (DHC 2020, Papendal, the Netherlands); December 2020, American Society of Hematology annual congress 2020 (ASH 2020, Virtual)

AWARDS - December 2020, Abstract achievement award (American Society of Hematology annual congress, ASH 2020, virtual)

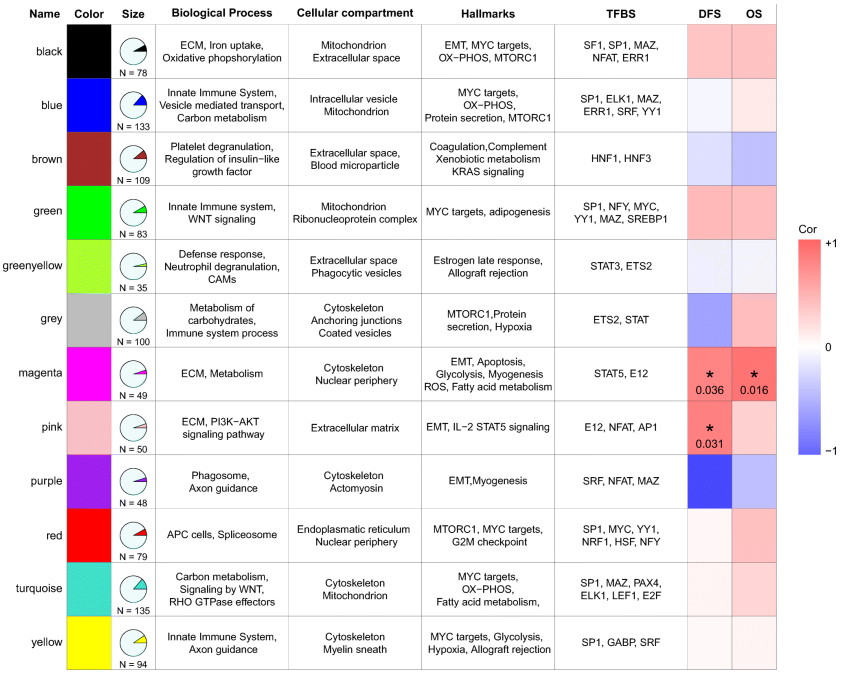
ACKNOWLEDGEMENTS - This project is supported by the Dutch Cancer Society (KWF-10355); the proteomics part of this study is supported by KWF grant 12516 (Phosphoproteomics and integrative analysis to enable precision medicine for anti-EGFR therapy in colorectal cancer).

Pancreatic Cancer

Co-expression analysis of pancreatic cancer proteome reveals biology and prognostic biomarkers

Mantini G, Vallés AM, Le Large TYS, Capula M, Funel N, Pham TV, Piersma SR, Kazemier G, Bijlsma MF, Giovannetti E, Jimenez CR

Cell Oncol (Dordr) 2020 Dec;43(6):1147-1159. doi: 10.1007/s13402-020-00548-y. PubMed PMID: 32860207.



Name	Color	Size	Biological Process	Cellular compartment	Hallmarks	TFBS	DFS	OS
black		N = 78	ECM, Iron uptake, Oxidative phopshorylation	Mitochondrion Extracellular space	EMT, MYC targets, OX−PHOS, MTORC1	SF1, SP1, MAZ, NFAT, ERR1		
blue		N = 133	Innate Immune System, Vesicle mediated transport, Carbon metabolism	Intracellular vesicle Mitochondrion	MYC targets, OX−PHOS, Protein secretion, MTORC1	SP1, ELK1, MAZ, ERR1, SRF, YY1		
brown		N = 109	Platelet degranulation, Regulation of insulin−like growth factor	Extracellular space, Blood microparticle	Coagulation,Complement Xenobiotic metabolism KRAS signaling	HNF1, HNF3		
green		N = 83	Innate Immune system, WNT signaling	Mitochondrion Ribonucleoprotein complex	MYC targets, adipogenesis	SP1, NFY, MYC, YY1, MAZ, SREBP1		
greenyellow		N = 35	Defense response, Neutrophil degranulation, CAMs	Extracellular space Phagocytic vesicles	Estrogen late response, Allograft rejection	STAT3, ETS2		
grey		N = 100	Metabolism of carbohydrates, Immune system process	Cytoskeleton Anchoring junctions Coated vesicles	MTORC1,Protein secretion, Hypoxia	ETS2, STAT		
magenta		N = 49	ECM, Metabolism	Cytoskeleton Nuclear periphery	EMT, Apoptosis, Glycolysis, Myogenesis ROS, Fatty acid metabolism	STAT5, E12	* 0.036	* 0.016
pink		N = 50	ECM, PI3K−AKT signaling pathway	Extracellular matrix	EMT, IL−2 STAT5 signaling	E12, NFAT, AP1	* 0.031	
purple		N = 48	Phagosome, Axon guidance	Cytoskeleton Actomyosin	EMT,Myogenesis	SRF, NFAT, MAZ		
red		N = 79	APC cells, Spliceosome	Endoplasmatic reticulum Nuclear periphery	MTORC1, MYC targets, G2M checkpoint	SP1, MYC, YY1, NRF1, HSF, NFY		
turquoise		N = 135	Carbon metabolism, Signaling by WNT, RHO GTPase effectors	Cytoskeleton Mitochondrion	MYC targets, OX−PHOS, Fatty acid metabolism,	SP1, MAZ, PAX4, ELK1, LEF1, E2F		
yellow		N = 94	Innate Immune System, Axon guidance	Cytoskeleton Myelin sneath	MYC targets, Glycolysis, Hypoxia, Allograft rejection	SP1, GABP, SRF		

Purpose: Despite extensive biological and clinical studies, including comprehensive genomic and transcriptomic profiling efforts, pancreatic ductal adenocarcinoma (PDAC) remains a devastating disease, with a poor survival and limited therapeutic options. The goal of this study was to assess co-expressed PDAC proteins and their associations with biological pathways and clinical parameters.

Methods: Correlation network analysis is emerging as a powerful approach to infer tumor biology from omics data and to prioritize candidate genes as biomarkers or drug targets. In this study, we applied a weighted gene co-expression network analysis (WGCNA) to the proteome of 20 surgically resected PDAC specimens (PXD015744) and confirmed its clinical value in 82 independent primary cases.

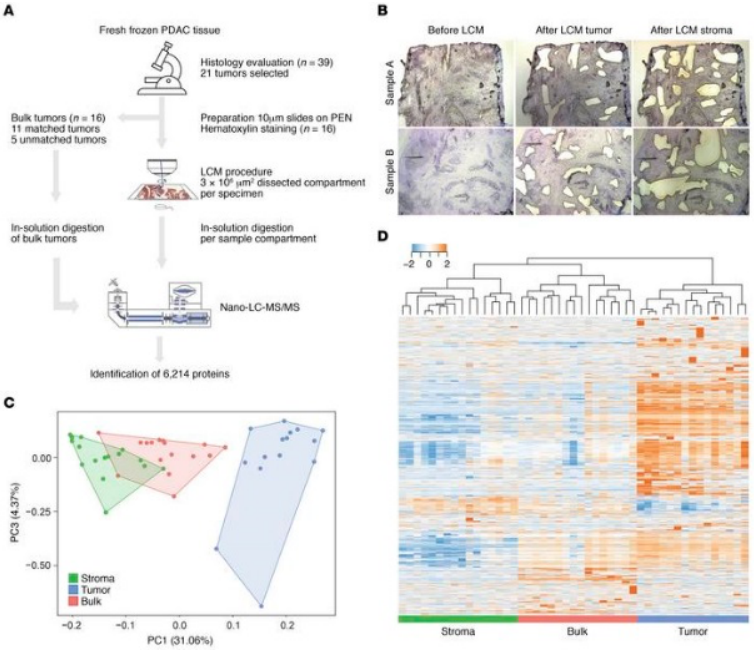
Results: Using WGCNA, we obtained twelve co-expressed clusters with a distinct biology. Notably, we found that one module enriched for metabolic processes and epithelial-mesenchymal-transition (EMT) was significantly associated with overall survival (p = 0.01) and disease-free survival (p = 0.03). The prognostic value of three proteins (SPTBN1, KHSRP and PYGL) belonging to this module was confirmed using immunohistochemistry in a cohort of 82 independent resected patients. Risk score evaluation of the prognostic signature confirmed its association with overall survival in multivariate analyses. Finally, immunofluorescence analysis confirmed co-expression of SPTBN1 and KHSRP in Hs766t PDAC cells.

Conclusions: Our WGCNA analysis revealed a PDAC module enriched for metabolic and EMT-associated processes. In addition, we found that three of the proteins involved were associated with PDAC survival.

Microdissected pancreatic cancer proteomes reveal tumor heterogeneity and therapeutic targets

Le Large TY, Mantini G, Meijer LL, Pham TV, Funel N, van Grieken NC, Kok B, Knol J, van Laarhoven HW, Piersma SR, Jimenez CR, Kazemier G, Giovannetti E, Bijlsma MF

JCI Insight 2020 Aug 6;5(15):e138290. doi: 10.1172/jci.insight.138290. PubMed PMID: 32634123.



Pancreatic ductal adenocarcinoma (PDAC) is characterized by a relative paucity of cancer cells that are surrounded by an abundance of nontumor cells and extracellular matrix, known as stroma. The interaction between stroma and cancer cells contributes to poor outcome, but how proteins from these individual compartments drive aggressive tumor behavior is not known. Here, we report the proteomic analysis of laser-capture microdissected (LCM) PDAC samples. We isolated stroma, tumor, and bulk samples from a cohort with long- and short-term survivors. Compartment-specific proteins were measured by mass spectrometry, yielding what we believe to be the largest PDAC proteome landscape to date. These analyses revealed that, in bulk analysis, tumor-derived proteins were typically masked and that LCM was required to reveal biology and prognostic markers. We validated tumor CALB2 and stromal COL11A1 expression as compartment-specific prognostic markers. We identified and functionally addressed the contributions of the tumor cell receptor EPHA2 to tumor cell viability and motility, underscoring the value of compartment-specific protein analysis in PDAC.

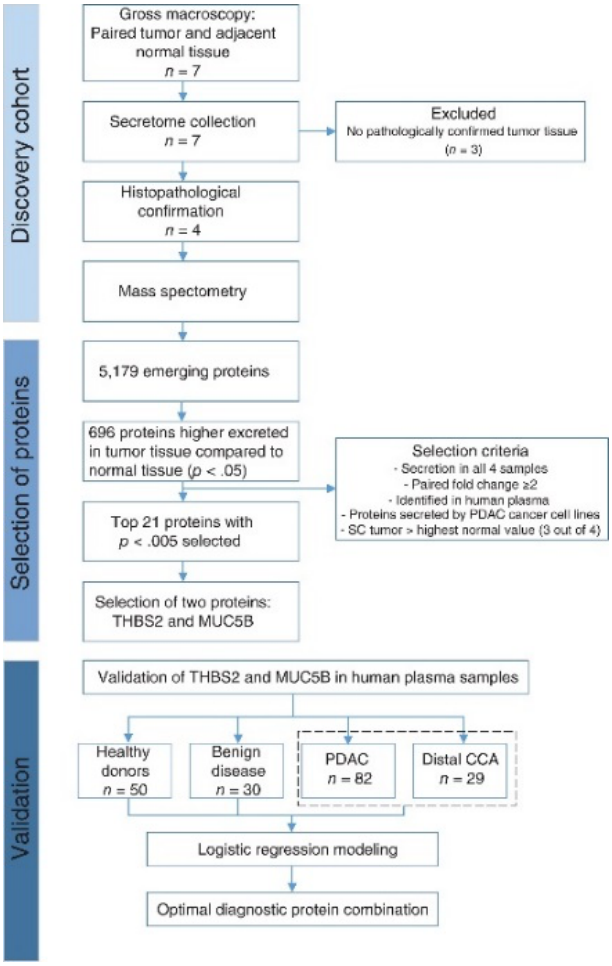
Combined Expression of Plasma Thrombospondin-2 and CA19-9 for Diagnosis of Pancreatic Cancer and Distal Cholangiocarcinoma: A Proteome Approach

Le Large TYS, Meijer LL, Paleckyte R, Boyd LNC, Kok B, Wurdinger T, Schelfhorst T, Piersma SR, Pham TV, van Grieken NCT, Zonderhuis BM, Daams F, van Laarhoven HWM, Bijlsma MF, Jimenez CR, Giovannetti E, Kazemier G

Oncologist 2020 Apr;25(4):e634-e643. doi: 10.1634/theoncologist.2019-0680. PubMed PMID: 31943574.

Background: Minimally invasive diagnostic biomarkers for patients with pancreatic ductal adenocarcinoma (PDAC) and distal cholangiocarcinoma (dCCA) are warranted to facilitate accurate diagnosis. This study identified diagnostic plasma proteins based on proteomics of tumor secretome.

Materials and methods: Secretome of tumor and normal tissue was collected after resection of PDAC and dCCA. Differentially expressed proteins were measured by mass spectrometry. Selected candidate biomarkers



and carbohydrate antigen 19-9 (CA19-9) were validated by enzyme-linked immunosorbent assay in plasma from patients with PDAC (n = 82), dCCA (n = 29), benign disease (BD; n = 30), and healthy donors (HDs; n = 50). Areas under the curve (AUCs) of receiver operator characteristic curves were calculated to determine the discriminative power.

Results: In tumor secretome, 696 discriminatory proteins were identified, including 21 candidate biomarkers. Thrombospondin-2 (THBS2) emerged as promising biomarker. Abundance of THBS2 in plasma from patients with cancer was significantly higher compared to HDs ($p < .001$, AUC = 0.844). Combined expression of THBS2 and CA19-9 yielded the optimal discriminatory capacity (AUC = 0.952), similarly for early- and late-stage disease (AUC = 0.971 and AUC = 0.911). Remarkably, this combination demonstrated a power similar to CA19-9 to discriminate cancer from BD (AUC = 0.764), and THBS2 provided an additive value in patients with high expression levels of bilirubin.

Conclusion: Our proteome approach identified a promising set of candidate biomarkers. The combined plasma expression of THBS2/CA19-9 is able to accurately distinguish patients with PDAC or dCCA from HD and BD.

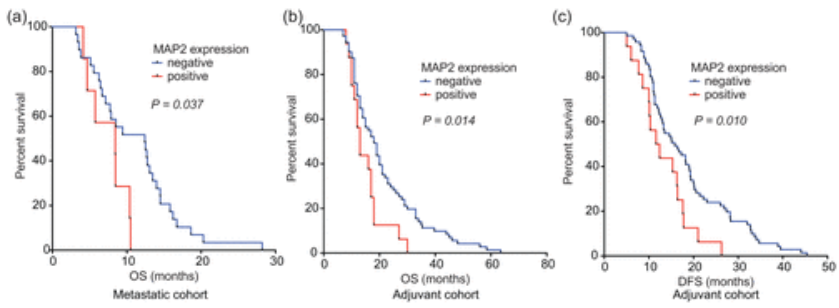
Implications for practice: The combined plasma expression of thrombospondin-2 and carbohydrate antigen 19-9 is able to accurately diagnose patients with pancreatic cancer and distal cholangiocarcinoma. This will facilitate minimally invasive diagnosis for these patients by distinguishing them from healthy individuals and benign diseases.

Proteomic analysis of gemcitabine-resistant pancreatic cancer cells reveals that microtubule-associated protein 2 upregulation associates with taxane treatment

Le Large TYS, El Hassouni B, Funel N, Kok B, Piersma SR, Pham TV, Olive KP, Kazemier G, van Laarhoven HWM, Jimenez CR, Bijlsma MF, Giovannetti E

Ther Adv Med Oncol 2019 May 10;11:1758835919841233. doi: 10.1177/1758835919841233. PubMed PMID: 31205498.

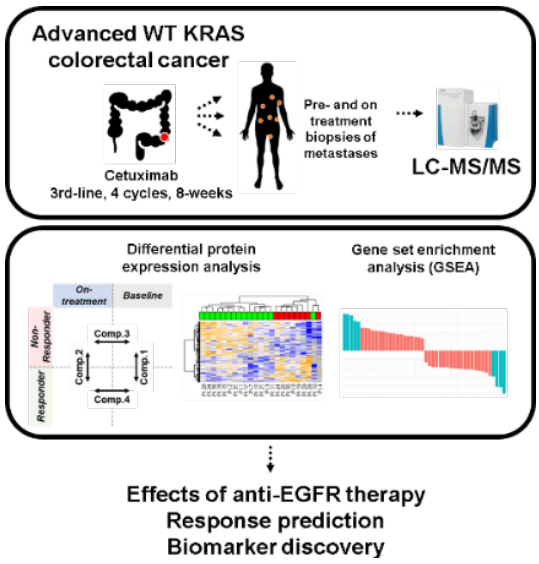
Background: Chemoresistance hampers the treatment of patients suffering from pancreatic ductal adenocarcinoma (PDAC). Here we aimed to evaluate the (phospho)proteome of gemcitabine-sensitive and gemcitabine-resistant PDAC cells to identify novel therapeutic targets and predictive biomarkers.



Methods: The oncogenic capabilities of gemcitabine-sensitive and resistant PDAC cells were evaluated *in vitro* and *in vivo*. Cultured cells were analyzed by label-free proteomics. Differential proteins and phosphopeptides

were evaluated by gene ontology and for their predictive or prognostic biomarker potential with immunohistochemistry of tissue microarrays.

Results: Gemcitabine-resistant cells had increased potential to induce xenograft tumours (p value < 0.001). Differential analyses showed that proteins associated with gemcitabine resistance are correlated with microtubule regulation. Indeed, gemcitabine-resistant cells displayed an increased sensitivity for paclitaxel *in vitro* (p < 0.001) and nab-paclitaxel had a strong anti-tumour efficacy *in vivo*. Microtubule-associated protein 2 (MAP2) was found to be highly upregulated (p = 0.002, fold change = 10) and phosphorylated in these resistant cells. Expression of MAP2 was correlated with a poorer overall survival in patients treated with gemcitabine in the palliative (p = 0.037) and adjuvant setting (p = 0.014).



Conclusions: These data show an explanation as to why the combination of gemcitabine with nab-paclitaxel is effective in PDAC patients. The identified gemcitabine-resistance marker, MAP2, emerged as a novel prognostic marker in PDAC patients treated with gemcitabine and warrants further clinical investigation.

Colorectal Cancer

Proteomics for cetuximab and panitumumab response prediction in metastatic colorectal cancer

S.L. Gerritse[1], F.R. Böttger[2], E.J. van Helden[3], C. Menke-van der Houven van Oordt[4], C.R. Jimenez[2], M. Labots[4], H.M.W. Verheul[1]

[1]Department of Medical Oncology, Radboud Institute for Health Sciences, Radboud University Medical Center, Nijmegen, The Netherlands
[2]Department of Medical Oncology, OncoProteomics Laboratory, Cancer Center Amsterdam, Amsterdam UMC, Vrije Universiteit, Amsterdam, The Netherlands
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INTRODUCTION - Treating *KRAS* and *NRAS* wild-type metastatic colorectal cancer (mCRC) patients with anti-EGFR therapy can be challenging due to suboptimal treatment responses and toxicity. Early detection of non-response to this treatment can facilitate early decision making.

AIM - The aim of this study was to assess whether proteomics could further improve patient selection for anti-EGFR MoAb therapy (cetuximab or panitumumab). In addition, we aimed to gain more insight into the underlying mechanisms for (acquired) resistance to anti-EGFR MoAb monotherapy.

APPROACH - The prospective phase I–II multicenter interventional IMPACT-CRC study (NCT02117466) was performed in patients with mCRC starting treatment with cetuximab 500 mg/m2 or panitumumab 6mg/kg. At baseline and day 34 (before the third cycle) patients underwent a biopsy of a metastatic tumor lesion. This approach allowed us to evaluate proteome profiles under the sole effect of anti-EGFR therapy. Global protein expression of 27 patients was profiled using label-free LC-MS/MS analysis.

RESULTS - Limma-based statistics between responders and non-responders and between pre- and on-treatment biopsies revealed discriminatory protein expression signatures. Strong differential biology related to developmental and proliferation processes distinguished responders from non-responders. Anti-EGFR therapy induced proteome changes were linked to metabolism and immune system. Furthermore, anti-EGFR therapy response prediction biomarker candidates as well as putative drug targets were identified.

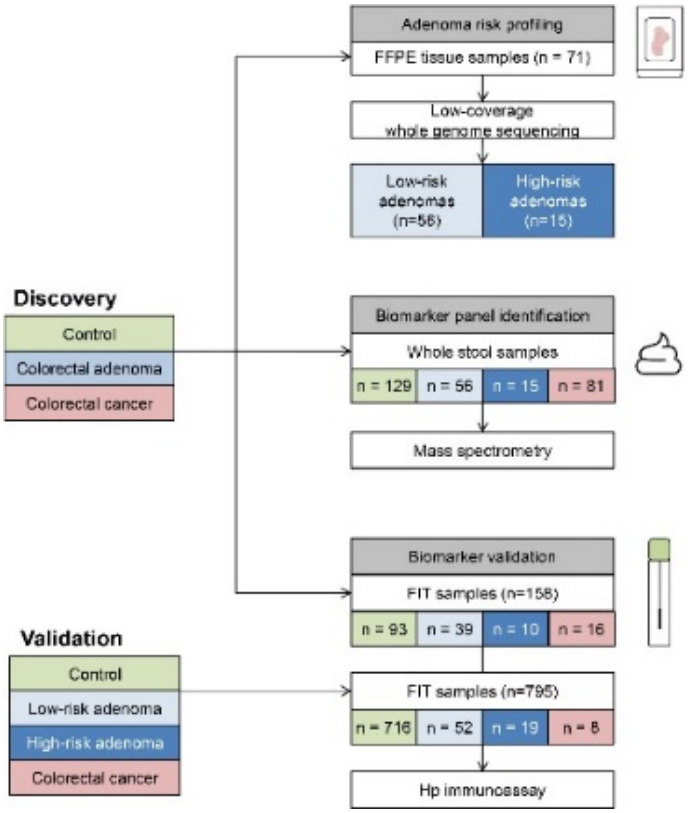
CONCLUSIONS - Our findings improve the understanding of signaling pathways in mCRC. Helping us to gain more insight into the underlying mechanisms for (acquired) resistance to anti-EGFR MoAb monotherapy and further optimization for this therapy.

ACKNOWLEDGEMENTS - This project is supported by the Dutch Cancer Society (Alpe d'huzes Grant IMPACT, RUG 2012-5565).

Proteins in stool as biomarkers for non-invasive detection of colorectal adenomas with high risk of progression

Komor MA, Bosch LJ, Coupé VM, Rausch C, Pham TV, Piersma SR, Mongera S, Mulder CJ, Dekker E, Kuipers EJ, van de Wiel MA, Carvalho B, Fijneman RJ, Jimenez CR, Meijer GA, de Wit M

J Pathol 2020 Mar;250(3):288-298. doi: 10.1002/path.5369. PubMed PMID: 31784980.

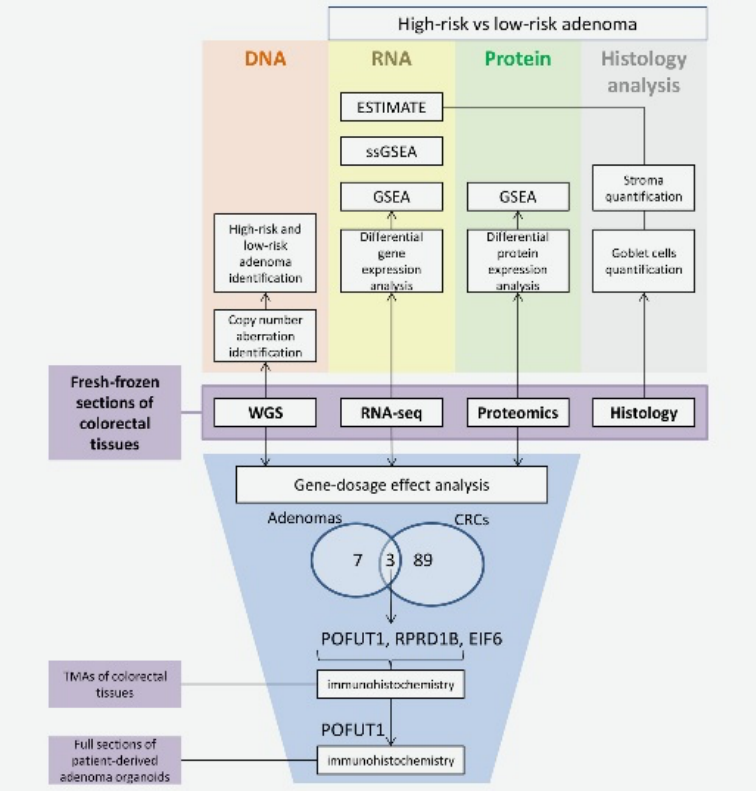


Screening to detect colorectal cancer (CRC) in an early or premalignant state is an effective method to reduce CRC mortality rates. Current stool-based screening tests, e.g. fecal immunochemical test (FIT), have a suboptimal sensitivity for colorectal adenomas and difficulty distinguishing adenomas at high risk of progressing to cancer from those at lower risk. We aimed to identify stool protein biomarker panels that can be used for the early detection of high-risk adenomas and CRC. Proteomics data (LC-MS/MS) were collected on stool samples from adenoma (n = 71) and CRC patients (n = 81) as well as controls (n = 129). Colorectal adenoma tissue samples were characterized by low-coverage whole-genome sequencing to determine their risk of progression based on specific DNA copy number changes. Proteomics data were used for logistic regression modeling to establish protein biomarker panels. In total, 15 of the adenomas (15.8%) were defined as high risk of progressing to cancer. A protein panel, consisting of haptoglobin (Hp), LAMP1, SYNE2, and ANXA6, was identified for the detection of high-risk adenomas (sensitivity of 53% at specificity of 95%). Two panels, one consisting of Hp and LRG1 and one of Hp, LRG1, RBP4, and FN1, were identified for high-risk adenomas and CRCs detection (sensitivity of 66% and 62%, respectively, at specificity of 95%). Validation of Hp as a biomarker for high-risk adenomas and CRCs was performed using an antibody-based assay in FIT samples from a subset of individuals from the discovery series (n = 158) and an independent validation series (n = 795). Hp protein was significantly more abundant in high-risk adenoma FIT samples compared to controls in the discovery (p = 0.036) and the validation series (p = 9e-5). We conclude that Hp, LAMP1, SYNE2, LRG1, RBP4, FN1, and ANXA6 may be of value as stool biomarkers for early detection of high-risk adenomas and CRCs.

NGS-ProToCol Consortium. Molecular characterization of colorectal adenomas reveals POFUT1 as a candidate driver of tumor progression

Komor MA, de Wit M, van den Berg J, Martens de Kemp SR, Delis-van Diemen PM, Bolijn AS, Tijssen M, Schelfhorst T, Piersma SR, Chiasserini D, Sanders J, Rausch C, Hoogstrate Y, Stubbs AP, de Jong M, Jenster G, Carvalho B, Meijer GA, Jimenez CR, Fijneman RJA

Int J Cancer 2020 Apr 1;146(7):1979-1992. doi: 10.1002/ijc.32627. PubMed PMID: 31411736.



Removal of colorectal adenomas is an effective strategy to reduce colorectal cancer (CRC) mortality rates. However, as only a minority of adenomas progress to cancer, such strategies may lead to overtreatment. The present study aimed to characterize adenomas by in-depth molecular profiling, to obtain insights into altered biology associated with the colorectal adenoma-to-carcinoma progression. We obtained low-coverage whole genome sequencing, RNA sequencing and tandem mass spectrometry data for 30 CRCs, 30 adenomas and 18 normal adjacent colon samples. These data were used for DNA copy number aberrations profiling, differential expression, gene set enrichment and gene-dosage effect analysis. Protein expression was independently validated by immunohistochemistry on tissue microarrays and in patient-derived colorectal adenoma organoids. Stroma percentage was determined by digital image analysis of tissue sections. Twenty-four out of 30 adenomas could be unambiguously classified as high risk (n = 9) or low risk (n = 15) of progressing to cancer, based on DNA copy number profiles. Biological processes more prevalent in high-risk than low-risk adenomas were related to proliferation, tumor microenvironment and Notch, Wnt, PI3K/AKT/mTOR and Hedgehog signaling, while metabolic processes and protein secretion were enriched in low-risk adenomas. DNA copy number driven gene-dosage effect in high-risk adenomas and cancers was observed for POFUT1, RPRD1B and EIF6. Increased POFUT1 expression in high-risk adenomas was validated in tissue samples and organoids. High POFUT1 expression was also associated with Notch signaling enrichment and with decreased goblet cells differentiation. In-depth molecular characterization of colorectal adenomas revealed POFUT1 and Notch signaling as potential drivers of tumor progression.

Signaling pathways involved in colorectal adenoma-to-carcinoma progression

Sanne Martens-de Kemp[1,2], Alex Henneman[1,2], Richard de Goeij-de Haas[2], Sander Piersma[2], Thang Pham[2], Gerrit Meijer[1], Beatriz Carvalho[1*], and Connie Jimenez[2*]
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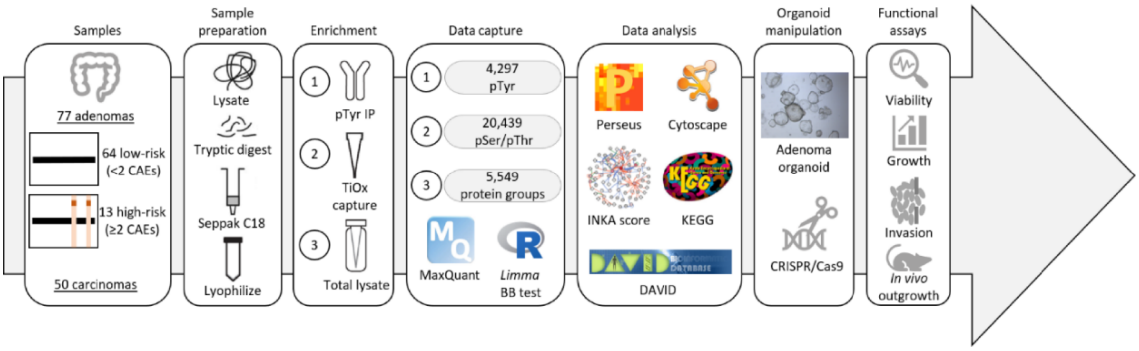
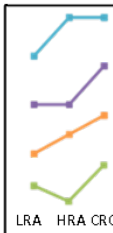
INTRODUCTION - Colorectal cancer (CRC) develops in a multi-step-process from normal epithelium, through a pre-malignant lesion (adenoma), into a malignant lesion (adenocarcinoma). A minority of about 5% of adenomas will ultimately progress into cancer. To improve clinical practice it is important to identify this subgroup. Specific copy number alterations are associated with the risk of progression (gain of 8q/13q/20q or loss of 8p/15q/17p/18q) [1]. Yet, the activation status of these and other genes, and the associated signaling pathways, during colorectal carcinogenesis are not fully known.

AIM - To unravel which signaling pathways are involved in colorectal adenoma-to-carcinoma progression via a comprehensive analysis of phosphoproteomes at different stages of colorectal carcinogenesis, in order to better understand the biology of this transition.



APPROACH - Biobank tissue material was collected for 77 adenomas and 50 CRCs. Adenomas were analyzed with shallow sequencing/MLPA, and those harboring ≥2 copy number aberrations that are correlated with progression were considered high-risk (HRA, n=13). Others were considered low-risk (LRA, n=64). Mass spectrometric profiling of proteins and their phosphorylation was performed on trypsin-digested tissue lysates and phosphopeptide-enriched fractions thereof. Phosphopeptide enrichment entailed either antibody-based (pTyr) or titanium dioxide-based (pSer/pThr) capture.

RESULTS - We identified 5,549 protein groups, 4,297 pTyr phosphopeptides, and 20,439 pSer/pThr phosphopeptides in total. Comparing LRA and CRC as the start and end points of the progression sequence, we found 462 pTyr phosphosites and 6,305 pSer/pThr phosphosites that were significantly differential and distinguished LRA from CRC in hierarchical clustering. Gene ontology analysis implicated a wide range of biological functions for proteins harboring these sites: 1. RTK signaling, protein translation, RNA splicing; 2. cell junction establishment/maintenance, cell adhesion, Golgi vesicle transport, oxidative phosphorylation, general RNA processes; and 3. entry into cell of other organism, movement in host environment, organelle organization, chromosome organization, RNA transport and ERBB pathway. Applying our INKA (Integrative Inferred Kinase Activity) algorithm [2], we concluded CDK1, CDK2, and PRKDC, among others, to be more active in CRC while many receptor tyrosine kinases more active in adenomas showed higher activity in HRA compared to LRA (eg., EGFR, INSR, EPHA2, EPHB1). CBL, a proto-oncogene-encoded E3 ubiquitin ligase involved in proteasome-mediated protein degradation and downmodulation of signaling, was significantly less phosphorylated in CRC. We hypothesize that this results in reduced EGFR ubiquitination and downmodulation, making CBL an interesting protein to test for its potential involvement in adenoma progression.



Trend	pTyr	pSer/pThr
Early phosphorylation	21	77
Late phosphorylation	13	82
Progressive phosphorylation	3	0
Reactive phosphorylation	2	5
Early dephosphorylation	0	134
Late dephosphorylation	108	757
Progressive dephosphorylation	1	3
Reactive dephosphorylation	4	74

To zoom in on differences between LRA, HRA, and CRC, we investigated specific phosphorylation trends Assessing early (de)phosphorylation, we found 232 phosphosites on 217 proteins to be significantly different between LRA and HRA. Network and gene ontology analysis revealed a protein cluster enriched for proteins involved in Hippo signaling, a tumor suppressor pathway [3] in which SCRIB functions as a scaffold in phosphorylation events that eventually result in the inactivation of the transcription factor YAP1 involved in cell proliferation and survival [4]. SCRIB and various other Hippo pathway members were less phosphorylated in CRC compared to (low-risk) adenomas. SCRIB phosphorylation may be necessary to inhibit cell proliferation by binding and nuclear exclusion of YAP1. The *SCRIB* gene is located on chromosome 8q, often gained in CRC, and there might be an interplay between gene dosage and protein phosphorylation status.

With late (de)phosphorylation trends, we found 960 phosphosites on 694 proteins to be significantly differential between HRA and CRC. We identified a protein cluster involved in the mitotic cell cycle, in line with the increased activity of CDKs in CRCs as found in our INKA analysis. It included PDS5B, a protein encoded by a putative tumor suppressor gene [5] and involved in sister chromatid separation during cell division. Defects in this process lead to aneuploidy, a phenomenon seen in ~85% of adenoma-carcinoma transitions [6]. PDS5B shows more phosphorylation in CRC than in adenomas, which might inactivate the protein. Where *PDS5B* is

frequently lost in many cancers, it is often amplified in CRC, and, like for CBL, gene dosage and PDS5B phosphorylation may have a relationship.

Three candidate proteins, CBL, SCRIB, and PDS5B, were selected for further analysis in our human adenoma organoid culture system, along with PTEN as a positive control for 'progression' upon knockout. Separate CRISPR-based gene knockouts were achieved for all four targets in at least one of three well-characterized organoid cultures, with multi-gene KOs being still under way. For oncogenic transformation assays in organoid cultures, with their challenging three-dimensional, gel matrix-supported makeup, we devised ways to monitor proliferation, growth, viability, and apoptosis. The single-gene KOs are currently under investigation for progression in this system, and preliminary results for CBL show that, as predicted, its inactivation leads to a more cancer-like phenotype. As organoids do not grow in vivo, ultimate analyses of oncogenic, invasive, and metastatic behaviors will be performed by organoid cell injection in mice.

CONCLUSIONS - This is the first large phosphoproteomics dataset of colorectal adenomas and carcinomas that exhibiting differences in kinase activities between benign and malignant lesions. Candidate driver genes that may normally be needed to suppress cancerous growth were found to have differential phosphorylation (CBL, SCRIB: early in adenoma progression, PDS5B: late in progression) and have been knocked out in adenoma-derived organoids. Once all organoid experiments have been successfully concluded, mouse studies will be initiated to shed more light on the role of these candidates in colorectal adenoma-to-carcinoma progression.

REFERENCES -

1. Hermsen M, Postma C, Baak J, et al. Colorectal adenoma to carcinoma progression follows multiple pathways of chromosomal instability. Gastroenterology 2002;123:1109–1119.
2. Beekhof R, Alphen C, Henneman AA, et al. INKA, an integrative data analysis pipeline for phosphoproteomic inference of active kinases. Mol Syst Biol 2019;15:613–22.
3. Sanchez-Vega F, Mina M, Armenia J, et al. Oncogenic Signaling Pathways in The Cancer Genome Atlas. Cell 2018;173:321–337.e10.
4. Mohseni M, Sun J, Lau A, et al. A genetic screen identifies an LKB1–MARK signalling axis controlling the Hippo–YAP pathway. Nat Cell Biol 2013;16:108–117.
5. Denes V, Pilichowska M, Makarovskiy A, et al. Loss of a cohesin-linked suppressor APRIN (Pds5b) disrupts stem cell programs in embryonal carcinoma: an emerging cohesin role in tumor suppression. Oncogene 2019:1–7.
6. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. Nature 1998;396:643–649.

Data independent *versus* data dependent acquisition mass spectrometry for proteomic classification of colorectal cancer subtypes

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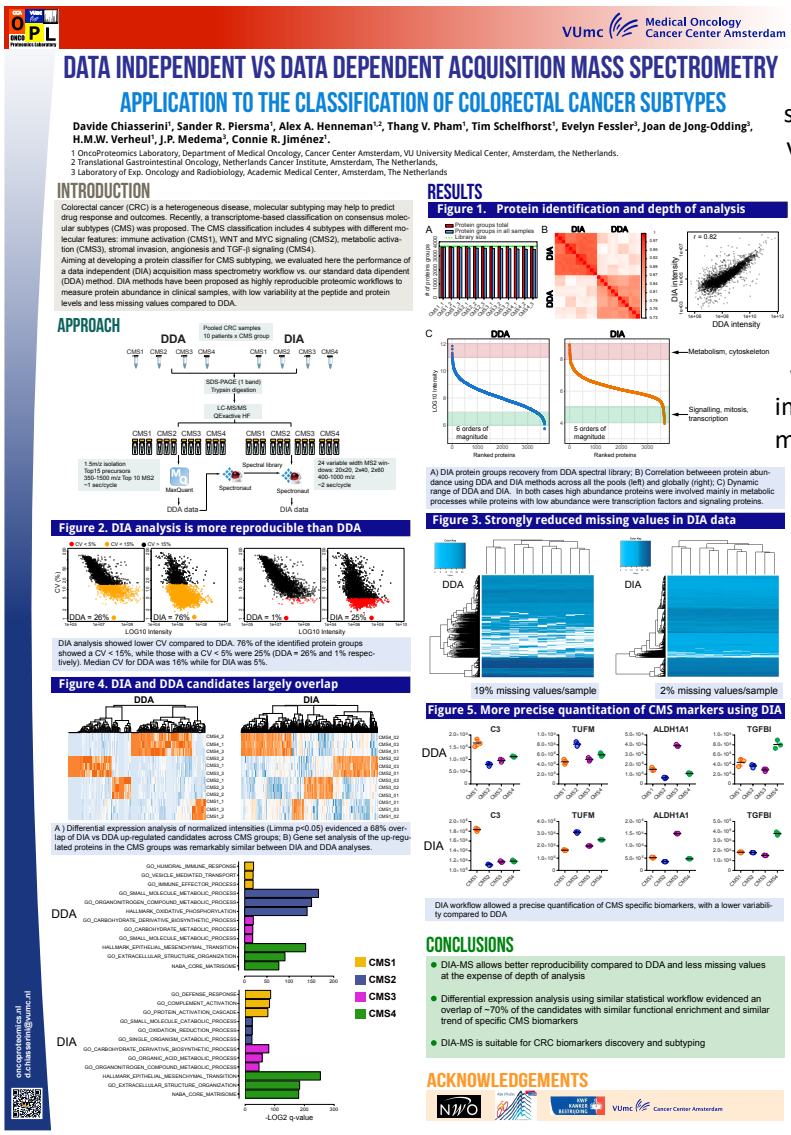
INTRODUCTION - Colorectal cancer (CRC) is a heterogeneous disease, and molecular subtyping may help to predict drug response and outcomes. Recently, a classification based on 4 consensus molecular subtypes (CMS) was proposed. Here we compared data dependent (DDA) and data independent (DIA) acquisition methods for distinguishing the four CMS subtypes, with the aim to build a proteomic classifier for CRC.

APPROACH - CRC tissues from the 4 CMS subtypes were lysed, digested and desalted. Four pools (CMS1,2,3,4) were constructed, including 10 patients per group. Single shot runs in triplicate were performed for both DDA and DIA using a 120 min gradient on a QExactive HF. For DDA, a top-15 method was used, while DIA data were acquired using variable acquisition windows of 20, 40 and 60 Da. DDA data were searched using the MaxQuant computational platform; while DIA data were searched using the Spectronaut software against a spectral library built with 12 DDA runs of the pools, including 3697 protein groups and 29793 peptides.

RESULTS - About 3600 protein groups were identified in DIA mode, showing an overlap of 90% with DDA



analysis. DIA showed a high level of data completeness, with an average number of missing values per sample of 2% (DDA=19%). Technical variation was also lower with DIA, with a median CV of 5%, vs 16% of DDA analysis. DIA analysis was able to detect 68% of the candidates differentiating the 4 CMS subtypes found with DDA. CMS1 subtype specific markers were associated with complement activation and immune processes, CMS2 with mitochondrial organization and oxidative metabolism, CMS3 with glucose metabolism and vesicle transport; while CMS4 with extracellular matrix and epithelial-mesenchymal transition.

CONCLUSIONS - DIA is a robust method for biomarker discovery in clinical settings, the high reproducibility makes it amenable for CRC proteomic subtyping.

Phosphoproteomics and integrative analysis to enable precision medicine for anti-EGFR therapy in colorectal cancer

Franziska Böttger[1,2], Robin Beekhof[1], Andrea Bertotti[3,4], Valentina Vurchio[3,4], Francesca Cottino[3], Eugenia R. Zanella[3], Giorgia Migliardi[3,4], Marco Viviani[3,4], Elena Grassi[3,4], Richard R. de Haas[1], Sander R. Piersma[1], Thang V. Pham[1], Jaco C. Knol[1], Alex A. Henneman[1], Mariette Labots[5], Henk M.W. Verheul[5,6], Livio Trusolino[3,4], Lodewyk Wessels[2], Connie R. Jimenez[1]

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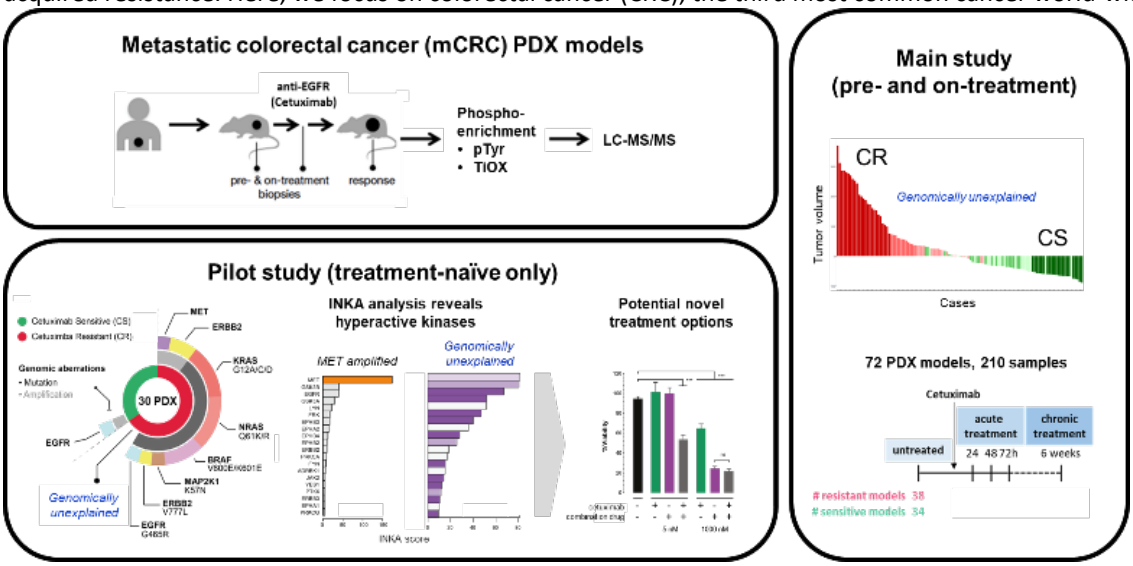
INTRODUCTION - Epidermal growth factor receptor (EGFR) is a well-exploited kinase target in precision medicine in several cancer types. Unfortunately, the efficacy of such therapies is limited due to primary and

acquired resistance. Here, we focus on colorectal cancer (CRC), the third most common cancer world-wide,



with 50% of patients developing metastasis (mCRC). mCRC treatment is hampered by therapy resistance, with clinical benefit from EGFR blockade only observed in 10% of patients. Even in molecularly stratified patients (*KRAS/NRAS/BRAF* wild-type), ~40% has no benefit and when taking into account all potential resistance mechanisms identified to date, an estimated ~30% still does not benefit from therapy. Moreover, there are no validated predictors of positive response to EGFR blockade. Using genomics, we have identified several kinases predictive of resistance in ~21% of *KRAS/NRAS/BRAF* wild-type patient-derived xenograft (PDX) models. As we have reached the limits of genomics, we now extend these findings, using (phospho-) proteomics as a complementary approach capturing protein activation states on a global scale. Kinases control protein activity and signaling through phosphorylation. Thus, unbiased profiling of protein phosphorylation by mass spectrometry (phospho-proteomics) is a powerful tool to uncover predictive markers and drug targets. Importantly, we will employ paired pre- and on-treatment profiles of tumors to pinpoint resistance mechanisms.

AIM - Our primary aim is to uncover aberrantly active signaling pathways and mechanisms underlying sensitivity and resistance to anti-EGFR monoclonal antibodies in *KRAS/NRAS/BRAF* wild-type mCRC. This will reveal novel predictors for improved therapy selection as well as signaling dependencies other than EGFR and alternative phosphokinases that can be targeted to circumvent resistance.

APPROACH - To evaluate the feasibility and utility of comprehensive proteomic and phospho-proteomic profiling in unraveling the molecular basis of sensitivity and resistance to cetuximab-mediated EGFR blockade and in identifying alternative drug targets, we first analysed a panel of 30 treatment-naive patient-derived xenograft models of mCRC by mass-spectrometry. Global proteome, pTyr- and TiOx-based phospho-proteomes were profiled. Using both group-based statistical approaches as well as single sample integrative inferred kinase activity (INKA) analysis, differential signaling events between cetuximab sensitive and resistant tumor were explored.

Subsequently, we focused on finding molecular drivers in genomically unexplained models using the same phosphoproteomics approach. To this end, we collected and processed a panel of 72 mCRC PDX models consisting of pairs of pre- and cetuximab-treated samples at different time points (210 samples in total).

RESULTS - Group-based statistics between cetuximab sensitive and resistant PDX models revealed a discriminatory phospho-site signature that could clearly separate sensitive from resistant tumors in cluster analysis. Functional analysis revealed biology related to cell-cell junction organization being aberrantly regulated. Furthermore, ranking of kinase activities in single samples could confirm the driver activity of eg, EGFR, MET and ERBB2 in respective genomically explained models, and revealed high activity of several other kinases in resistant *RAS/RAF* wild-type tumors with genomically unexplained resistance. Inhibition of these hyperactive kinases alone or in combination with cetuximab resulted in growth reduction of corresponding PDX-derived organoids and PDX models.

CONCLUSIONS - Here, we highlight the added benefit of phospho-proteomics in understanding the biology of anti-EGFR treatment and response prediction. We showcase the feasibility of phospho-proteomics coupled to

single sample INKA analysis in providing a powerful read-out of relevant kinase activities for individualized (combination) treatment. Currently, we are working on extending the findings of genomic studies and the pilot phospho-proteomics study using pre- and on-treatment samples to find molecular drivers in a large panel of genomically unexplained models. Dedicated integration with mutation, copy number and RNA expression data, as well as functional validation in PDX-matched organoids will be performed.

REFERENCE - Beekhof, R., Bertotti, A., Böttger, F., Vurchio, V., Cottino, F., Zanella, E.R., Migliardi, G., Viviani, M., Grassi, E., Henneman, A.A., et al. Phosphoproteomics identifies targets and markers associated with sensitivity and resistance to EGFR blockade in colorectal cancer. Manuscript in preparation

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Lung Cancer

Identification of protein biomarkers for prediction of response to platinum-based treatment regimens in non-small cell lung cancer

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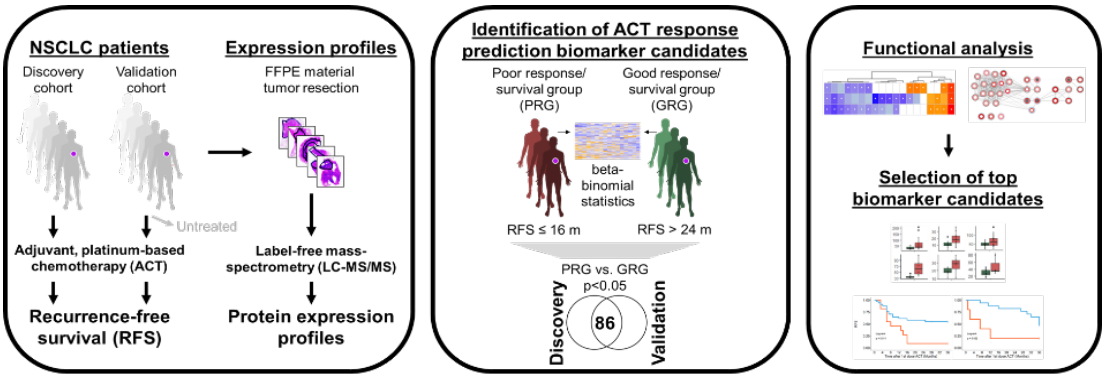
INTRODUCTION - The majority of stage IIA-IIIA non-small cell lung cancer (NSCLC) patients eligible for surgery are treated with adjuvant platinum-based chemotherapy (ACT) in a one-size-fits-all approach. However, a significant number of patients do not benefit due to acquired or intrinsic drug resistance. No predictive patient selection biomarker is currently available for ACT.



AIM - The aim of this study was to identify protein markers that can aid in pinpointing NSCLC patients likely to derive clinical benefit from ACT.

APPROACH - Using global, label-free GeLC-MS/MS-based proteomics, we have profiled archived primary tumor resection material (FFPE) of 45 NSCLC patients who received ACT, and then correlated protein expression profiles to clinical outcome in order to develop a predictor of recurrence-free survival (RFS). Using the same methodology, an independent validation cohort consisting of 32 patients that either received ACT or were left

untreated following resection, was profiled. Most promising ACT response prediction candidates were identified by data integration.

RESULTS - Proteomic profiling of 45 NSCLC patient samples identified almost 5000 proteins. Unsupervised cluster analysis revealed a poor response/survival sub-cluster that displayed a strong epithelial-mesenchymal transition (EMT) signature and high stromal score. Cisplatin sensitivity and resistance profiles beyond this stromal subpopulation were defined by applying beta-binomial statistics. We identified and validated proteins involved in pathways relevant to the mechanism of action of cisplatin, such as RNA processing, DNA damage repair and drug metabolism.

CONCLUSIONS - In the current study, protein profiles reflecting sensitivity and resistance to cisplatin were identified in 2 independent, multi-center cohorts of NSCLC patients in the ACT setting. Predictive and prognostic potential was determined by analysis of untreated resection samples. In addition, potential novel drug targets were identified. Currently, candidate predictive platinum-drug response proteins are being validated using immunohistochemistry (IHC) as an independent, clinically applicable protein assay.

REFERENCES - Böttger, F., Radonic, T., Bahce, I., Monkhorst, K., Piersma, S.R., Pham, T. V., Thunnissen, E., Smit, E.F., Dingemans, A.-M.C., Hillen, L.M., et al. Manuscript in preparation.

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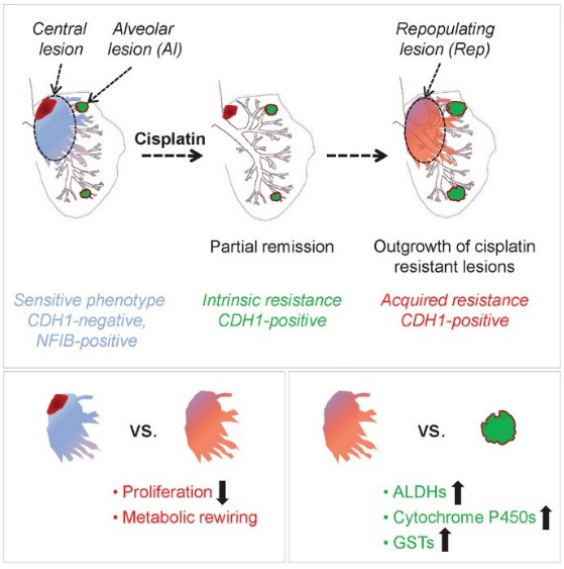
Tumor Heterogeneity Underlies Differential Cisplatin Sensitivity in Mouse Models of Small-Cell Lung Cancer

Böttger F, Semenova EA, Song JY, Ferone G, van der Vliet J, Cozijnsen M, Bhaskaran R, Bombardelli L, Piersma SR, Pham TV, Jimenez CR, Berns A

Cell Rep 2019 Jun 11;27(11):3345-3358.e4. doi: 10.1016/j.celrep.2019.05.057. PubMed PMID: 31189116.



Small-cell lung cancer is the most aggressive type of lung cancer, characterized by a remarkable response to chemotherapy followed by development of resistance. Here, we describe SCLC subtypes in Mycl- and Nfib-driven GEMM that include CDH1-high peripheral primary tumor lesions and CDH1-negative, aggressive intrapulmonary metastases. Cisplatin treatment preferentially eliminates the latter, thus revealing a striking differential response. Using a combined transcriptomic and proteomic approach, we find a marked reduction in proliferation and metabolic rewiring following cisplatin treatment and present evidence for a distinctive metabolic and structural profile defining intrinsically resistant populations. This offers perspectives for effective combination therapies that might also hold promise for treating human SCLC, given the very similar response of both mouse and human SCLC to cisplatin.

Proteome analysis of non-small cell lung cancer cell line secretomes and patient sputum reveals biofluid biomarker candidates for cisplatin response prediction

Böttger F, Schaaij-Visser TB, de Reus I, Piersma SR, Pham TV, Nagel R, Brakenhoff RH, Thunnissen E, Smit EF, Jimenez CR

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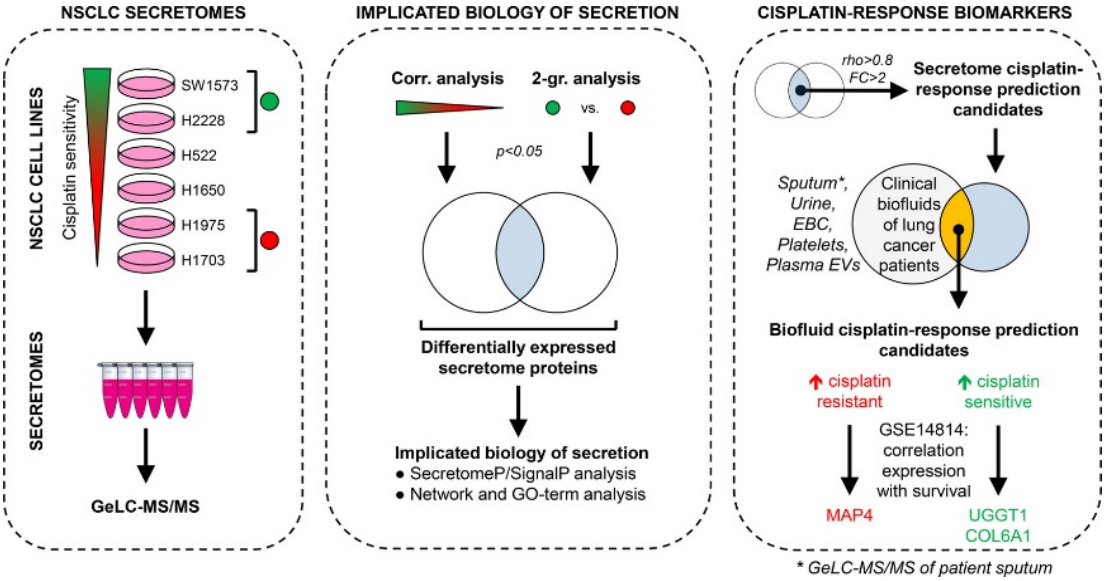
Molecular markers are urgently needed to select non-small cell lung cancer (NSCLC) patients most likely to benefit from platinum-based chemotherapies. Of particular interest are proteins that can be found in biofluids like sputum for non-invasive detection. Therefore, we profiled the secretomes of 6 NSCLC cell lines with varying

IC50-values for cisplatin, using label-free GeLC-MS/MS-based proteomics. Out of a total dataset of 2610 proteins, 304 proteins showed significant differences in expression levels between cisplatin sensitive and insensitive cell lines. Functional data mining revealed that the secretion of typically extracellular factors was associated with a higher sensitivity towards cisplatin, while cisplatin insensitivity correlated with increased secretion of theoretically intra-cellular proteins. Stringent statistical analysis and quantitative filtering yielded 58 biomarker candidates, 34 of which could be detected in clinical biofluids of lung cancer patients such as sputum using label-free LC-MS/MS-based proteomics. To assess performance of these biofluid biomarker candidates, we correlated protein expression with patient survival using a publically available clinical gene expression data set (GSE14814). We thus identified 3 top candidates with potential predictive value in determining cisplatin response (UGGT1, COL6A1 and MAP4) for future development as non-invasive biomarkers to guide treatment decisions.



SIGNIFICANCE: Platinum-based chemotherapies are still the standard of care for NSCLC and other lung cancer types in the clinic today. However, due to chemoresistance, many patients suffer from the toxic side effects of these treatments without gaining any benefit in terms of survival. To date, no molecular biomarkers are available to predict clinical outcome of platinum-based chemotherapy. Because proteins present the functional read-out of genetic, epigenetic and translational events in the cell, a protein test is likely to be particularly suitable for response prediction. Of high relevance are proteins that are shed or secreted from cells, for example at primary tumor sites, and can be found in easily accessible biofluids like sputum for non-invasive detection. Here, we report the proteome profiling of the conditioned media (secretomes) of a panel of NSCLC cell lines in relation to cisplatin IC50 values, as a pre-clinical model, and of patient sputum as a clinical, lung cancer relevant biofluid. Using this approach in conjunction with exploration of the predictive potential in a transcriptome lung cancer patient dataset, we reveal biofluid biomarker candidates that, with further validation, may be used for non-invasive cisplatin response prediction in the future.

Breast Cancer

Discovery and clinical validation of novel protein biomarkers for homologous recombination deficient breast cancer

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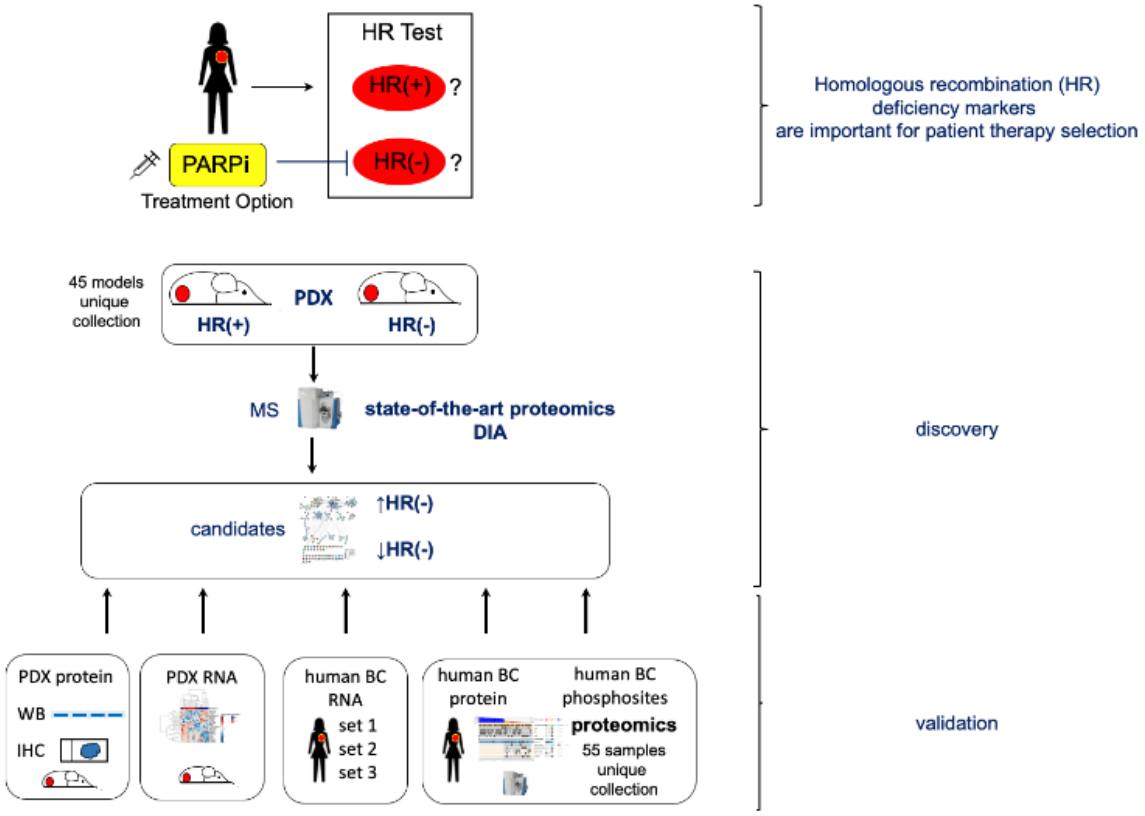
INTRODUCTION - Triple negative breast cancers (TNBC) show very poor prognosis, with chemotherapy as the only standard treatment option. However, because of a *BRCA1* mutation and consequent deficiency in homologous recombination (HR) repair of DNA double-strand breaks, a subgroup of TNBC tumors is sensitive to DNA damaging drugs or PARP1 inhibitors interfering with DNA repair. Therefore, identifying biomarkers that can additionally distinguish breast cancers that lack *BRCA* mutations but are HR deficient is of importance for personalized therapy. In order to provide a basis for future development of a protein test that is aimed at patient selection for PARP inhibitor treatment, we used patient-derived xenograft (PDX) mouse models and mass spectrometry based proteomics to identify candidate protein biomarkers for HR deficient breast cancer.



METHODS - Untreated tumor biopsies and samples harvested 24h after cisplatin treatment were collected from 45 PDX models with known HR status. Overall, 94 tissues were analyzed by proteomic and phosphoproteomic characterization using label-free single-shot LC-MS/MS on a Q Exactive HF mass spectrometer.

RESULTS - In total, we identified 6208 poteins and 10480 high-confidence phosphosites on average for each sample. Gene set enrichment analysis of proteomic data linked proteins with enhanced levels in the HR deficient group to mitotic spindle and DNA repair pathways (e.g. XRCC5/6). Moreover, we observed downregulation of interferon alpha and gamma response pathways, the ER phagosome pathway, and antigen processing and presentation in HR deficient samples.

For promising candidates, these findings were verified via immunoblotting using the same lysates. Next, we established immunohistochemical staining procedures for selected candidates, and currently our PDX tissue collection is stained for verification of the proteomics results.

For our phosphoproteomic data, using phosphosite-specific signature analysis, we found for example activity of cell cycle kinases changed in the HR deficient situation.

CONCLUSION - Our study indicates a compromised antigen processing and presentation phenotype for HR deficient tumors and thus links DNA repair and immune defense. We have started now to validate our findings via proteomics profiling of 55 well-annotated human breast cancer samples with known HR status.

Integrative Proteomics and Phosphoproteomics Profiling of Response to Dual HER2-Blockade with Pertuzumab and Trastuzumab in HER2+ Breast Cancer Cell Models

Paula González-Alonso[1,2], Sander R Piersma[1], Jaco C Knol[1], Alex Henneman[1], Thang V Pham[1], Richard R de Haas[1], Ester Martín-Aparicio[2], Sandra Zazo[2], Melani Luque[2], Marta Sanz-Álvarez[2], Juan Madoz-Gúrpide[2], Ana Rovira[3], Joan Albanell[3], Pilar Eroles[4], Ana Lluch[4], Connie R Jimenez[1*], Federico Rojo[2*]

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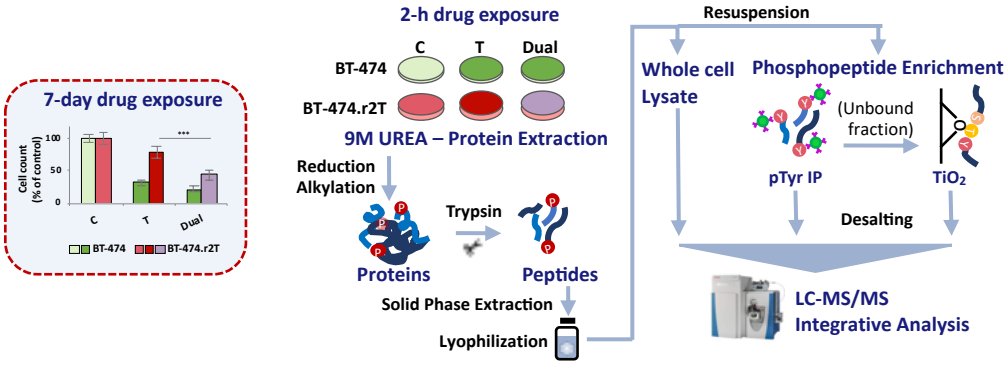
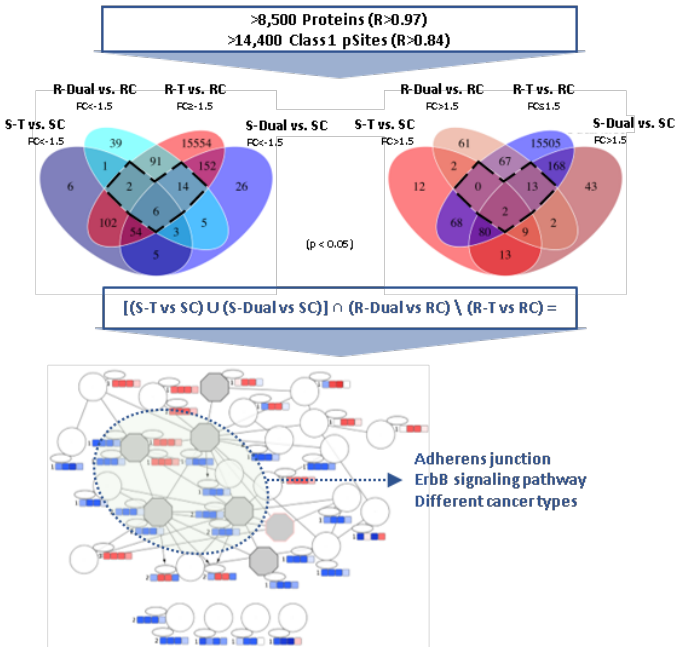


INTRODUCTION – While HER2-enriched (HER2+) breast cancers targeted by trastuzumab frequently acquire resistance to this therapy after some months of treatment exposure, dual HER2-blockade with pertuzumab and trastuzumab significantly improves the prognosis of this disease. Although evidence is emerging of pertuzumab-based therapy eventually become ineffective due to acquired resistance development, and there is an urgent need for alternative therapies. Many trastuzumab-resistance mechanisms modify protein phosphorylation profiles involved in oncogenic signaling networks. Mapping multiproteomic profiles using quantitative global proteomics and phosphoproteomics allows unraveling novel mechanisms of resistance and surrogate biomarkers of dual HER2-blockade response as potential druggable targets for directed therapy in acquired trastuzumabresistant HER2+ breast cancer.



APPROACH - Global proteomics, pTyr-based and TiOx-based phosphoproteomics were performed of HER2+ BT-474 breast cancer cell lines sensitive and with acquired resistance to trastuzumab in duplicate at baseline and after 2 h drug treatment with trastuzumab alone or in combination with pertuzumab.

RESULTS - Phosphoproteome profiling of sensitive and resistant HER2+ breast cancer cell lines+/- (combination) drug treatment yielded a large dataset consisting of 14.471 class I phosphosites on 8,565 phosphoproteins including 388 protein kinases, with a good reproducibility between 2 replicates (average Pearson correlation coefficient >0.97 for proteins and >0.84 for Class 1 pSites. We first defined the resulting overlap between baseline and trastuzumab-exposed parental and resistant cells as the top signaling reprograming in acquired trastuzumab resistance.

We further prioritized the most relevant signaling events observed after effective antiproliferative anti-HER2 therapy in breast cancer as the molecular mechanisms determining differential sensitivity to short-term exposure to trastuzumab alone or combined with pertuzumab. A resulting PPI network was generated with the overlapping candidates between differential regulation after any treatment in parental cells and after dual treatment in resistant cells, which resulted unmodified or reversely regulated after trastuzumab in resistant cells. In contrast to the effect of trastuzumab alone, dual drug treatment of resistant cell lines showed overlapping regulation profiles with either single or dual drug regulation in sensitive cells, including restoration of inhibition or activation of EGFR/HER2-MAPK proliferation signaling and adherens junction proteins.

CONCLUSIONS - Regulation events arising from the dual treatment of cells with acquired trastuzumab resistance might be crucial to initial drug sensitivity. Phosphoproteomics of the BT-474 HER2+ breast cancer cell model has shown wide impact of ErbB-MAPK signaling on the trastuzumab resistant phenotype, with multiple KEGG pathways affected including adherens junction and different types of cancer. Data analysis is on-going to select biomarkers as potential drug targets for novel combination treatment of HER2+ breast cancer cell lines with acquired resistance to trastuzumab.

Renal Cancer

Loss of FLCN tumor suppressor triggers an interferon response signature in kidney epithelial cells

Iris Glykofridis[1,2], Jesper Balk[1], Jaco Knol[2], Thang Pham[2], Sander Piersma[2], Renee de Menezes[3], Saskia van Mil[1], Hanne Meijers-Heijboer[1], Connie Jimenez*[2], Rob Wolthuis*[1]

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INTRODUCTION - Folliculin (FLCN) is a tumor suppressor gene and loss of function mutations predispose to an increased risk of renal cancer in Birt-Hogg-Dubé syndrome. However, understanding of the mechanisms and molecular pathways by which FLCN loss leads to oncogenic transformation of renal cells is insufficiently clear.

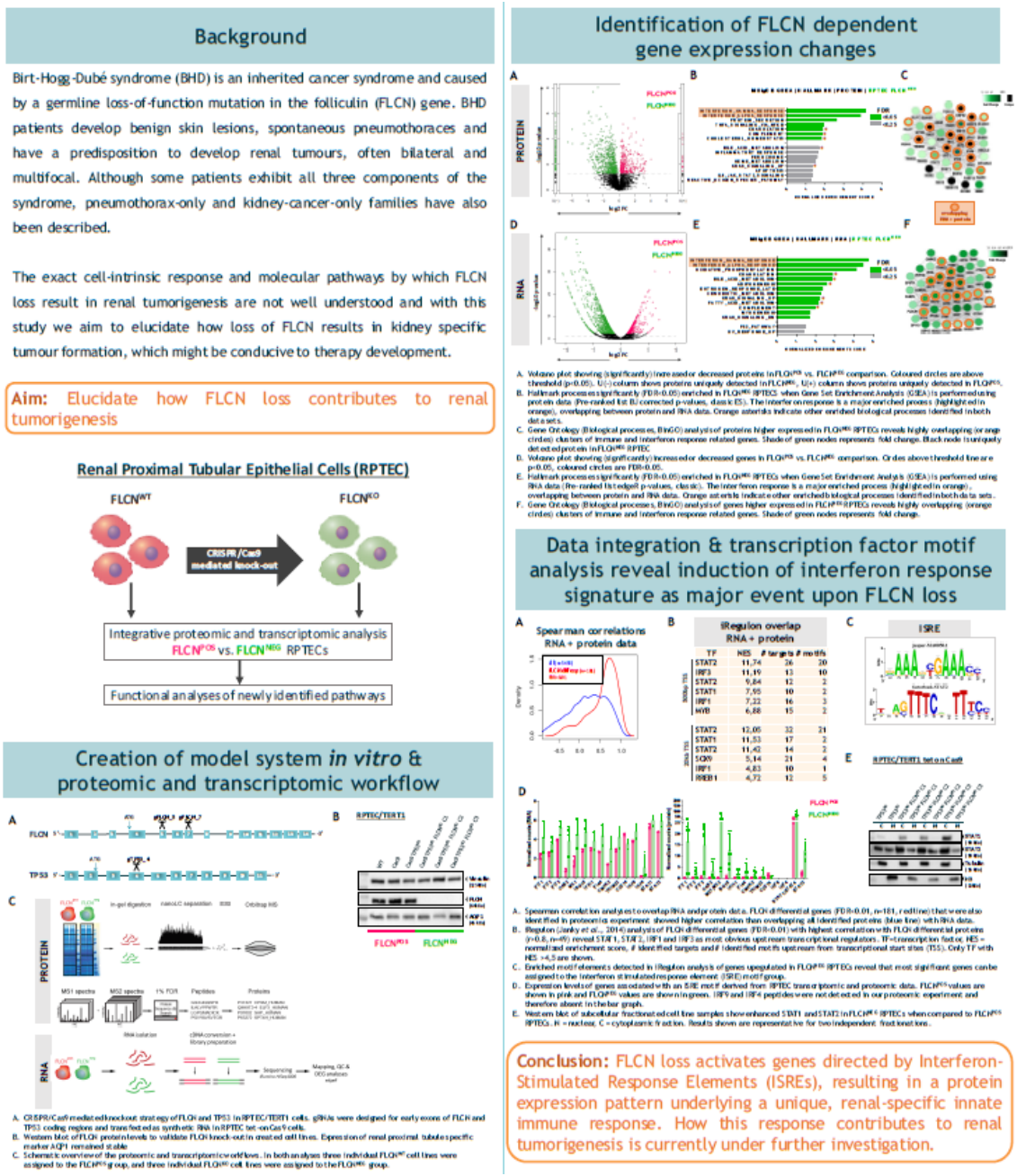
AIM - We aim to reveal the entire spectrum of FLCN-dependent molecular transcriptomic and proteomic alterations in the context of a kidney cell culture model.

APPROACH - Here, we created a novel renal epithelial cell based isogenic model system to study the exact effect of FLCN loss. By means of CRISPR/Cas9 we knocked out FLCN expression in renal proximal tubular epithelial cells (RPTEC/TERT1), which are of the cell type that is regarded as the origin of renal cell carcinoma. By RNA sequencing and label-free GeLC-MS/MS-based proteomics we determined the unique transcriptomes and functionally relevant proteomes of the generated isogenic cell line pairs ($FLCN^{NEG}$ vs. $FLCN^{POS}$). Gene Ontology and Gene Set Enrichment Analyses revealed a broad spectrum of biological processes regulated by FLCN, which were functionally studied.

RESULTS - The analyses confirm earlier observations regarding FLCN-mediated regulation of the TFE3 transcription factor and, moreover, point towards regulation of STAT1/STAT2 and induction of a kidney tubular interferon response signature. Mechanistically, FLCN loss promotes STAT2 recruitment to chromatin and slows cellular proliferation.

CONCLUSIONS - Our integrated analysis identifies STAT1/2 signaling as a novel target of FLCN in renal cells and BHD tumors. STAT1/2 activation appears to counterbalance TFE3-directed hyper-proliferation and may influence the immune response. These findings shed light on unique roles of FLCN in human renal tumorigenesis and pinpoint novel prognostic biomarkers.

REFERENCE - Manuscript in revision at eLife.



Phosphoproteomic analyses of FLCN knock-out human renal tubular epithelial cells

Iris Glykofridis[1,2], Alex Henneman[2], Richard Goeij-de Haas[2], Jesper Balk[1], Thang Pham[2], Sander Piersma[2], Rob Wolthuis*[1] , Connie Jimenez*[2]

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INTRODUCTION - Germline inactivating mutations in Folliculin (FLCN) cause Birt–Hogg–Dubé (BHD) syndrome, a rare autosomal dominant disorder predisposing to kidney tumors. To address if FLCN is involved in protein and receptor phosphorylation we determined complete phosphoproteomic profiles of $FLCN^{POS}$ and $FLCN^{NEG}$ human renal tubular epithelial cells.

AIM - Our aim is to address how FLCN is involved in protein and receptor phosphorylation and study how loss of FLCN protein expression contributes to renal tumorigenesis.

APPROACH - Differential phosphorylation of peptides between $FLCN^{POS}$ and $FLCN^{NEG}$ renal proximal tubular epithelial cells (RPTECs) were examined using pTyr and TiOx based phosphopeptide enrichment followed by label-free LC-MS/MS. Kinase activity inference analyses (InKA) and phosphosite specific signature analyses (PTM-SEA) revealed a broad spectrum of differentially phosphorylated proteins, which were functionally studied.

RESULTS - For $FLCN^{POS}$ and $FLCN^{NEG}$ RPTECs Tyrosine-phosphorylation was observed for 2042 peptides, with 976 phosphorylated proteins. For Serine and Threonine, 13702 phosphorylated peptides levels were identified, with 3353 phosphorylated proteins. Kinase activity inference analyses and phosphosite specific signature analyses confirm previously described effects of FLCN loss on (kinase) phosphorylation of proteins involved in EGFR, MAPK and MET signaling. Moreover, we identified novel proteins and receptors of which the phosphorylation state is dependent on FLCN expression, which are currently under further investigation.

CONCLUSIONS - Our results confirmed previously known roles for EGFR, MAPK and MET signaling in $FLCN^{NEG}$ cells and also elucidated novel regulators that are differentially phosphorylated upon FLCN loss. Together, this phosphoproteomic study reveals FLCN dependent changes in phosphorylation that might contribute to renal tumorigenesis and warrant further investigation of specific drug targets to prevent renal tumorigenesis in Birt-Hogg-Dubé patients.

Identification of the pan-cancer extracellular vesicle surface proteome and its application to detect prostate cancer in urine

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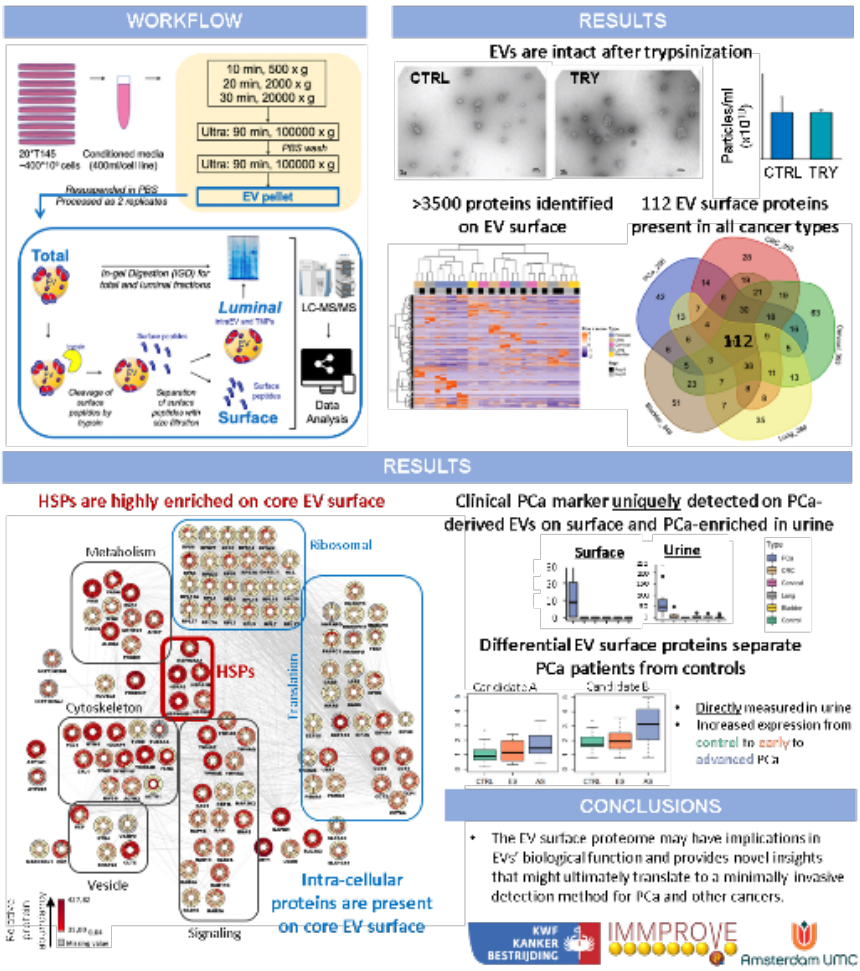

INTRODUCTION - Cancer cells secrete extracellular vesicles (EVs) in biofluids, and cancer-EVs can be isolated from urine. Determination of cancer-associated EVs in urine might therefore provide a non-invasive diagnosis method not only for proximal cancers such as prostate cancer (PCa), but also for cancers of distant organs. To directly capture and detect cancer type-specific EVs, an understanding of the EV surface protein constituents is needed.

AIM - Investigation of the EV surface proteome *in vitro* using mass spectrometry in a pan-cancer cell line panel.

METHODS/APPROACH - Ultracentrifuge-isolated EVs from pan-cancer cell line panel (n = 16; PCa: PC3, DU145, LNCaP, LAPC4, 22RV1; Colorectal cancer: CaCo2, HCT116, HT29, Lovo92; Cervical cancer: HeLa, SiHa, Caski; Lung cancer: H522, H1650, H1703; Bladder cancer: RT112, T24) were treated with trypsin in duplicate, after which the surface peptides were separately quantified from the luminal and total fraction using label-free LC-MS/MS. To explore clinical relevance, urinary EVs of multiple cancer patients including prostate (n=15), lung (n=15), colorectal (n=15), cervical (n=15) and bladder cancer (n=6), and controls (n = 15) were isolated using Vn96 peptide (ME-kit)-mediated capture, via binding to heat-shock proteins (HSPs) at the outer-surface of EVs; and protein profiles were measured using label-free LC-MS/MS. The expression of 2 PCa-associated EV surface proteins were subsequently validated by ELISA in 32 PCa and control urine samples.

RESULTS - We identified >3500 proteins on the surface of the EVs. 112 proteins were present in both replicates of all the cell lines inspected independent of cancer type. Examination of the core EV surface proteome of 112 proteins revealed the enrichment of HSPs on the outer EV membrane. In addition to vesicle-related and metabolic proteins, intracellular DNA/RNA-binding protein complexes such as nuclear and ribosomal proteins were also detected at the EV surface. To determine whether EV surface proteins can be detected in cancer

patients, we isolated urinary EVs from a pan-cancer cohort using a clinically applicable EV-capture kit based on the presence of HSPs on the EV membrane. This method thus potentially enriches for cancer secreted EVs. Several PCa-specific proteins were uniquely expressed on the surface of prostate-derived cell lines, and were also highly enriched in the urinary EVs of prostate cancer patients. To further study their clinical applicability, 2 surface candidates were selected ($p<0.05$; fold change>2) for ELISA measurement in 4 ml unprocessed urine (without EV isolation) and showed an increased expression in an independent cohort of 32 PCa urine samples.

CONCLUSIONS - The identified *in vitro* EV surface proteome may have implications in EVs' biological function and provides novel insights that might ultimately translate to a minimally-invasive detection method for PCa, as well as distant cancers.

REPORTS

- Poster presentations: EAU 2020; HUPO 2020
- Oral presentation: NLSEV 2020

The urinary extracellular vesicle proteome of prostate cancer reveals distinct expression patterns and promising biomarkers

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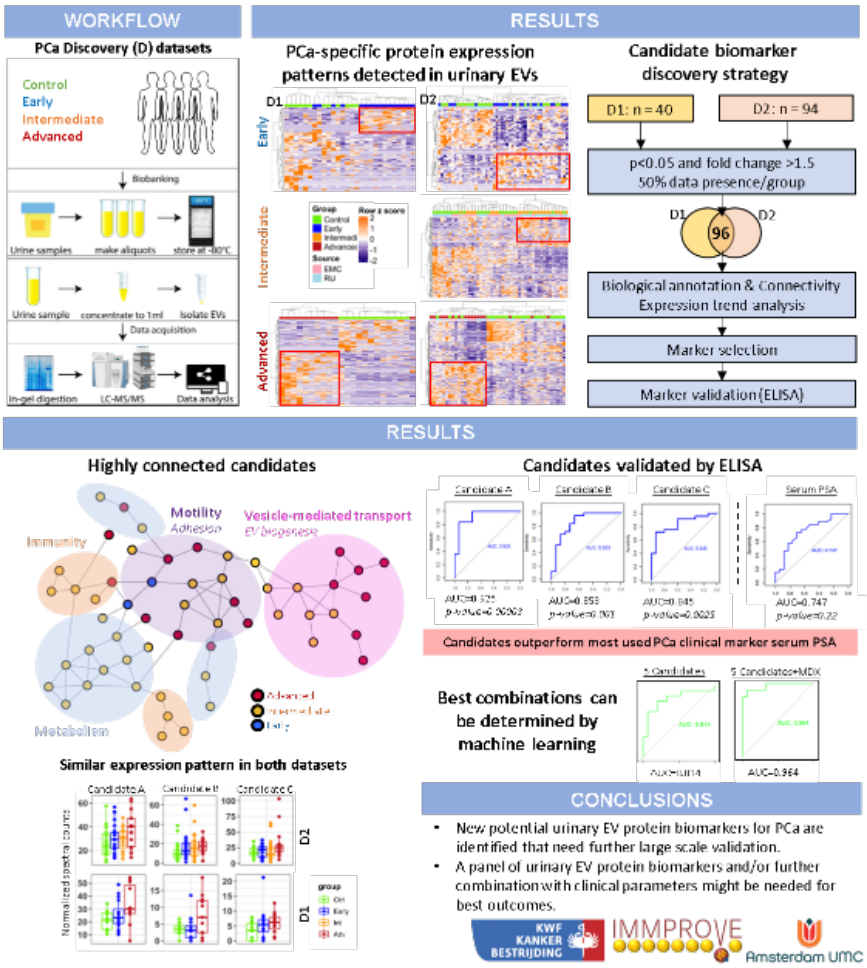
INTRODUCTION - To improve prostate cancer (PCa) diagnosis, an accurate minimally-invasive test is preferred. PCa-associated factors can be found in urine, including in secreted extracellular vesicles (EVs). EVs harbor molecular components of the cell from where they originate, therefore proteomics profiling of urinary EVs may provide a sensitive and accurate source for PCa biomarkers.

AIM - Identification of urinary EV protein biomarkers for PCa diagnosis

METHODS/APPROACH - Urine was collected after signed informed consent with approval by the local medical ethical committee. Urinary EVs were isolated using Vn96 peptide (ME kit) mediated capture, via binding to heat-shock proteins at the outer-surface of EVs. Proteomics was performed of two PCa cohorts (cohort 1: n=40; VUmc: control (age-matched n=12), early stage PCa (n=17) and advanced stage PCa (n=11); and cohort 2: n=94, Erasmus MC and Radboud UMC: control (age-matched n=28), early stage PCa (n=24), intermediate stage PCa (n=24) and advanced stage PCa (n=18)). Three candidate proteins were validated using ELISA in an third independent cohort of 60 samples.

RESULTS - We identified 3000-4000 proteins in the urinary EVs of the two independent PCa discovery cohorts, respectively. PCa patients showed a distinct urinary EV protein expression profile in both datasets, with a clearer separation from controls in the smaller cohort. A total of 96 proteins that were found to be significantly ($p<0.05$) upregulated in PCa patients in both datasets were enriched for known cancer-related pathways including vesicle-secretion, motility, adhesion, immunity and metabolism. Candidate proteins were selected based on multiple filters including significance ($p<0.05$), fold change (>1.5), data presence and involvement in (prostate)cancer development/growth. 5 candidates underwent validation using ELISA in an independent patient cohort of 60 samples in urinary EVs isolated from 2 ml of urine. To normalize the expression of the candidate proteins, we measured urine PSA and creatinine for prostate-secreted correction, CD63 and CD9 for EV number correction, and total protein concentration for input correction. When corrected for protein concentration, 3 of the candidates significantly separated ($p<0.05$) PCa patients from control (AUC=0.845-0.925); showing improvement on the most widely used PCa marker in the clinic (serum PSA) (AUC=0.747). Combination of candidates with each other and/or clinical markers further enhanced the performance of the biomarkers (AUC=964).



CONCLUSIONS - New potential urinary biomarkers for PCa were identified and validated using proteomics and immunological assays in 3 clinical cohorts. Validation of urine PCa-biomarkers require normalization (e.g. PSA, EV-number and protein concentration) in order to accurately measure the proteins for clinical application. This urinary EV protein signature will be validated by immunoassays in another large independent cohort of samples.

REPORTS

- Poster presentations: EAU 2020; HUPO 2020
- Oral presentation: NLSEV 2020

Longitudinal landscape of urinary EV proteome reveals stable protein expression patterns within and between individuals

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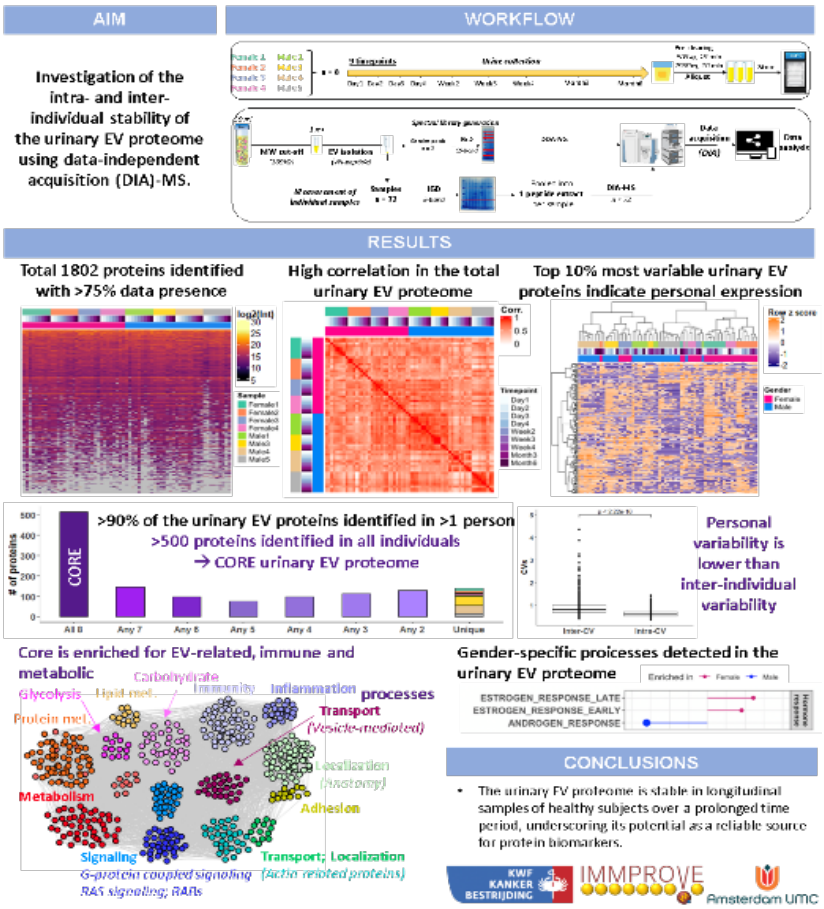
INTRODUCTION - Urinary extracellular vesicles (EVs) are an attractive source for the detection of disease biomarkers. However, little is known about the consistency and stability of urinary EV proteins within and between individuals over a longer time period. For the potential use of urinary EVs as diagnostic biomarker source, it is essential to know how stable the global proteome is over a longer period of time.

AIM - Investigation of the intra- and inter-individual stability of urinary EV proteome over a period of 6 months.

METHODS/APPROACH - We profiled the urinary EV proteome of 8 healthy individuals at 9 timepoints over 6 months (total 72 samples) using data-independent-acquisition (DIA)-MS. Data analysis was performed using CV analysis, cytoscape network analysis, and gender specific Gene-Set-Enrichment analysis.

RESULTS - We identified total 1802 proteins with high correlation amongst all samples. Unsupervised cluster analysis yielded person-specific profiles that overall were very stable in each person, as well as between individuals. 90% of the proteins were detected in more than one person. Furthermore, day-to-day variability was lower than inter-individual variability (median CV 0.6 versus 0.7). The core interaction network of 516 proteins identified in all individuals revealed sub-networks of EV-related proteins involved in RAB-related



signaling and cytoskeletal transport, and sub-clusters enriched for glycolytic activity, protein synthesis, and immune function. Finally, gender-specific expression patterns were identified in the urinary EV proteome, mostly related to hormonal and reproduction pathways.

CONCLUSIONS - Our findings indicate that the urinary EV proteome is stable in longitudinal samples of healthy subjects over a prolonged time period, underscoring its potential as a reliable source for protein biomarkers.

Proteome profiling of tissue-derived extracellular vesicles and soluble secretome reveals non-invasive candidate markers involved in colorectal cancer proliferation and progression

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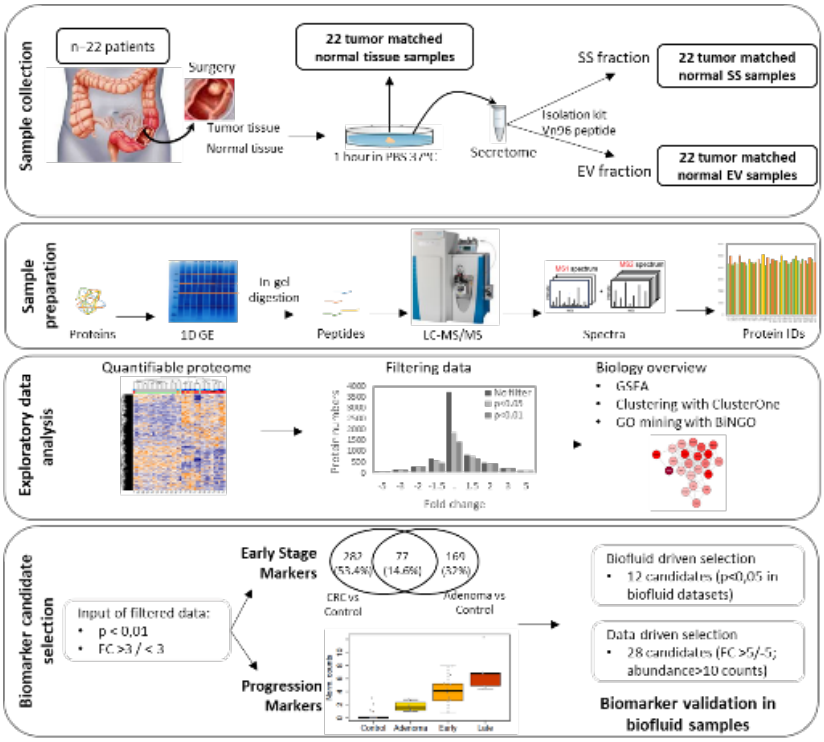
INTRODUCTION - The cancer secretome is of high interest for non-invasive biomarker discovery. One component of the secretome comprises small extracellular vesicles (EVs) that are secreted by most cell types, and implicated in intercellular communication. Both cancer cell soluble secretome (SS) and EV protein components are detectable in biofluids, thereby providing liquid biopsies of high interest for biomarker discovery.

AIM - Multi-compartment secretome and tissue proteomics was employed to identify candidate biomarkers for potential non-invasive diagnostics of colorectal cancer.

METHODS - Tumor and adjacent normal tissue secretome was collected ex vivo from 22 patients with colorectal cancer. Mass spectrometry-based proteomics was performed on tissue, SS and EV fractions isolated using Vn96 peptide.

RESULTS - The multi-compartment CRC dataset comprised 3745 EV proteins and 2157 SS proteins. Comparative analysis revealed 455 EV proteins with increased and 182 with decreased abundance in cancer EVs ($p<0.01$; FC>3 or <-3) and 771 differential SS proteins (653 up- and 118 down-regulated; $p<0.01$; FC>3 or <-3). Candidate



markers with potential for early detection were selected based on differential in both normal versus adenoma (n=4 pairs) and normal versus cancer (n=18 pairs) comparisons, yielding 172 EV proteins and 138 SS proteins. EV proteins were biologically involved in RNA splicing, DNA repair and replication, and translation, which are common features of colorectal cancer. Surprisingly, SS proteins were also implicated in these processes. Furthermore, 64 proteins exhibited a trend profile of increasing/decreasing abundance in control, adenoma, early-, and advanced stage groups. These candidate proteins are potential progression markers and were involved in gene expression and translational elongation. To determine whether these proteins have any diagnostic potential, different external datasets were used, in which the proteome was measured in blood, stool or urine samples of control versus colorectal cancer patients. Of all selected candidate EV proteins, 12 showed a significant and unidirectional deregulation in all biofluids. Analysis on the soluble secretome fraction is ongoing.

CONCLUSIONS -We report a unique clinical dataset of cancer tissue and secreted proteome. We demonstrate that direct profiling of cancer secreted EVs reveals candidate proteins with high biomarker potential. Furthermore, more notable deregulated proteins were found compared to tissue. Future analysis will aim to evaluate the biomarker potential of the selected candidate proteins by measurement within biofluids.

ACKNOWLEDGEMENTS - SECRET ITN (Marie Skłodowska-Curie grant agreement No 859962).

Exploration of the platelet proteome in patients with early-stage cancer

Sabrkhany S, Kuijpers MJE, Knol JC, Olde Damink SWM, Dingemans AC, Verheul HM, Piersma SR, Pham TV, Griffioen AW, Oude Egbrink MGA, Jimenez CR

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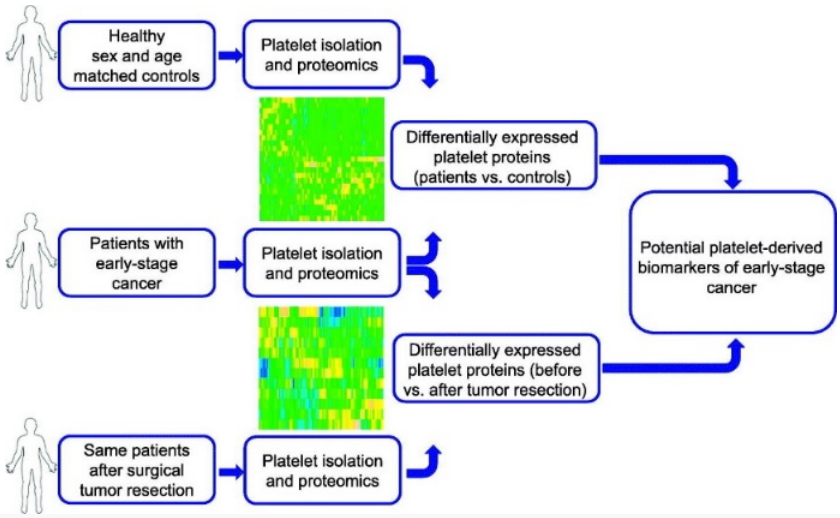

doi:10.1016/j.jprot.2018.02.011.PubMed PMID: 29432918.

Platelets play an important role in tumor growth and, at the same time, platelet characteristics are affected by cancer presence. Therefore, we investigated whether the platelet proteome harbors differentially expressed

proteins associated with early-stage cancer. For this proof-of-concept study, patients with early-stage lung (n = 8) or head of pancreas cancer (n = 4) were included, as were healthy sex- and age-matched controls for both subgroups. Blood samples were collected from controls and from patients before surgery. Furthermore, from six of the patients, a second sample was collected two months after surgery. NanoLC-MS/MS-based proteomics of gel-fractionated platelet proteins was used for comparative spectral count analyses of patients to controls and before to after surgery samples. The total platelet proteome dataset included 4384 unique proteins of which 85 were significantly (criteria Fc > 1.5 and p < 0.05) changed in early-stage cancer compared to controls. In addition, the levels of 81 platelet proteins normalized after tumor resection. When filtering for the most discriminatory proteins, we identified seven promising platelet proteins associated with early-stage cancer. In conclusion, this pioneering study on the platelet proteome in cancer patients clearly identifies platelets as a new source of candidate protein biomarkers of early-stage cancer.

Biological significance: Currently, most blood-based diagnostics/biomarker research is performed in serum or plasma, while the content of blood cells is usually neglected. It is known that especially blood platelets, which are the main circulating pool of many bioactive proteins, such as growth factors, chemokines, and cytokines, are a potentially rich source of biomarkers. The current study is the first to measure the effect of early-stage cancer on the platelet proteome of patients. Our study demonstrates that the platelet proteome of patients with early-stage lung or head of pancreas cancer differs considerably compared to that of healthy individuals of matched sex and age. In addition, the platelet proteome of cancer patients normalized after surgical resection of the tumor. Exploiting platelet proteome differences linked to both tumor presence and disease status, we were able to demonstrate that the platelet proteome can be mined for potential biomarkers of cancer.

Method Development (MS, wet lab)

Feasibility of phosphoproteomics on leftover samples after RNA extraction with guanidinium thiocyanate

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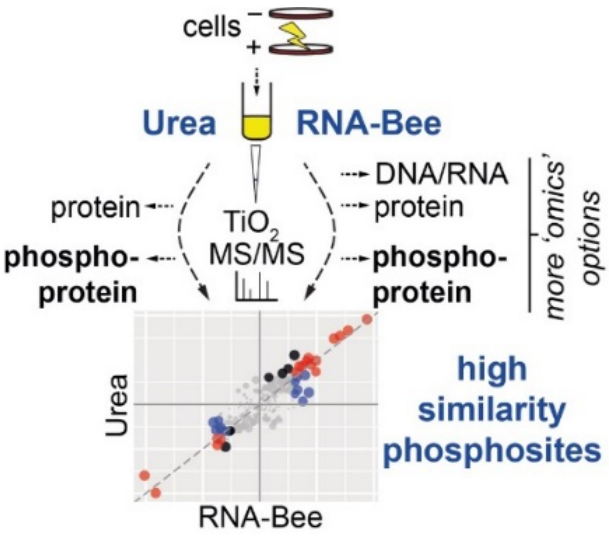

INTRODUCTION - In daily practice, different types of biomolecules are usually extracted for large-scale 'omics' analysis with tailored protocols. However, when sample material is limited, an all-in-one strategy is preferable. While lysis of cells and tissues with urea is the accepted standard for phosphoproteomic applications, DNA, RNA and proteins can be simultaneously extracted from small samples using acid guanidinium thiocyanate- phenol-chloroform (AGPC). Use of AGPC for mass spectrometry (MS)-based phosphoproteomics has been reported, but not benchmarked.

METHODS - Using high-resolution tandem mass spectrometry, a TiOx phosphoproteomic workflow and recent bioinformatic tools such as inferred kinase activity (INKA) analysis and phosphosite signature enrichment analysis (PTM-SEA), we compared urea- with AGPC-based protein extraction, profiling phosphorylations in the DNA damage response pathway after ionizing irradiation of U2OS cells as proof of principle.

RESULT - On average we identified circa 9000 phosphosites per sample with both extraction methods. Moreover, we observed high similarity of phosphosite characteristics (e.g. 94% shared class 1 identifications) and deduced kinase activities (e.g. ATM, ATR, CHEK1/2, PRKDC).

CONCLUSIONS - AGPC-based sample extraction can replace standard cell lysates for phosphoproteomic workflows and may thus be an attractive way to obtain input material for multiple omics workflows, yielding several data types from a single sample.

Optimization of phosphopeptide enrichment methods for clinical phosphoproteomics

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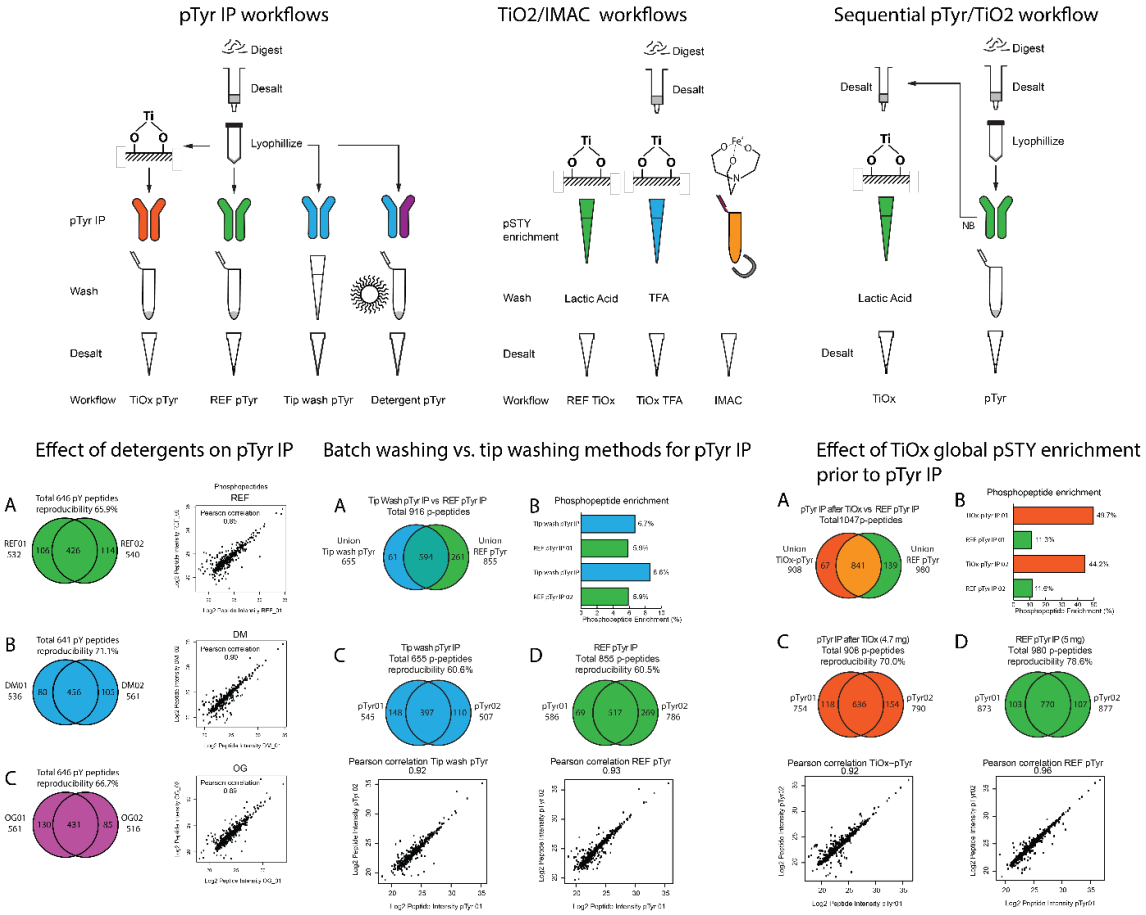
INTRODUCTION - In large-scale phosphoproteomics experiments phosphopeptides need to be enriched from cell or tissue lysate after tryptic digestion. Global phosphopeptide enrichment (phosphoserine, phosphothreonine and phosphotyrosine) is achieved using affinity purification materials including titanium dioxide beads (TiO_2) or immobilized metal affinity chromatography (IMAC) resins like Ti(IV)-IMAC and Fe(III)-IMAC. Phosphotyrosine-containing peptides are enriched specifically using selective sequence-context independent antibodies like 4G10, pY20 and pTyr-1000.



AIM - Development of optimal global and p-Tyr phosphopeptide enrichment methods for (clinical) tissue phosphoproteomics applications.

APPROACH - CRC cell line digest HCT116 was used for all method optimizations. For TiO_2 global phosphopeptide enrichment the effect of different washing buffers was assessed and compared to Fe(III) IMAC. Subsequently a method for robotic IMAC enrichment of phosphopeptides on the BRAVO AssayMap

liquid handling system was developed. For pTyr immunoprecipitation optimization the effect of mild detergents and the effect of global phosphopeptide pre-enrichment on pTyr yield and enrichment were assessed.

RESULTS - The TiO2 global phosphopeptide enrichment method identifies 8958 phosphopeptides from 500 µg protein input with 68.1 % ID reproducibility and a Pearson correlation of 0.92. The magnetic Fe(III)-IMAC beads method identifies 9047 phosphopeptides with 71.3% ID reproducibility and a Pearson correlation of 0.88. Overall performance of both methods is highly comparable, with IMAC providing the highest enrichment specificity. On the BRAVO robotic platform an average number of 9760 phosphopeptides was enriched (n=4) with an ID reproducibility of 80% and a Pearson r of >0.95 at an input level of 200 µg peptides. pTyr enrichment in the presence of 1% octyl glucoside or dodecyl maltoside did not improve enrichment efficiency, nor did modification of the wash protocol or pre-enrichment of phosphopeptides using TiO_2. The improved speed of the Q Exactive HF compared to the Q Exactive did have a large impact on phosphopeptide detection, increasing the number of identified pTyr peptides from 650 to 875 at 5 mg peptide input.

CONCLUSIONS - Implementation of global phosphopeptide enrichment on the BRAVO robotic platform both increased reproducibility as well as depth of phosphoproteome coverage at reduced peptide input. Alternate washing protocols did not improve pTyr enrichment but the increased detection rate of the Q Exactive HF increased the number of phosphopeptides significantly. pTyr enrichment methods using the BRAVO platform will be explored in 2021.

Method development for a Bravo liquid handling platform

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In 2020 an Agilent Bravo liquid handling platform was installed in the OPL. The Bravo can be used to automate protein digestion, peptide clean-up and phosphopeptide enrichment in a 96-well format. Bravo uses pipet tip-style cartridges and pre-set protocols (AssayMap) in a user-friendly format. We have explored automated enrichment of phosphopeptides using Fe(III) IMAC (immobilized-metal affinity chromatography) cartridges allowing enrichment from upto 200 µg peptide input. Elution conditions were modified (5% NH_4OH with 30% ACN) to achieve optimal phosphopeptide yield and enrichment. Impressive performance was observed with 9760 phosphopeptides enriched from 200 µg peptides with an ID reproducibility of 80%, a Pearson r of >0.95 and an enrichment of 72% (n=4). In 2021 pTyr IP wil be implemented on the Bravo using protein A cartridges to immobilize pTyr-1000 anti-pTyr mAb using the AssayMap protocol developed by Cell Signaling. Initial experiments will aim at pTyr peptide enrichment from 1-2 mg protein digest and downstream sample clean-up using either C18 or IMAC cartridges in 96-well format.

Urine storage protocol that is feasible with extracellular vesicle research and proteomics

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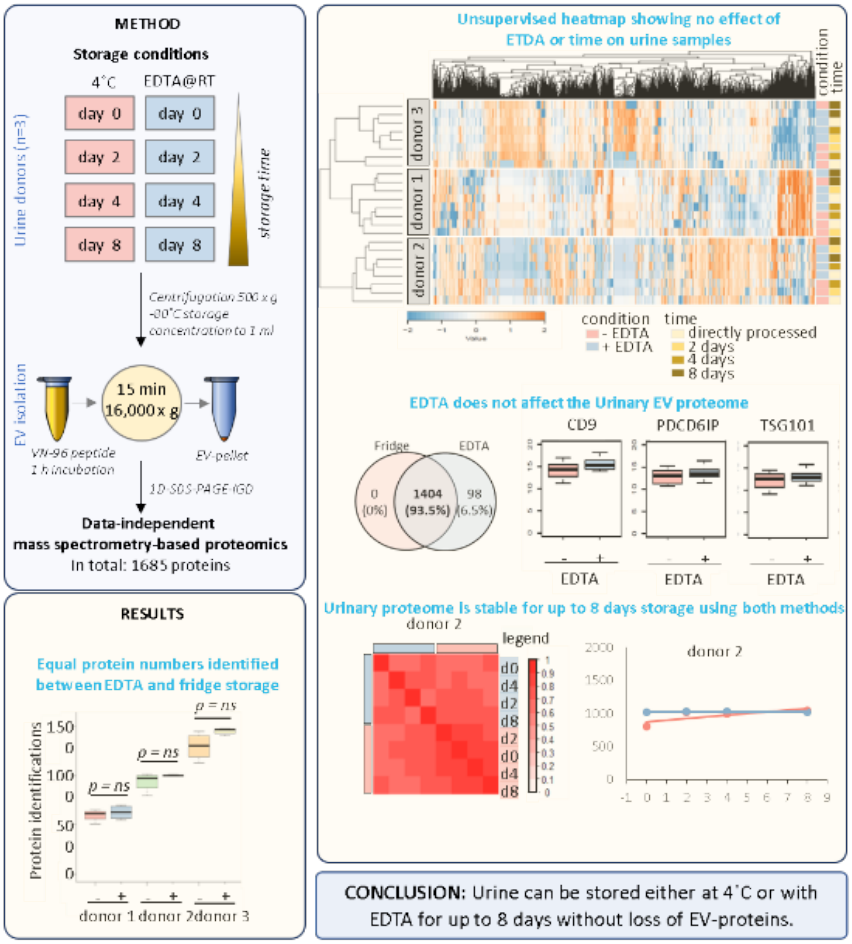
INTRODUCTION - Urine is an emerging biofluid for the identification of biomarkers that can detect disease. In particular extracellular vesicles (EVs) have gained increased interest as a biomarker source, because the content is protected against the outside environment. The advantage of urine as a biomarker source is that urine can be collected multiple times a day, in large volumes and it can be collected at home. Collection at home, however, usually causes a delay in the collection and processing time. Till date, little is known about the

effect of short-term storage of urinary EVs and their proteome. It is important to know the most optimal urine storage conditions until processing or downstream analysis in order to collect samples at distant hospitals, or at home.

APPROACH - In the current study, we investigated two different storage protocols, first urine stored at 4˚C without any preservative, and secondly with the addition of 40 mM EDTA at room temperature. For both conditions it was evaluated whether storage at 0, 2, 4 and 8 days leads to a change in the number of vesicles and the global urinary EV proteome using data-independent acquisition mass spectrometry-based proteomics. Using Rho-correlation analysis, we examined which proteins had a reduced abundancy in time to identify proteins that are possibly degraded.



RESULTS - We show that EDTA does not affect the urinary EV number or global proteome. For urine stored up to 1 week either at 4˚C of at RT with the addition of EDTA does not result in a significant reduction in EV numbers nor the protein profiles, with only <5 % of the identified proteins showing a reduction in abundancy in time.

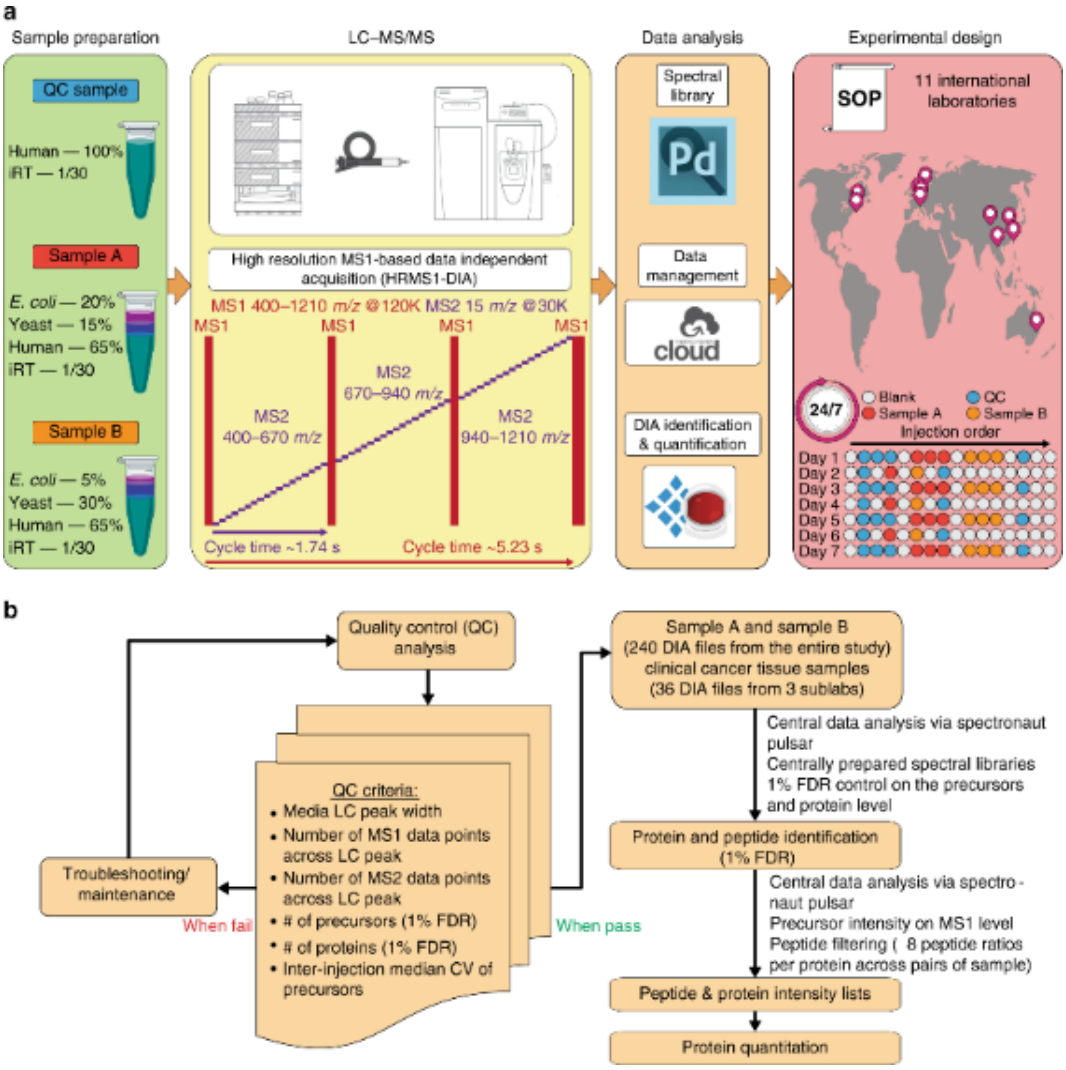
CONCLUSION - Urine can be stored either at 4˚C or with EDTA for up to 8 days without any significant loss in proteins. Furthermore, both tested storage methods are feasible for EV proteome biomarker research.

Standardization and Harmonization of Distributed Multi-National Proteotype Analysis supporting Precision Medicine Studies

Xuan Y, Bateman NW, Gallien S, Goetze S, Zhou Y, Navarro P, Hu M, Parikh N, Hood BL, Conrads KA, Loosse C, Kitata RB, Piersma SR, Chiasserini D, Zhu H, Hou G, Tahir M, Macklin A, Khoo A, Sun X, Crossett B, Sickmann A, Chen YJ, Jimenez CR, Zhou H, Liu S, Larsen MR, Kislinger T, Chen Z, Parker BL, Cordwell SJ, Wollscheid B, Conrads TP

Nat Commun 2020 Oct 16;11(1):5248. doi: 10.1038/s41467-020-18904-9. PubMed PMID: 33067419.

Cancer has no borders: Generation and analysis of molecular data across multiple centers worldwide is necessary to gain statistically significant clinical insights for the benefit of patients. Here we conceived and standardized a proteotype data generation and analysis workflow enabling distributed data generation and evaluated the quantitative data generated across laboratories of the international Cancer Moonshot consortium. Using harmonized mass spectrometry (MS) instrument platforms and standardized data acquisition procedures, we demonstrate robust, sensitive, and reproducible data generation across eleven international sites on seven consecutive days in a 24/7 operation mode.



The data presented from the high-resolution MS1-based quantitative data-independent acquisition (HRMS1-DIA) workflow shows that coordinated proteotype data acquisition is feasible from clinical specimens using such standardized strategies. This work paves the way for the distributed multi-omic digitization of large clinical specimen cohorts across multiple sites as a prerequisite for turning molecular precision medicine into reality.

Method Development (Bioinformatics)

Assessment of quantitation and statistical methods for DIA mass spectrometry-based proteomics data

Thang V. Pham[1], Frank Rolfs[1], Jim Termeulen[1], Alex A. Henneman[1], Sander R. Piersma[1], Connie R. Jimenez[1]

[1]Department of Medical Oncology, OncoProteomics Laboratory, Cancer Center Amsterdam, Amsterdam UMC, Vrije Universiteit, Amsterdam, The Netherlands

INTRODUCTION - The data-independent acquisition (DIA) approach in mass spectrometry-based proteomics has emerged as a promising alternative to data-dependent acquisition (DDA) because of its ability to provide a more complete data matrix by combining unbiased, broad range precursor ion fragmentation and targeted data extraction. For biomarker discovery studies, it is necessary to assess the performance of downstream analysis methods in a comparative analysis.

AIM - To evaluate the performance of methods for protein quantification and significance analysis.

APPROACH - Three datasets were used. In the Bruderer 15 dataset (Bruderer et al., 2015) (n=24), 12 proteins were spiked at different concentrations for each of the 8 samples in the dataset where each sample was measured in triplicates. In the Bruderer 17 datasets (Bruderer et al., 2017) (n=6), three species were mixed in a stable human proteome. The moonshot dataset (Xuan et al.) (n =120) contains mixture of human, yeast and E. coli proteomes at different concentrations. The R package iq package (Pham et al., Bioinformatics 2020) was employed for protein quantification, offering four different methods meanInt, topN, median polish and MaxLFQ. In our first assessment, the t-test and limma were considered for statistical significance analysis.

RESULTS - For quantitative analysis, MaxFLQ outperforms other methods for all datasets, confirming previous result on the Bruderer 15 dataset. The limma method is better than the t-test method on the Bruderer 17 datasets and equal for the moonshot dataset in terms of area under the curve.

CONCLUSIONS - The preliminary result shows that MaxLFQ protein quantification and limma statistics provide an effective approach for biomarker discovery studies. Ongoing analysis includes assessment of the effect of sample size, missing data, preprocessing software tools, additional statistical tests and phosphoproteomics experiments.

REFERENCES -

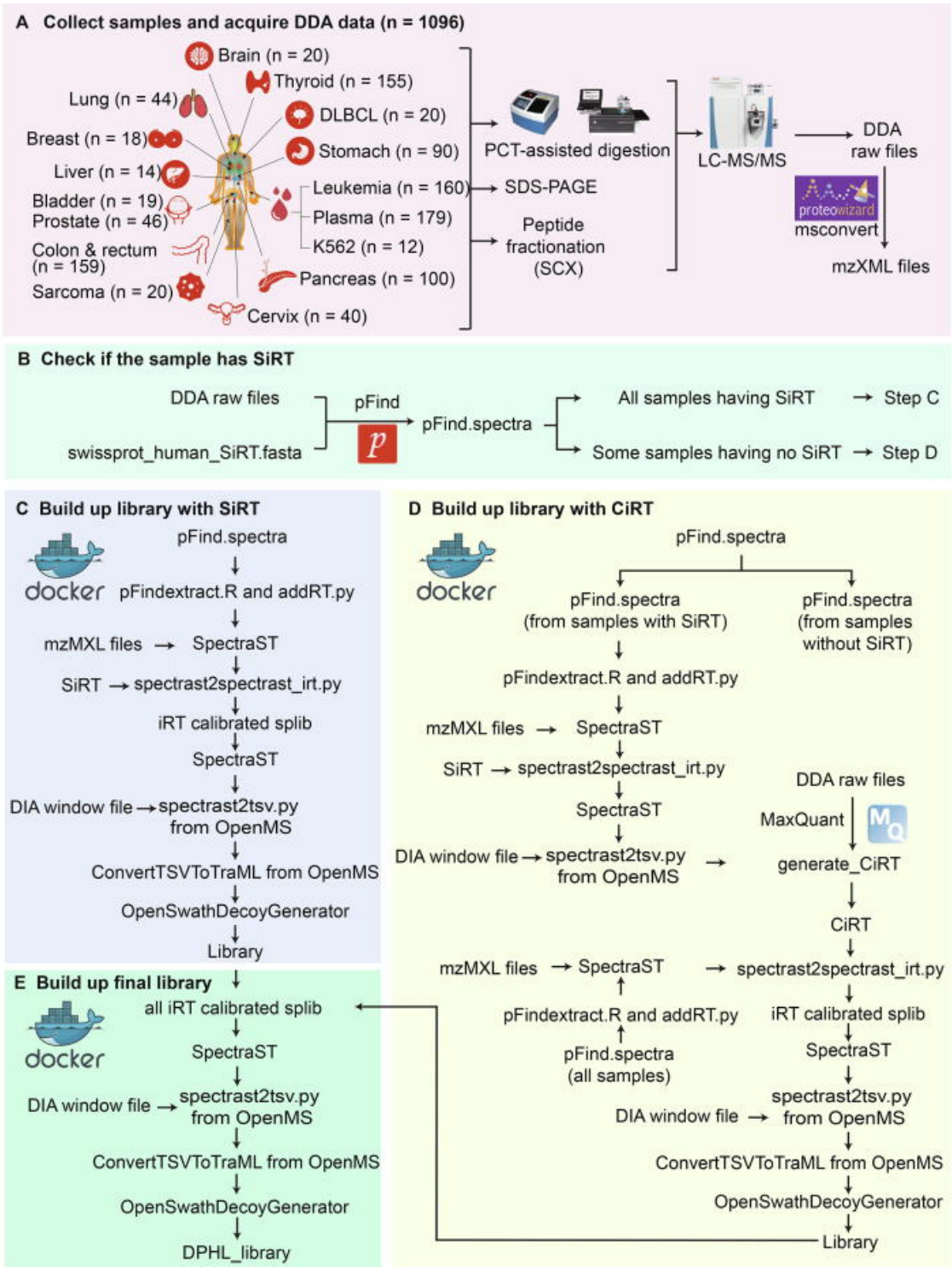
- Bruderer R et al., Extending the limits of quantitative proteome profiling with data-independent acquisition and application to acetaminophen-treated three-dimensional liver microtissues. Mol Cell Proteomics 2015 May;14(5):1400-10.
- Bruderer R et al., Optimization of Experimental Parameters in Data-Independent Mass Spectrometry Significantly Increases Depth and Reproducibility of Results. Mol Cell Proteomics 2017 Dec;16(12):2296-2309.
- Xuan Y et al., Standardization and Harmonization of Distributed Multi-National Proteotype Analysis supporting Precision Medicine Studies. Nat Commun 2020 Oct 16;11(1):5248.
- Pham TV et al., iq: an R package to estimate relative protein abundances from ion quantification in DIA-MS-based proteomics. Bioinformatics 2020 Apr 15;36(8):2611-2613.
- Pham TV et al., Assessment of quantitation and statistical methods for DIA mass spectrometry-based proteomics data. Abstract at the 19th Human Proteome Organization World Congress, 2020.

DPHL: A DIA Pan-human Protein Mass Spectrometry Library for Robust Biomarker Discovery.

Zhu T, Zhu Y, Xuan Y, Gao H, Cai X, Piersma SR, Pham TV, Schelfhorst T, Haas RRGD, Bijnsdorp IV, Sun R, Yue L, Ruan G, Zhang Q, Hu M, Zhou Y, Van Houdt WJ, Lelarge TYS, Cloos J, Wojtuszkiewicz A, Koppers-Lalic D, Böttger F, Scheepbouwer C, Brakenhoff RH, van Leenders GJLH, Ijzermans JNM, Martens JWM, Steenbergen RDM, Grieken NC, Selvarajan S, Mantoo S, Lee SS, Yeow SJY, Alkaff SMF, Xiang N, Sun Y, Yi X, Dai S, Liu W, Lu T, Wu Z, Liang X, Wang M, Shao Y, Zheng X, Xu K, Yang Q, Meng Y, Lu C, Zhu J, Zheng J, Wang B, Lou S, Dai Y, Xu C, Yu C, Ying H, Lim TK, Wu J, Gao X, Luan Z, Teng X, Wu P, Huang S, Tao Z, Iyer NG, Zhou S, Shao W, Lam H, Ma D, Ji J, Kon OL, Zheng S, Aebersold R, Jimenez CR, Guo T.

Genomics Proteomics Bioinformatics 2020 Apr;18(2):104-119. doi: 10.1016/j.gpb.2019.11.008. PubMed PMID: 32795611.

To address the increasing need for detecting and validating protein biomarkers in clinical specimens, mass spectrometry (MS)-based targeted proteomic techniques, including the selected reaction monitoring (SRM), parallel reaction monitoring (PRM), and massively parallel data-independent acquisition (DIA), have been developed. For optimal performance, they require the fragment ion spectra of targeted peptides as prior knowledge. In this report, we describe a MS pipeline and spectral resource to support targeted proteomics studies for human tissue samples.

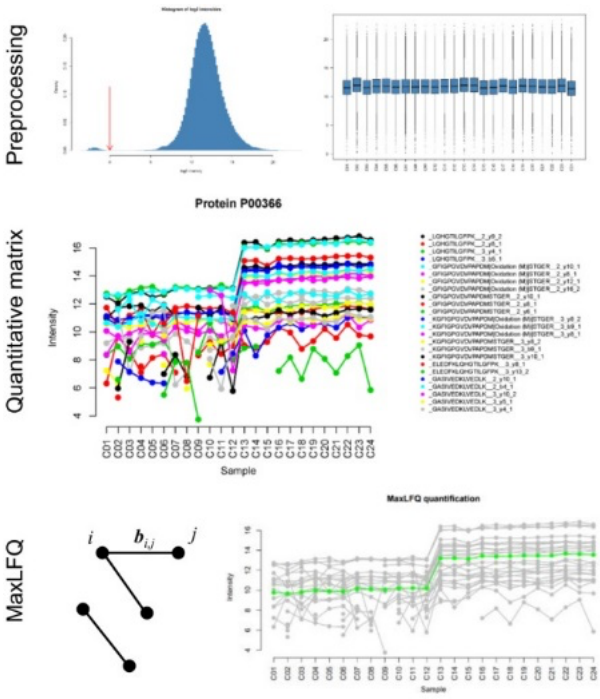


To build the spectral resource, we integrated common open-source MS computational tools to assemble a freely accessible computational workflow based on Docker. We then applied the workflow to generate DPHL, a comprehensive DIA pan-human library, from 1096 data-dependent acquisition (DDA) MS raw files for 16 types of cancer samples. This extensive spectral resource was then applied to a proteomic study of 17 prostate cancer (PCa) patients. Thereafter, PRM validation was applied to a larger study of 57 PCa patients and the differential expression of three proteins in prostate tumor was validated. As a second application, the DPHL spectral resource was applied to a study consisting of plasma samples from 19 diffuse large B cell lymphoma (DLBCL) patients and 18 healthy control subjects. Differentially expressed proteins between DLBCL patients and healthy control subjects were detected by DIA-MS and confirmed by PRM. These data demonstrate that the DPHL supports DIA and PRM MS pipelines for robust protein biomarker discovery. DPHL is freely accessible at https://www.iprox.org/page/project.html?id=IPX0001400000.

iq: an R package to estimate relative protein abundances from ion quantification in DIA-MS-based proteomics

Pham TV, Henneman AA, Jimenez CR

Bioinformatics 2020 Apr 15;36(8):2611-2613. doi: 10.1093/bioinformatics/btz961. PubMed PMID: 31909781.



Summary: We present an R package called iq to enable accurate protein quantification for label-free data-independent acquisition (DIA) mass spectrometry-based proteomics, a recently developed global approach with superior quantitative consistency. We implement the popular maximal peptide ratio extraction module of the MaxLFQ algorithm, so far only applicable to data-dependent acquisition mode using the software suite MaxQuant. Moreover, our implementation shows, for each protein separately, the validity of quantification over all samples. Hence, iq exports a state-of-the-art protein quantification algorithm to the emerging DIA mode in an open-source implementation.

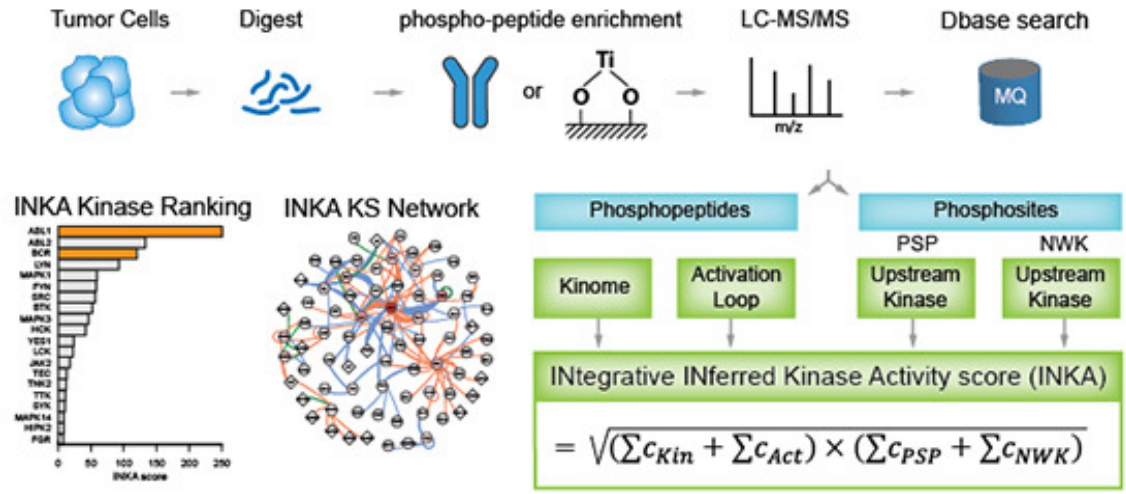
Availability and implementation: The open-source R package is available on CRAN, https://github.com/tvpham/iq/releases and oncoproteomics.nl/iq.

INKA, an integrative data analysis pipeline for phosphoproteomic inference of active kinases

Beekhof R, van Alphen C, Henneman AA, Knol JC, Pham TV, Rolfs F, Labots M, Henneberry E, Le Large TY, de Haas RR, Piersma SR, Vurchio V, Bertotti A, Trusolino L, Verheul HM, Jimenez CR

Mol Syst Biol 2019 2019 Apr 12;15(4):e8250. doi: 10.15252/msb.20188250. PubMed PMID: 30979792.

Identifying hyperactive kinases in cancer is crucial for individualized treatment with specific inhibitors. Kinase activity can be discerned from global protein phosphorylation profiles obtained with mass spectrometry-based phosphoproteomics. A major challenge is to relate such profiles to specific hyperactive kinases fueling growth/progression of *individual* tumors.



Hitherto, the focus has been on phosphorylation of either kinases or their substrates. Here, we combined label-free kinase-centric and substrate-centric information in an Integrative Inferred Kinase Activity (INKA) analysis. This multipronged, stringent analysis enables ranking of kinase activity and visualization of kinase-substrate networks in a single biological sample. To demonstrate utility, we analyzed (i) cancer cell lines with known oncogenes, (ii) cell lines in a differential setting (wild-type versus mutant, +/- drug), (iii) pre- and on-treatment tumor needle biopsies, (iv) cancer cell panel with available drug sensitivity data, and (v) patient-derived tumor xenografts with INKA-guided drug selection and testing. These analyses show superior performance of INKA over its components and substrate-based single-sample tool KARP, and underscore target potential of high-ranking kinases, encouraging further exploration of INKA's functional and clinical value.

Collaborative Oncology Research

Phosphoproteomic evaluation of the signaling pathways modulated by tryptanthrin in skin cancer cells

Mohan Shankar G[1], Richard Goeij de Haas[2], Alex A. Henneman[2], Sander R. Piersma[2], Thang V Pham[2], Connie R Jimenez[2], Ruby John Anto[1]

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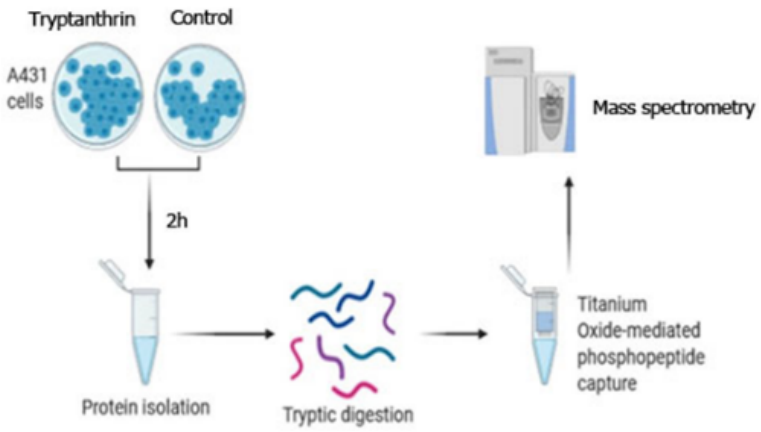
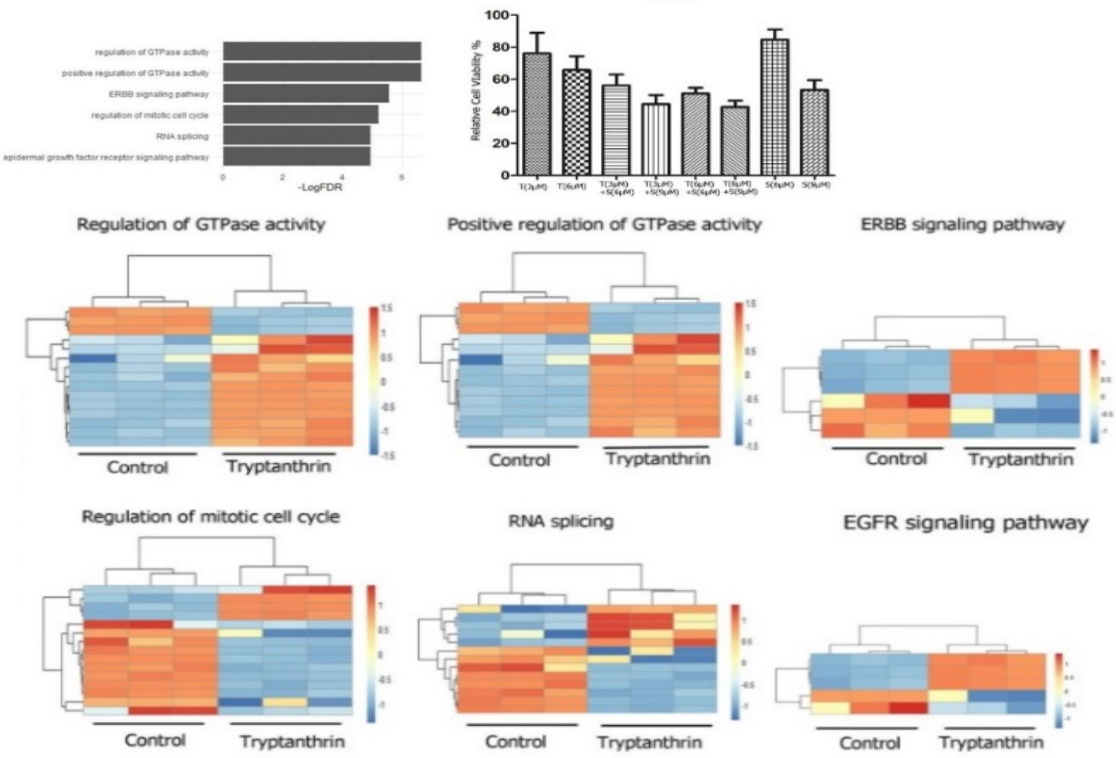
[2]Department of Medical Oncology, OncoProteomics Laboratory, Cancer Center Amsterdam, Amsterdam UMC, Vrije Universiteit, Amsterdam, The Netherlands

INTRODUCTION - Cutaneous squamous cell carcinoma (cSCC) is the second most prevalent type of Non-melanoma skin cancer (NMSC). Though majority of these tumors are benign, a small subset have metastatic potential and pose a risk of recurrence. Over-expression of epidermal growth factor receptor (EGFR) is correlated to poor prognosis of cSCC. Though results from clinical trials shows the effectiveness of targeting EGFR, there is significant scope for improvement of therapeutic outcome. A previous study from our lab has shown that tryptanthrin, an indoloquinazoline alkaloid, is effective in suppressing development of skin cancer in mouse models (Mohan Shankar et al, 2020). This study also demonstrates that tryptanthrin is as effective as 5-FU, a clinically used drug against NMSC, in exerting anti-proliferative effect against A431, an EGFR-over-expressing epidermoid squamous cell carcinoma cell line. However, the signaling pathways modulated by the compound were not comprehensively explored. This information might help in identifying effective therapeutic combinations, with tryptanthrin being as one of the treatment agents.

AIM - To understand the signaling modulation induced by tryptanthrin in A431 cells.

APPROACH - We assessed the signaling modulation induced by the compound using titanium dioxide based phosphoproteomics.





RESULTS - We have found that treatment of A431 cells with tryptanthrin (6µM) has induced differential phosphorylation of 134 peptides that comprise of 111 proteins (p-value: ≤0.05, Fold change: ≥2 or ≤-2). This list was used for gene ontology analysis to understand the biological processes that might be regulated by the compound. The analysis showed significant enrichment of proteins that function in GTPase activation, ERBB signaling, mitotic cell cycle, EGFR signaling and RNA splicing. Among these, the compound majorly induced phosphorylation of proteins involved in GTPase activation, while majority of the proteins that function in mitotic cell cycle regulation and RNA splicing were down-regulated. The compound induced phosphorylation of some of the proteins involved in ERBB and EGFR signaling. Moreover, other receptor tyrosine kinases were not affected by the compound. Hence, we tested the efficacy of a combination of tryptanthrin and sorafenib, a multi-kinase inhibitor, in inducing cytotoxicity in these cells. We have observed that the combination, comprising subtoxic concentrations of individual drugs, exerts cytotoxicity in an additive to mild synergistic mode.

CONCLUSION - The study has identified that a combination of tryptanthrin and sorafenib at subtoxic concentrations, is effective against EGFR-over-expressing Non-melanoma skin cancer.

Identification of signaling pathways regulating cancer cell plasticity and cutaneous SCC progression

Laura Lorenzo-Sanz[1*], Marta López-Cerdá[1*], Victoria da Silva-Diz[1,2], Rosa M Penin[3], Diana Pérez-Sidelnikova[4], Oriol Bermejo[4], Connie R. Jimenez[5], Purificación Muñoz[1]

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[2]Present address: Rutgers Cancer Institute of New Jersey, Rutgers University, New Brunswick, New Jersey, USA
[3]Pathology Service, Hospital Universitario de Bellvitge/IDIBELL, Barcelona, Spain
[4]Plastic Surgery Unit, Hospital Universitario de Bellvitge/IDIBELL, Barcelona, Spain
[5]Department of Medical Oncology, OncoProteomics Laboratory, Cancer Center Amsterdam, Amsterdam UMC, Vrije Universiteit, Amsterdam, The Netherlands
[*]contributed equally

INTRODUCTION - Cutaneous squamous cell carcinoma (cSCC) is the second most frequent skin cancer. Most cases are surgically treated, but around 5-8% of patients develop recurrent and aggressive tumors with enhanced metastasis risk, which reduce patient survival. Early cSCCs conserve epithelial differentiation features (WD-SCCs and MD-SCCs, which comprise most of diagnosed cSCCs), but advanced cSCCs loss epithelial differentiation traits and eventually acquire mesenchymal-like features (PD/S-SCCs), the latest representing the

most recurrent and metastatic cSCCs. High risk and metastatic cSCCs have been classically treated with radiotherapy and/or conventional chemotherapy. These therapies have limited clinical benefits. Currently no molecular biomarkers of cSCC progression are available.

Our previous studies demonstrated that cancer cells from WD-SCCs conserve epithelial differentiation traits. During long-term tumor growth (serial engraftments of these tumors in mice), WD-SCCs evolved to MD/PD-SCCs, which are formed by a mixed population of epithelial EpCAM^{+} and mesenchymal EpCAM^{-} cancer cells. Isolation and molecular characterization of these cell populations showed that EpCAM^{+} cells from MD/PD-SCCs acquire a strong plasticity that enable them to switch to EpCAM^{-} cells in response to microenvironment-derived factors. Subsequent growth of these mixed MD/PD-SCCs gave rise to mesenchymal-like PD/S-SCCs that were exclusively formed by full mesenchymal EpCAM^{-} tumor cells. Therefore, our previous results indicate that aggressive PD/S-SCCs are generated by the progression of epithelial-like cSCCs to mesenchymal-like cSCCs and the acquisition of cancer cell plasticity plays a key role in this process.

AIM - Our major aim is to identify signalling pathways driving cancer cell plasticity and progression toward the mesenchymal state, in order to identify kinase signalling that are responsible of promoting SCC progression.

APPROACH - We compared the large-scale and phospho-tyrosine phosphoproteomes by mass spectrometry-based profiling of full epithelial cancer cells, plastic EpCAM^{+} cancer cells, mesenchymal-like EpCAM^{-} and full mesenchymal cancer cells, which were isolated from mouse WD-SCCs, MD/PD-SCCs and PD/S-SCCs, respectively, and shortly in vitro cultured. Cell lysates from three cell biological replicates of each stage of cSCC progression were processed following the phosphoproteome workflow set up in Jimenez's laboratory. For global phosphopeptide analysis, desalted peptides were enriched for phosphopeptides using TiO2 beads. For phosphotyrosine identification, phosphopeptide immunoprecipitation was performed using agarose bead-coupled phosphotyrosine antibodies P-Tyr-1000. NanoLC–MS/MS was performed using a Q-Exactive-HF mass spectrometer. For each sample, phosphoproteomic data analysis was based on phosphopeptide identification, quantification (intensity-based and spectral-counting based) and normalization of phosphosite intensities. Statistical testing together with cluster analysis of differential phosphopeptides was performed in R Studio. The phospho analysis has been focused on ID mapping (activation loop phosphorylation and kinase motif analysis) and downstream data mining including GO analysis, STRING 10.0 database and Cytoscape 3.4 analysis for protein-protein interaction and signalling pathways networks.

RESULTS - To obtain a general overview of SCC progression, we performed cluster analysis of differential phosphopeptides, which revealed that the phosphoproteome of each cancer cell population cluster tightly and was distinct from other stages. We identified 299 peptides whose phosphorylation in Ser, Thr and Tyr residues changed significantly during the transition from epithelial to plastic state, of which 67 showed changes in pTyr residues (43 peptides showed an increased pTyr and 24 peptides showed a decreased pTyr in plastic cancer cells as compared to epithelial cancer cells). In addition, we identified alterations in 748 phosphopeptides and 257 peptides whose phosphorylation in Tyr residues significantly change during the switch from epithelial to mesenchymal state.

CONCLUSIONS - These results indicate that during the progression from epithelial to mesenchymal cancer cell state multiple signaling pathways may be up and down-regulated, promoting cSCC progression. Currently, we are determining the relevance of some of the identified signaling pathways in promoting the acquisition of cancer cell plasticity and cSCC progression, in order to design targeted therapies that block the generation of highly aggressive and metastatic mesenchymal-like cSCCs.

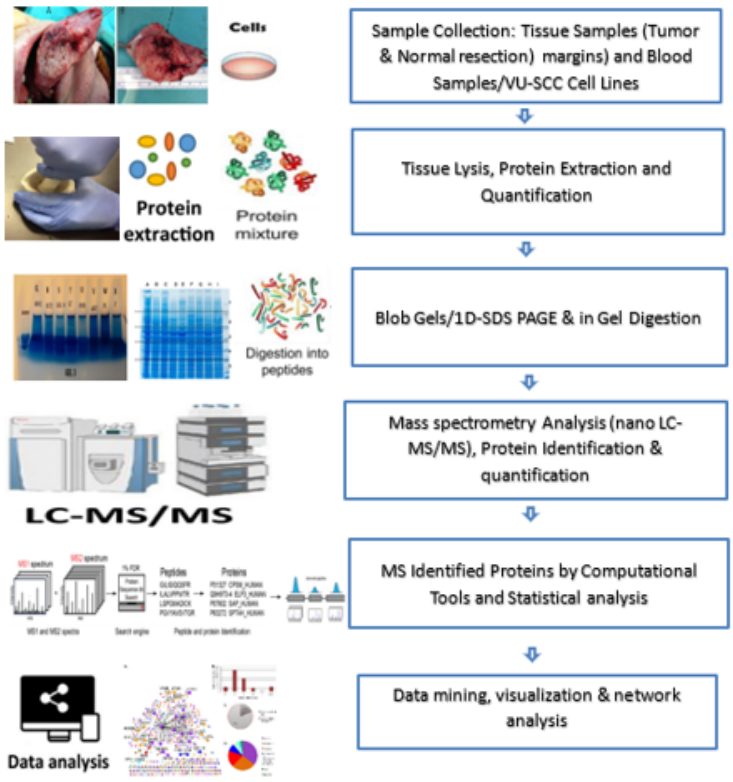
Identification of Protein markers in Oral Squamous Cell Carcinoma (OSCC)

Madiha Mumtaz[1], Irene V. Bijnsdorp[2], Thang V. Pham[2], Sander R. Piersma[2], Connie R. Jimenez[2], Akhtar, M. Waheed[1], Samiullah[3]

[1]School of Biological Sciences, University of the Punjab, Lahore, Pakistan
[2]Department of Medical Oncology, OncoProteomics Laboratory, Cancer Center Amsterdam, Amsterdam UMC, Vrije Universiteit, Amsterdam, The Netherlands[3]Shalamar Medical and Dental College, Lahore, Pakistan

INTRODUCTION - Oral squamous cell carcinoma (OSCC) is a main reason of oral cancer mortality and morbidity. The worldwide 8th most prevalent mouth cancer incidence is mainly more among men. Cancer of oral cavity in central south Asia, ranks among third most common kinds of cancer. The discovery of initial markers to differentiate normal from malignant cells in clinical diagnosis of oral squamous cell carcinoma (OSCC) would be of critical importance because this malignancy has poor prognosis.

AIM - An early and accurate diagnosis of oral squamous cell carcinoma (OSCC) may reduce morbidity and mortality of the patients. To improve the clinical outcome in OSCC patients, the present study is aimed at identifying robust biomarkers for early OSCC diagnosis and also knowledge about the expression and interactions of proteins in both normal and malignant tissues may significantly enhance understanding of the mechanisms of disease progression and pathogenesis.



APPROACH- In order to look for early-stage protein markers, a systematic protein profiling approach involving electrophoresis coupled with mass spectrometric analyses of 17 paired human malignant and adjacent normal mucosa OSCC samples is performed. Protein profiles in 1D-gel-separated fractions (blob gel/sample) were measured by label-free LC-MS/MS-based proteomics by spectral counting on a QExactive-platform. Protein identification and quantification was performed using MaxQuant, data analysis using the beta binomial test, Cytoscape, Clue Go+Pedia, Enricher, iRegulon and secretory proteins were also predicted using SignalP5.0/SecrotomeP2.0-database.

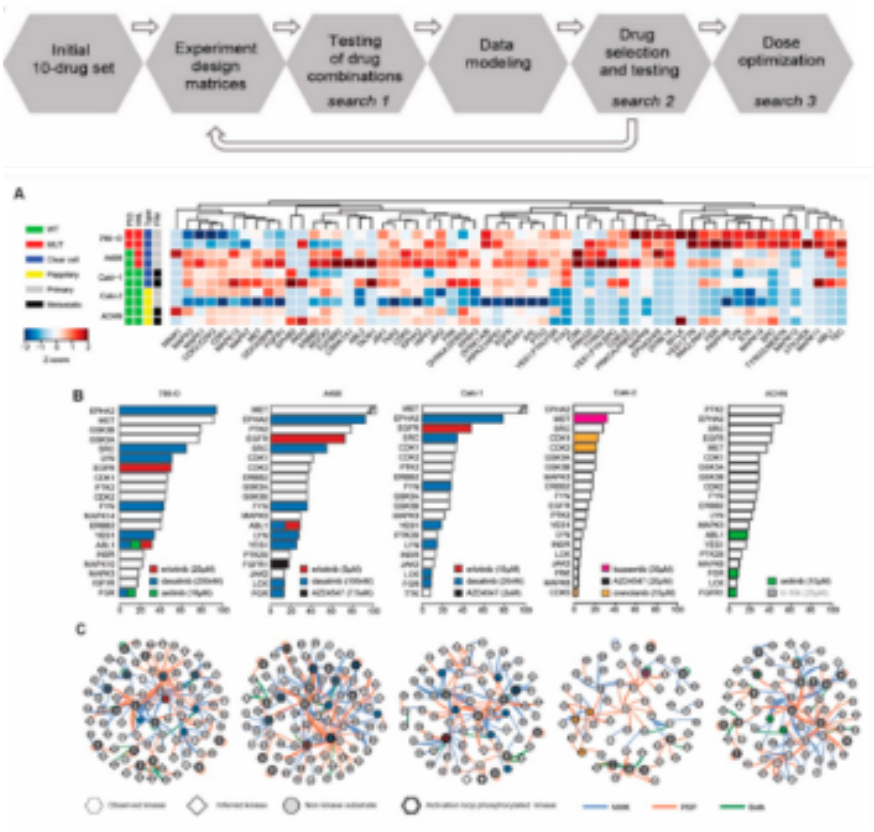
RESULTS - Total identified proteins were 5,123, out of which 299 proteins were differentially expressed (205 upregulated and 94 down regulated) by fixing the fold change barrier to ≥2 at a nominally significant p-value <0.01 and several biological processes significantly were enriched with these proteins. Enrichment analyses of gene ontologies and pathways revealed enrichment of terms associated with Myogenesis, Fatty Acid Metabolism and KRAS Signaling DN among the downregulated proteins in patients, whereas terms related to Protein Secretion, Unfolded Protein Response, Spliceosomal complex assembly, Protein localization to endosome and Interferon Gamma Response were enriched among upregulated proteins and transcription factors regulating these major pathways targeting many proteins were also discussed. We identified the range of proteins both classically and non-classically secreted. Additionally, we found tumor suppressor miRNAs which target many proteins/genes networks and possibly suggestive to be key riboswitches in regulatory cascade of OSCC.

CONCLUSIONS - Our investigation identified key proteins involved in important pathways is likely to provide insight into the mechanism of OSCC progression which may lead to design precise OSCC diagnostics and therapeutics strategies.

Integrating Phenotypic Search and Phosphoproteomic Profiling of Active Kinases for Optimization of Drug Mixtures for RCC Treatment.

Van Beijnum JR, Weiss A, Berndsen RH, Wong TJ, Reckman LC, Piersma SR, Zoetemelk M, de Haas R, Dormond O, Bex A, Henneman AA, Jimenez CR, Griffioen AW, Nowak-Sliwinska P.

Cancers (Basel) 2020 Sep 21;12(9):2697. doi: 10.3390/cancers12092697. PubMed PMID: 32967224.

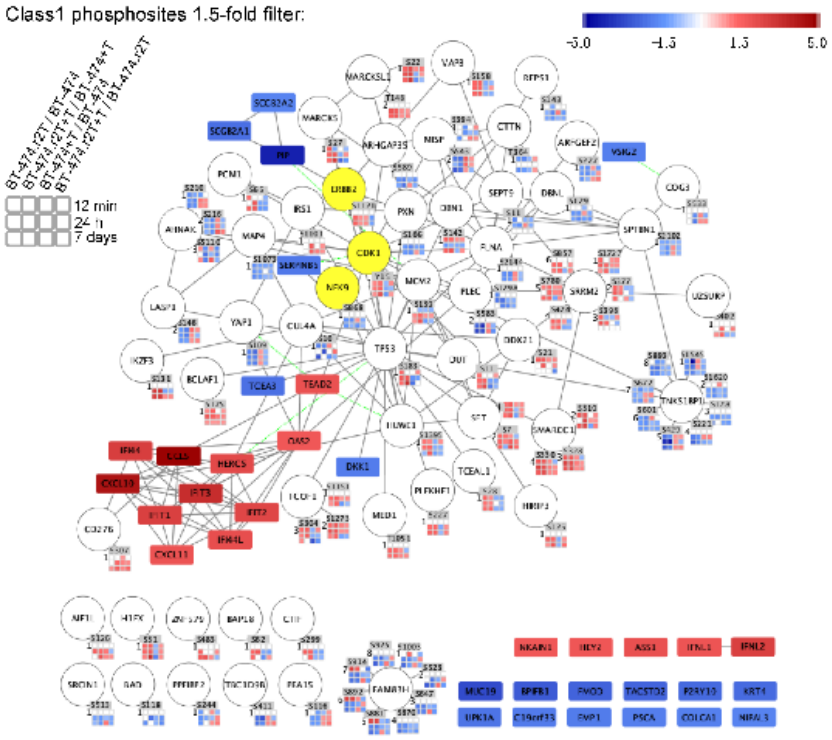


Combined application of multiple therapeutic agents presents the possibility of enhanced efficacy and reduced development of resistance. Definition of the most appropriate combination for any given disease phenotype is challenged by the vast number of theoretically possible combinations of drugs and doses, making extensive empirical testing a virtually impossible task. We have used the streamlined-feedback system control (s-FSC) technique, a phenotypic approach, which converges to optimized drug combinations (ODC) within a few experimental steps. Phosphoproteomics analysis coupled to kinase activity analysis using the novel INKA (integrative inferred kinase activity) pipeline was performed to evaluate ODC mechanisms in a panel of renal cell carcinoma (RCC) cell lines. We identified different ODC with up to 95% effectivity for each RCC cell line, with low doses (ED_{5-25}) of individual drugs. Global phosphoproteomics analysis demonstrated inhibition of relevant kinases, and targeting remaining active kinases with additional compounds improved efficacy. In addition, we identified a common RCC ODC, based on kinase activity data, to be effective in all RCC cell lines under study. Combining s-FSC with a phosphoproteomic profiling approach provides valuable insight in targetable kinase activity and allows for the identification of superior drug combinations for the treatment of RCC.

The Hippo Pathway Transducers YAP1/TEAD Induce Acquired Resistance to Trastuzumab in HER2-Positive Breast Cancer

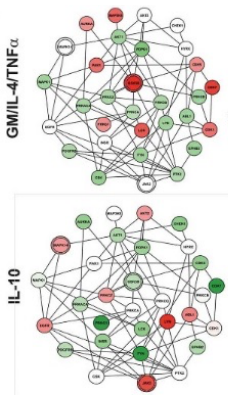
González-Alonso P, Zazo S, Martín-Aparicio E, Luque M, Chamizo C, Sanz-Álvarez M, Minguez P, Gómez-López G, Cristóbal I, Caramés C, García-Foncillas J, Eroles P, Lluch A, Arpí O, Rovira A, Albanell J, Piersma SR, Jimenez CR, Madoz-Gúrpide J, Rojo F

Cancers (Basel) 2020 Apr 29;12(5):1108. doi: 10.3390/cancers12051108. PubMed PMID: 32365528.



Trastuzumab is the first-line targeted therapeutic drug for HER2-positive breast cancer, leading to improved overall survival. However, acquired resistance inevitably occurs. We aimed to identify, quantify, and assess the mechanisms of acquired resistance to trastuzumab. We aimed to identify, quantify, and assess the mechanisms of acquired resistance to trastuzumab. We established an acquired trastuzumab-resistant model in vitro from BT-474, a trastuzumab-sensitive, HER2-amplified breast-cancer cell line. A multi-omic strategy was implemented to obtain gene, proteome, and phosphoproteome signatures associated with acquired resistance to trastuzumab in HER2-positive breast cancer, followed by validation in human clinical samples. YAP1 dephosphorylation and *TEAD2* overexpression were detected as significant alterations in the Hippo pathway in trastuzumab-resistant breast cancer. Because of the emerging role of these proteins as mediators of normal growth and tumorigenesis, we assessed the exogenous modulation of their activity, either by in vitro gene silencing or by pharmacological inhibition of the YAP1/TEAD complexes, both in vitro and in vivo. Moreover, we identified increased signaling through the Hippo pathway in human samples after progression following trastuzumab treatment. Finally, YAP1/TAZ nuclear accumulation in malignant cells in HER2 breast tumor was significantly associated with worse progression-free and overall survival in metastatic HER2-positive breast-cancer patients. Our results suggest the involvement of Hippo signaling in acquired trastuzumab resistance in breast cancer. Additionally, we provide novel evidence for a potential breast-cancer treatment strategy based on dual targeting of HER2 and Hippo pathway effectors, which may improve the antitumor activity of trastuzumab and help overcome resistance.

Constitutively active GSK3ß as a means to bolster dendritic cell functionality in the face of tumour-mediated immune suppression



López González M, Oosterhoff D, Lindenberg JJ, Milenova I, Lougheed SM, Martiáñez T, Dekker H, Quixabeira DCA, Hangalapura B, Joore J, Piersma SR, Cervera-Carrascon V, Santos JM, Scheper RJ, Verheul HMW, Jiménez CR, Van De Ven R, Hemminki A, Van Beusechem VW, De Gruijl TD

Oncoimmunology 2019 Jul 19;8(10):e1631119. doi: 10.1080/2162402X.2019.1631119. PubMed PMID: 31646076.

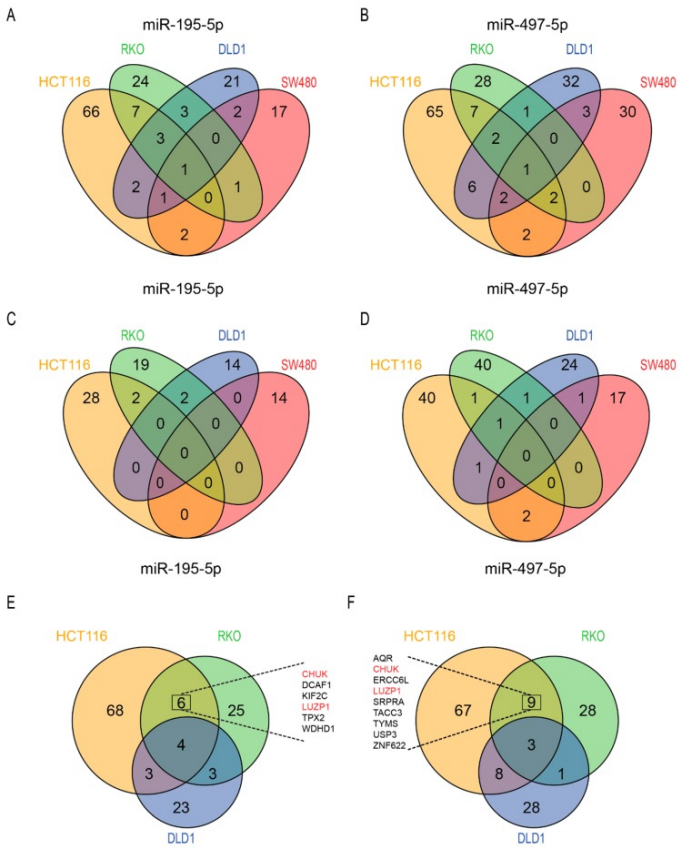
In patients with cancer, the functionality of Dendritic Cells (DC) is hampered by high levels of tumor-derived suppressive cytokines, which interfere with DC development and maturation. Poor DC development can limit the efficacy of immune checkpoint blockade and *in vivo* vaccination approaches. Interference in intracellular signaling cascades downstream from the receptors of major tumor-associated suppressive cytokines like IL-10 and IL-6, might improve DC development and activation, and thus enhance immunotherapy efficacy. We performed exploratory functional screens on arrays consisting of >1000 human kinase peptide substrates to identify pathways involved in DC development and its inhibition by IL-10 or IL-6. The resulting alterations in phosphorylation of the kinome substrate profile pointed to glycogen-synthase kinase-3β (GSK3β) as a pivotal kinase in both DC development and suppression. GSK3β inhibition blocked human DC differentiation *in vitro*, which was accompanied by decreased levels of IL-

12p70 secretion, and a reduced capacity for T cell priming. More importantly, adenoviral transduction of monocytes with a constitutively active form of GSK3β induced resistance to the suppressive effects of IL-10 and melanoma-derived supernatants alike, resulting in improved DC development, accompanied by up-regulation of co-stimulatory markers, an increase in CD83 expression levels in mature DC, and diminished release of IL-10. Moreover, adenovirus-mediated intratumoral manipulation of this pathway in an *in vivo* melanoma model resulted in DC activation and recruitment, and in improved immune surveillance and tumor control. We propose the induction of constitutive GSK3β activity as a novel therapeutic means to bolster DC functionality in the tumor microenvironment.

Proteomic Analysis of miR-195 and miR-497 Replacement Reveals Potential Candidates that Increase Sensitivity to Oxaliplatin in MSI/P53wt Colorectal Cancer Cells

Poel D, Boyd LNC, Beekhof R, Schelfhorst T, Pham TV, Piersma SR, Knol JC, Jimenez CR, Verheul HMW, Buffart TE

Cells 2019 Sep 19;8(9):1111. doi: 10.3390/cells8091111. PubMed PMID: 31546954.

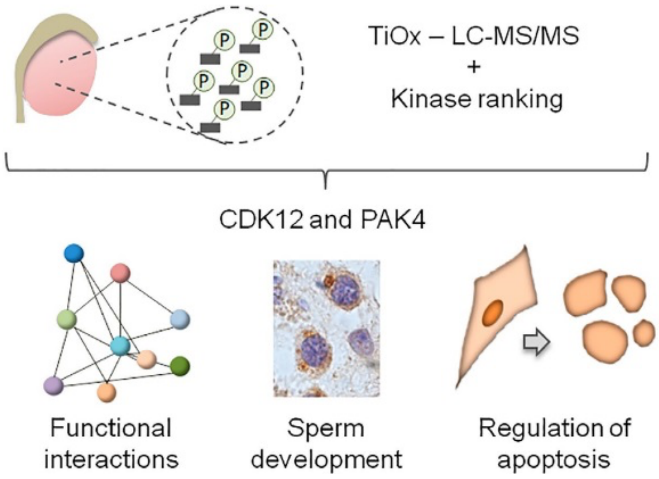


Most patients with advanced colorectal cancer (CRC) eventually develop resistance to systemic combination therapy. miR-195-5p and miR-497-5p are downregulated in CRC tissues and associated with drug resistance. Sensitization to 5-FU, oxaliplatin, and irinotecan by transfection with miR-195-5p and miR-497-5p mimics was studied using cell viability and clonogenic assays in cell lines HCT116, RKO, DLD-1, and SW480. In addition, proteomic analysis of transfected cells was implemented to identify potential targets. Significantly altered proteins were subjected to STRING (protein-protein interaction networks) database analysis to study the potential mechanisms of drug resistance. Cell viability analysis of transfected cells revealed increased sensitivity to oxaliplatin in microsatellite instable (MSI)/P53 wild-type HCT116 and RKO cells. HCT116 transfected cells formed significantly fewer colonies when treated with oxaliplatin. In sensitized cells, proteomic analysis showed 158 and 202 proteins with significantly altered expression after transfection with miR-195-5p and miR-497-5p mimics respectively, of which CHUK and LUZP1 proved to be coinciding downregulated proteins. Resistance mechanisms of these proteins may be associated with nuclear factor kappa-B signaling and G1 cell-cycle arrest. In conclusion, miR-195-5p and miR-497-5p replacement enhanced sensitivity to oxaliplatin in treatment naïve MSI/P53 wild-type CRC cells. Proteomic analysis revealed potential miRNA targets associated with the cell-cycle which possibly bare a relation with chemotherapy sensitivity.

Human Testis Phosphoproteome Reveals Kinases as Potential Targets in Spermatogenesis and Testicular Cancer

Castillo J, Knol JC, Korver CM, Piersma SR, Pham TV, de Goeij-de Haas RR, van Pelt AMM, Jimenez CR, Jansen BJH

Mol Cell Proteomics 2019 Mar 15;18(Suppl 1):S132-S144. doi: 10.1074/mcp.RA118.001278. PubMed PMID: 30683686.



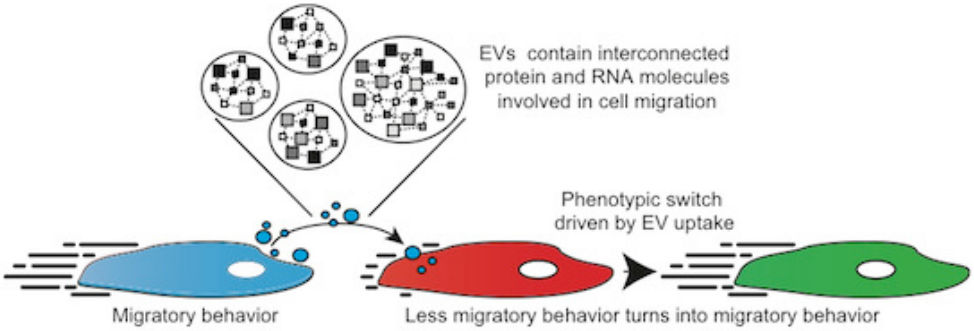
Spermatogenesis is a complex cell differentiation process that includes marked genetic, cellular, functional and structural changes. It requires tight regulation, because disturbances in any of the spermatogenic processes would lead to fertility deficiencies as well as disorders in offspring. To increase our knowledge of signal transduction during sperm development, we carried out a large-scale identification of the phosphorylation events that occur in the human male gonad. Metal oxide affinity chromatography using TiO_2 combined with LC-MS/MS was conducted to profile the phosphoproteome of adult human testes with full spermatogenesis. A total of 8187 phosphopeptides derived from 2661 proteins were identified, resulting in the most complete report of human testicular phosphoproteins to date. Phosphorylation events were enriched in proteins functionally related to spermatogenesis, as well as to highly active processes in the male gonad, such as transcriptional and translational regulation, cytoskeleton organization, DNA packaging, cell cycle and apoptosis. Moreover, 174 phosphorylated kinases were identified. The most active human protein kinases in the testis were predicted both by the number of phosphopeptide spectra identified and the phosphorylation status of the kinase activation loop. The potential function of cyclin-dependent kinase 12 (CDK12) and p21-activated kinase 4 (PAK4) has been explored by *in silico*, protein-protein interaction analysis, immunodetection in testicular tissue, and a functional assay in a human embryonal carcinoma cell line. The colocalization of CDK12 with Golgi markers suggests a potential crucial role of this protein kinase during sperm formation. PAK4 has been found expressed in human spermatogonia, and a role in embryonal carcinoma cell response to apoptosis has been observed. Together, our protein discovery analysis confirms that phosphoregulation by protein kinases is highly active in sperm differentiation and opens a window to detailed characterization and validation of potential targets for the development of drugs modulating male fertility and tumor behavior.

Cancer cells copy migratory behavior and exchange signaling networks via extracellular vesicles

Steenbeek SC, Pham TV, de Ligt J, Zomer A, Knol JC, Piersma SR, Schelfhorst T, Huisjes R, Schiffelers RM, Cuppen E, Jimenez CR*, van Rheenen J*

*Shared senior authors

EMBO J 2018 Aug 1;37(15):e98357. doi: 10.15252/embj.201798357. PubMed PMID: 29907695.



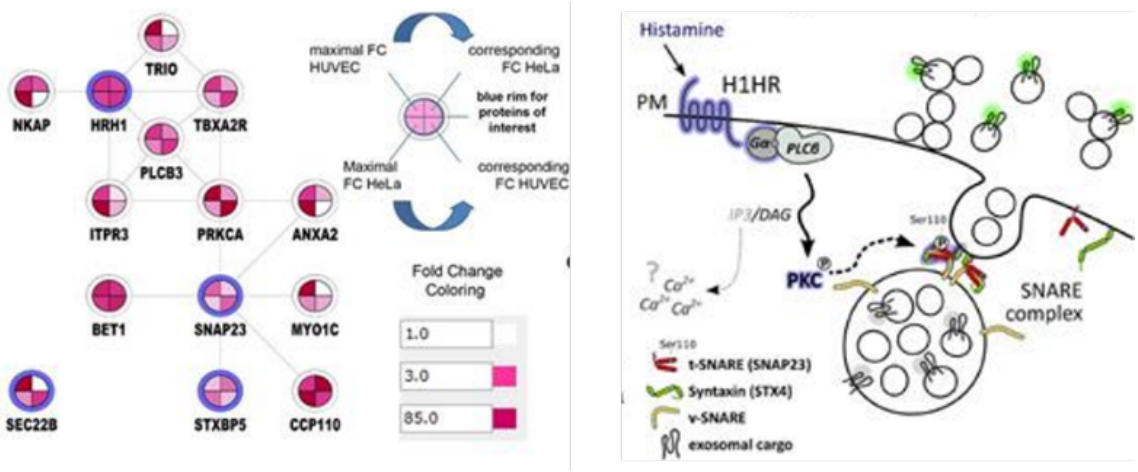
Recent data showed that cancer cells from different tumor subtypes with distinct metastatic potential influence each other's metastatic behavior by exchanging biomolecules through extracellular vesicles (EVs). However, it is debated how small amounts of cargo can mediate this effect, especially in tumors where all cells are from one subtype, and only subtle molecular differences drive metastatic heterogeneity. To study this, we have characterized the content of EVs shed *in vivo* by two clones of melanoma (B16) tumors with distinct metastatic potential. Using the Cre-LoxP system and intravital microscopy, we show that cells from these

distinct clones phenocopy their migratory behavior through EV exchange. By tandem mass spectrometry and RNA sequencing, we show that EVs shed by these clones into the tumor microenvironment contain thousands of different proteins and RNAs, and many of these biomolecules are from interconnected signaling networks involved in cellular processes such as migration. Thus, EVs contain numerous proteins and RNAs and act on recipient cells by invoking a multi-faceted biological response including cell migration.

Quantifying exosome secretion from single cells reveals a modulatory role for GPCR signaling

Verweij FJ, Bebelman MP, Jimenez CR, Garcia-Vallejo JJ, Janssen H, Neefjes J, Knol JC, de Goeij-de Haas R, Piersma SR, Baglio SR, Verhage M, Middeldorp JM, Zomer A, van Rheenen J, Coppolino MG, Hurbain I, Raposo G, Smit MJ, Toonen RFG, van Niel G, Pegtel DM

J Cell Biol 2018 Mar 5;217(3):1129-1142. doi: 10.1083/jcb.201703206. PubMed PMID: 29339438.



Exosomes are small endosome-derived extracellular vesicles implicated in cell-cell communication and are secreted by living cells when multivesicular bodies (MVBs) fuse with the plasma membrane (PM). Current techniques to study exosome physiology are based on isolation procedures after secretion, precluding direct and dynamic insight into the mechanics of exosome biogenesis and the regulation of their release. In this study, we propose real-time visualization of MVB-PM fusion to overcome these limitations. We designed tetraspanin-based pH-sensitive optical reporters that detect MVB-PM fusion using live total internal reflection fluorescence and dynamic correlative light-electron microscopy. Quantitative analysis demonstrates that MVB-PM fusion frequency is reduced by depleting the target membrane SNAREs SNAP23 and syntaxin-4 but also can be induced in single cells by stimulation of the histamine H1 receptor (H1HR). Interestingly, activation of H1R1 in HeLa cells increases Ser110 phosphorylation of SNAP23, promoting MVB-PM fusion and the release of CD63-enriched exosomes. Using this single-cell resolution approach, we highlight the modulatory dynamics of MVB exocytosis that will help to increase our understanding of exosome physiology and identify druggable targets in exosome-associated pathologies.

Collaborative Neurology Research

Proteomic profiling of extracellular vesicles derived from cerebrospinal fluid of patients with Alzheimer's disease: in search of potential biomarkers

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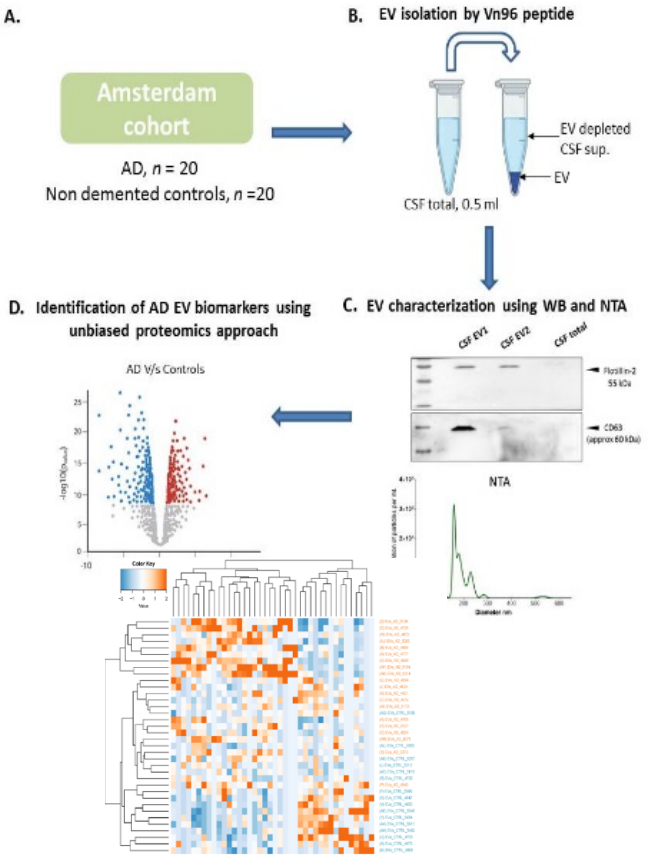

INTRODUCTION - Extracellular vesicles (EVs) are nano-sized vesicles released from cells to the cerebrospinal fluid (CSF). EVs have been reported to play a role in the pathogenesis of Alzheimer's disease (AD) and are promising source of novel biomarkers.

AIM - We aimed to characterize the proteome of CSF EVs to identify proteins and pathways which are altered in AD EVs compared to EVs from non-demented controls.

APPROACH - CSF samples were obtained from the Amsterdam Dementia cohort comprising AD patients (n=20) and non-demented controls (n=20) (see left Fig.). EV isolation was carried out on 0.5 mL of CSF using the peptide-affinity method that precipitates EVs based on binding to heat shock proteins on the vesicle surface[1,2]. This was followed by characterization of EVs using Western blotting and nanoparticle tracking analyzer (NTA). High-resolution mass spectrometry was used to characterize the proteome of CSF EVs[3], while functional annotations were investigated using enrichment analysis.

RESULTS - 617 proteins were identified in total in all the CSF EV samples including proteins involved in EV biogenesis, heat shock proteins, and tetraspanins. 41 proteins were differentially regulated in AD versus controls, out of which 29 were upregulated and 12 were downregulated. Majority of the differentially regulated proteins were related to inflammation, complement pathway and metabolism.

CONCLUSION - EVs derived from CSF may reveal novel biomarker candidates for AD.

REFERENCES -

1. Ghosh A, Davey M, Chute IC, Griffiths SG, Lewis S, Chacko S, Barnett D, Crapoulet N, Fournier S, Joy A, Caissie MC, Ferguson AD, Daigle M, Meli MV, Lewis SM, Ouellette RJ. Rapid isolation of extracellular vesicles from cell culture and biological fluids using a synthetic peptide with specific affinity for heat shock proteins. PLoS One. 2014 Oct 17;9(10):e110443.

2. Knol JC, de Reus I, Schelfhorst T, Beekhof R, de Wit M, Piersma SR, Pham TV, Smit EF, Verheul HMW, Jiménez CR. Peptide-mediated 'miniprep' isolation of extracellular vesicles is suitable for high-throughput proteomics. EuPA Open Proteom. 2016 Feb 22;11:11-15.

3. Chiasserini D, van Weering JR, Piersma SR, Pham TV, Malekzadeh A, Teunissen CE, de Wit H, Jiménez CR. Proteomic analysis of cerebrospinal fluid extracellular vesicles: a comprehensive dataset. J Proteomics. 2014 Jun 25;106:191-204.

Clinical proteomics of cerebrospinal fluid to identify and validate biomarkers for progression of Mild Cognitive Impairment stages to Alzheimer's Disease

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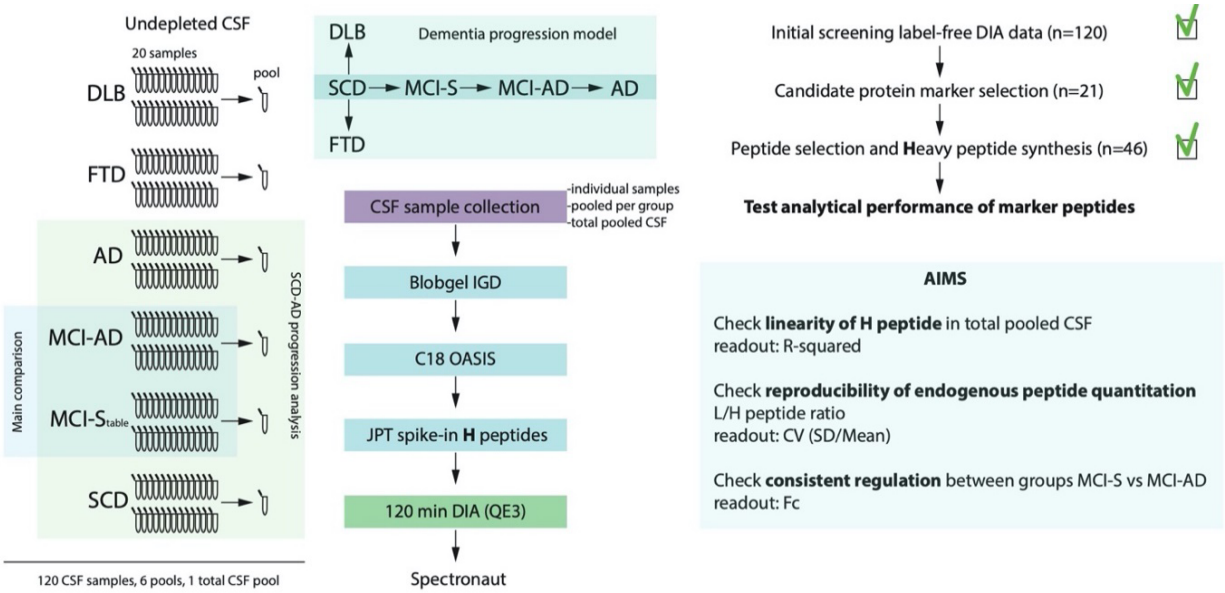
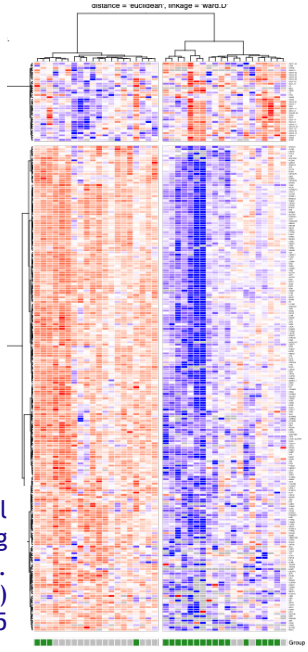


INTRODUCTION - Cerebrospinal fluid (CSF) protein biomarkers such as amyloid-beta, tau and phospho-tau have provided an invaluable tool for diagnosis of Alzheimer's Disease (AD). Their performance is noteworthy, yet there is still a need for additional markers for prognosis, patient stratification in clinical trials, and response to treatment. Previously we used proteomics of CSF to identify 15 potential protein biomarkers, including 4 top candidates discriminating AD patients from controls and mild cognitive impairment (MCI) cases that progress to AD from those that do not.

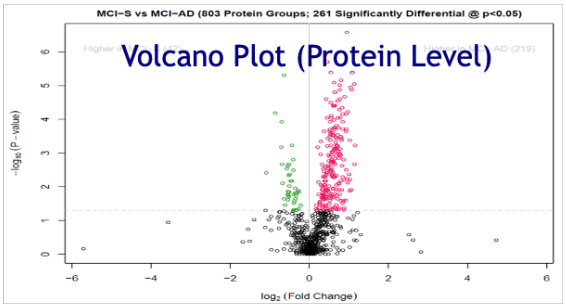
AIM - Validation of 15 promising AD biomarker candidates using next generation high-throughput mass spectrometry of CSF. Applying a data-independent MS approach (DIA), we will also be able to identify and validate new biomarkers.

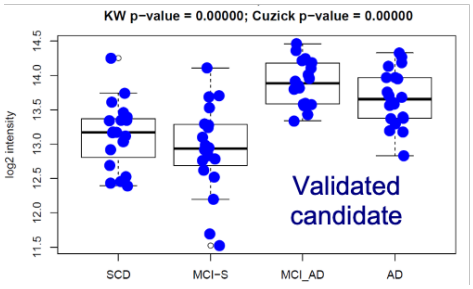
APPROACH - We will perform quantitative DIA-MS protein profiling of CSF from two independent well-characterized dementia cohorts of 120 samples that include samples of confirmed AD (n=20), controls (subjective memory complaint, SCD, n=20) and precursor stages (MCI stable, n=20 and MCI progressing to AD, n=20), as well as other dementias (dementia with Lewy bodies, DLB, n=20 and frontotemporal dementia, FTD, n=20).

RESULTS - Currently. we have performed proteomics on cohort 1 (n=120). In total 803 proteins were identified in undepleted CSF. Statistical analysis (Mann-Whitney) showed that 10 of the 15 predefined candidates were significantly differential between the two MCI groups ($p < 0.05$). Additional AD progression analysis (Kruskal-Wallis test for multi-group analysis and one-sided Cuzick test for trend analysis) was performed on the SCD / MCI-Stable / MCI-AD / AD disease progression stages. Six of the 15 predefined candidate biomarker proteins were also statistically significant in the progression analysis including ALDOA, CHI3L1 and GDA. In addition, multiple additional promising candidates were identified.
To enable more precise quantitation, for each validated protein marker, the best responding two peptides in DIA-MS were selected as internal reference peptides. Spike-in experiments of different amounts of heavy labelled variants of these peptides has resulted in the development of highly linear assays for our validated CSF protein markers.



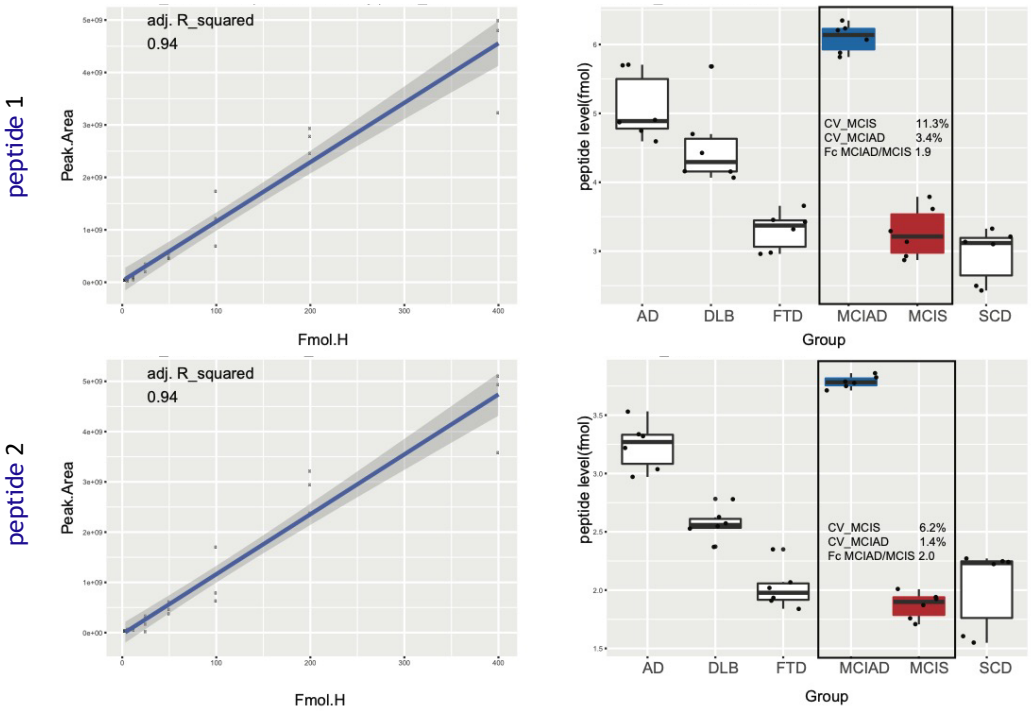
Hierarchical clustering MCI-S (green) vs. MCI-AD (grey) p<0.05





Heavy peptide response linearity

Peptide quantification reproducibility



To test reproducibility and robustness of candidate peptide quantification using DIA-MS, heavy labeled reference peptides were spiked (10, 50 and 200 fmol) in tryptic CSF digest pools per patient group (example shown for one candidate). Heavy to light ratios were determined by DIA-MS using Spectronaut. The endogenous (light) peptide quantities were evaluated in the groups (6 groups, pooled CSF of n=20 pt/group). Coefficients of variation (CV) and fold changes of the endogenous candidate biomarker peptides were calculated for the MCI-AD versus MCI-S comparison.

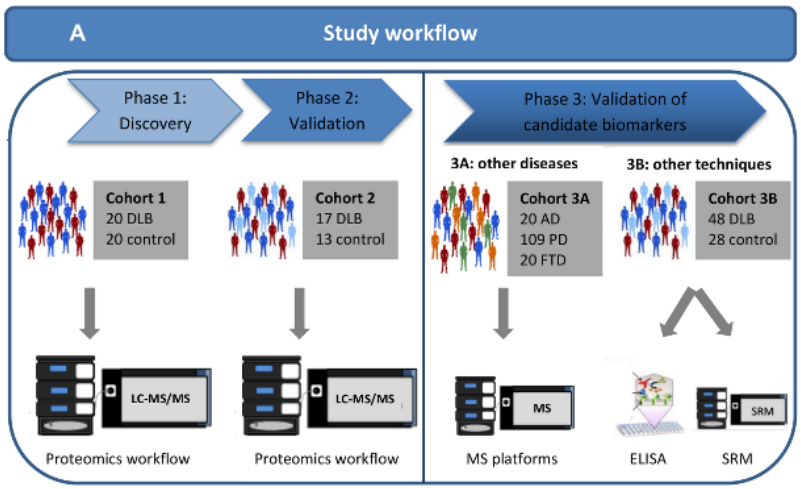
OUTLOOK - A biomarker panel based on a panel of CSF proteins reflecting diverse AD biology is expected to have prognostic value and enable appropriate selection of cases at an early stage for clinical trials with experimental drugs and will aid in developing effective treatments. This MS-based clinically applicable test will be portable to other laboratories employing DIA-MS.

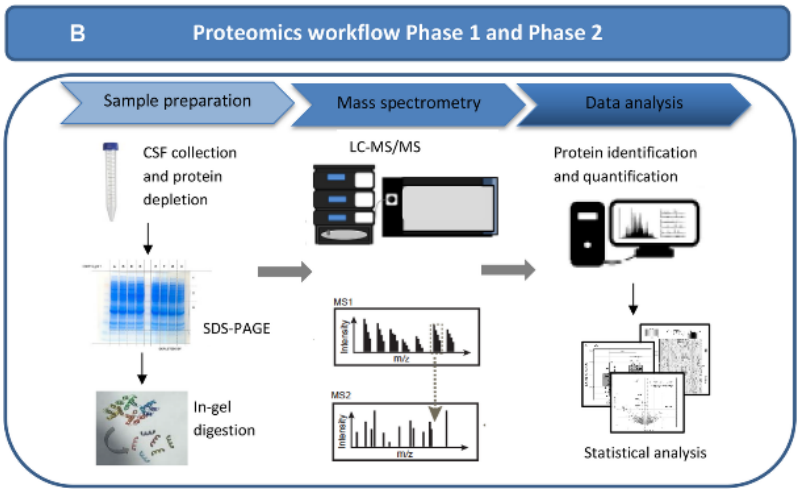
ACKNOWLEDGEMENTS - Supported by Weston Brain Institute.

Identification of novel cerebrospinal fluid biomarker candidates for dementia with Lewy bodies: a proteomic approach

Van Steenoven I, Koel-Simmelink MJA, Vergouw LJM, Tijms BM, Piersma SR, Pham TV, Bridel C, Ferri GL, Cocco C, Noli B, Worley PF, Xiao MF, Xu D, Oeckl P, Otto M, van der Flier WM, de Jong FJ, Jimenez CR, Lemstra AW, Teunissen CE

Mol Neurodegener 2020 Jun 18;15(1):36. doi: 10.1186/s13024-020-00388-2. PubMed PMID: 32552841.





Background: Diagnosis of dementia with Lewy bodies (DLB) is challenging, largely due to a lack of diagnostic tools. Cerebrospinal fluid (CSF) biomarkers have been proven useful in Alzheimer's disease (AD) diagnosis. Here, we aimed to identify novel CSF biomarkers for DLB using a high-throughput proteomic approach.

Methods: We applied liquid chromatography/ tandem mass spectrometry with label-free quantification to identify biomarker candidates to individual CSF samples from a well-characterized cohort comprising patients with DLB (n = 20) and controls (n = 20). Validation was performed using (1) the identical proteomic workflow in an independent cohort (n = 30), (2) proteomic data from patients with related neurodegenerative diseases (n = 149) and (3) orthogonal techniques in an extended cohort consisting of DLB patients and controls (n = 76). Additionally, we utilized random forest analysis to identify the subset of candidate markers that best distinguished DLB from all other groups.

Results: In total, we identified 1995 proteins. In the discovery cohort, 69 proteins were differentially expressed in DLB compared to controls ($p < 0.05$). Independent cohort replication confirmed VGF, SCG2, NPTX2, NPTXR, PDYN and PCSK1N as candidate biomarkers for DLB. The downregulation of the candidate biomarkers was somewhat more pronounced in DLB in comparison with related neurodegenerative diseases. Using random forest analysis, we identified a panel of VGF, SCG2 and PDYN to best differentiate between DLB and other clinical groups (accuracy: 0.82 (95%CI: 0.75-0.89)). Moreover, we confirmed the decrease of VGF and NPTX2 in DLB by ELISA and SRM methods. Low CSF levels of all biomarker candidates, except PCSK1N, were associated with more pronounced cognitive decline ($0.37 < r < 0.56$, all $p < 0.01$).

Conclusion: We identified and validated six novel CSF biomarkers for DLB. These biomarkers, particularly when used as a panel, show promise to improve diagnostic accuracy and strengthen the importance of synaptic dysfunction in the pathophysiology of DLB.

Brain endothelial cell expression of SPARCL-1 is specific to chronic multiple sclerosis lesions and is regulated by inflammatory mediators in vitro

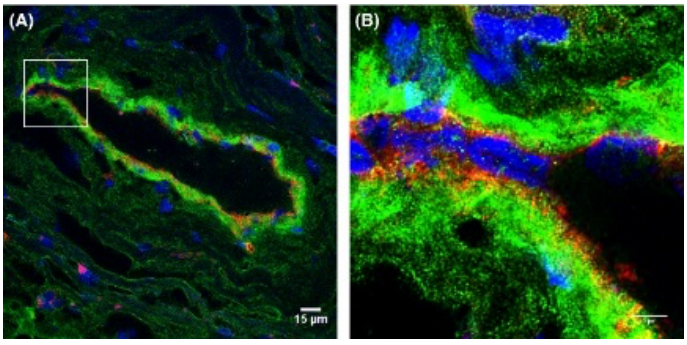
Bridel C, Koel-Simmelink MJA, Peferoen L, Derada Troletti C, Durieux S, Gorter R, Nutma E, Gami P, Iacobaeus E, Brundin L, Kuhle J, Vrenken H, Killestein J, Piersma SR, Pham TV, De Vries HE, Amor S, Jimenez CR, Teunissen CE

Neuropathol Appl Neurobiol 2018 Jun;44(4):404-416. doi: 10.1111/nan.12412. 28543098 PubMed PMID: 28543098.

Aims: Cell matrix modulating protein SPARCL-1 is highly expressed by astrocytes during CNS development and following acute CNS damage. Applying NanoLC-MS/MS to CSF of RRMS and SPMS patients, we identified SPARCL-1 as differentially expressed between these two stages of MS, suggesting a potential as CSF biomarker to differentiate RRMS from SPMS and a role in MS pathogenesis.

Methods: This study examines the potential of SPARCL-1 as CSF biomarker discriminating RRMS from SPMS in three independent cohorts (n = 249), analyses its expression pattern in MS lesions (n = 26), and studies its regulation in cultured human brain microvasculature endothelial cells (BEC) after exposure to MS-relevant inflammatory mediators.

Results: SPARCL-1 expression in CSF was significantly higher in SPMS compared to RRMS in a Dutch cohort of 76 patients. This finding was not replicated in 2 additional cohorts of MS patients from Sweden (n = 81) and Switzerland (n = 92). In chronic MS lesions, but not active lesions or NAWM, a vessel expression pattern of SPARCL-1 was observed in addition to the expression by astrocytes. EC were found to express SPARCL-1 in chronic MS lesions, and SPARCL-1 expression was regulated by MS-relevant inflammatory mediators in cultured human BEC.



Conclusions: Conflicting results of SPARCL-1's differential expression in CSF of three independent cohorts of RRMS and SPMS patients precludes its use as biomarker for disease progression. The expression of SPARCL-1 by BEC in chronic MS lesions together with its regulation by inflammatory mediators in vitro suggest a role for SPARCL-1 in MS neuropathology, possibly at the brain vascular level.

Collaborative Microbiology Research

Understanding the virulence of pathogenic mycobacteria to find new therapies for tuberculosis

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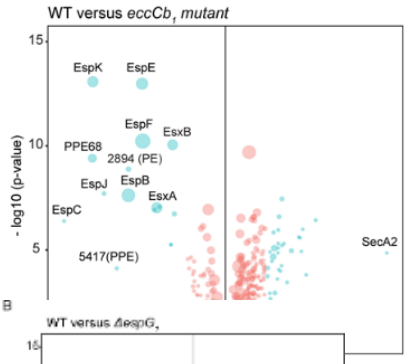
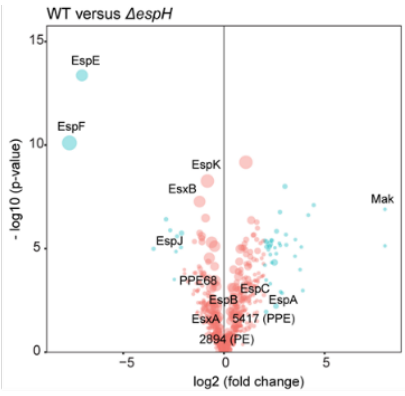
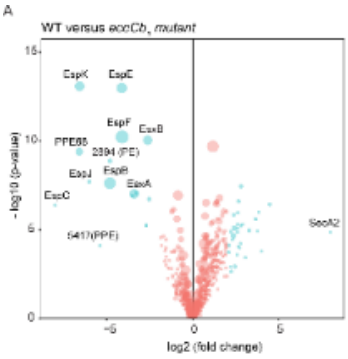
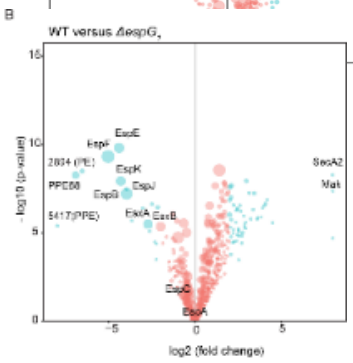
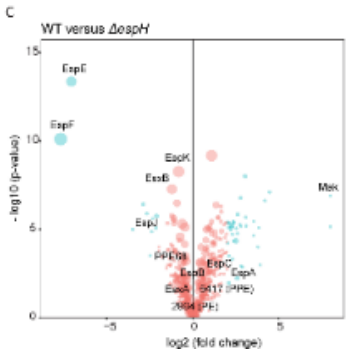
INTRODUCTION -
M. tuberculosis is the causative agent of tuberculosis, a major infectious disease that still causes enormous mortality and morbidity worldwide. *M. tuberculosis* a facultative intracellular pathogen that has an intimate relationship with

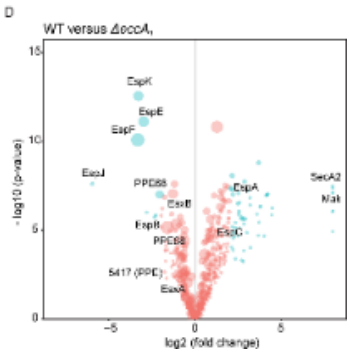












host macrophages. Proteins secreted by the type VII secretion pathwyas, also called ESX systems, play important roles in the interaction with the host and the interaction with the macrophage host cell in particular, for instance by orchestrating the escape from the phagosome into the cytosol of the macrophage. However, the exact role of the type VII secretion substrates is unknown, due to their number and complicated interdependency for secretion. We study the five different type VII secretion systems (named ESX-1 through ESX-5), their substrates and the function of these substrates in virulence. We hypothesize that understanding the function of tehse substrates will help to identify new mechanisms to block infection of this pathogen.

AIM - The aim is to understand the working of the type VII secretion systems and their substrates in pathogenic mycobacteria.

APPROACH - In the last years we have focused on the ESX-1 system, which is required for phagosome escape and the ESX-5 system, which secrets most substrates. For ESX-1 we produced a new set of mutants in the fish pathogen *M. marinum* and studied the effect of these mutations on virulence and protein secretion. For ESX-5 we focused on a specific subset of substrates, the PE-PGRS proteins to see if we could find a common factor that is required for their secretion.

RESULTS - We produced mutants in genes coding for *M. marinum* accessory ESX-1 proteins EccA1, EspG1 and EspH, i.e. proteins that are neither substrates nor structural components. Proteomic analysis revealed that EspG1 is crucial for ESX-1 secretion, since all detectable ESX-1 substrates were absent from the cell surface and culture supernatant in an espG1 mutant. Deletion of eccA1 resulted in minor secretion defects, but interestingly, the severity of these secretion defects was dependent on the culture conditions. Finally, espH deletion showed a partial secretion defect; whereas several ESX-1 substrates were secreted in normal amounts, secretion of EsxA and EsxB was diminished and secretion of EspE and EspF was fully blocked. Interaction studies showed that EspH binds EspE and therefore could function as a specific chaperone for this substrate. Despite the observed differences in secretion, hemolytic activity was lost in all *M. marinum* mutants, implying that hemolytic activity is not strictly correlated with EsxA secretion. Surprisingly, while EspH is essential for successful infection of phagocytic host cells, deletion of espH resulted in a significantly increased virulence phenotype in zebrafish larvae, linked to poor granuloma formation and extracellular outgrowth. Together, these data show that different sets of ESX-1 substrates play different roles at various steps of the infection cycle of *M. marinum*.

The most recently evolved type VII secretion system is ESX-5 and through this system dozens of substrates belonging to the PE and PPE families, which are named for conserved proline and glutamic acid residues close

to the amino terminus, are secreeted. However, the role of these proteins remains largely elusive. We show that mutations of ppe38 completely block the secretion of two large subsets of ESX-5 substrates, that is, PPE-MPTR and PE_PGRS, together comprising >80 proteins. Importantly, hypervirulent clinical *M. tuberculosis* strains of the Beijing lineage have such a mutation and a concomitant loss of secretion. Restoration of PPE38-dependent secretion partially reverted the hypervirulence phenotype of a Beijing strain, and deletion of ppe38 in moderately virulent *M. tuberculosis* increased virulence. This indicates that these ESX-5 substrates have an important role in virulence attenuation. Phylogenetic analysis revealed that deletion of ppe38 occurred at the branching point of the 'modern' Beijing sublineage and is shared by Beijing outbreak strains worldwide, suggesting that this deletion may have contributed to their success and global distribution. The emergence of hypervirulent modern Beijing strains has been associated with increased population densities during and after the industrial revolution, where hypervirulence could be an advantageous trait. This feature can be used to identify clades with potential hypervirulence.

REFERENCES -

1. EspH is a hypervirulence factor for Mycobacterium marinum and essential for the secretion of the ESX-1 substrates EspE and EspF. Phan TH, van Leeuwen LM, Kuijl C, Ummels R, van Stempvoort G, Rubio-Canalejas A, Piersma SR, Jiménez CR, van der Sar AM, Houben ENG, Bitter W. PLoS Pathog. 2018 14:e1007247.

2. Mutations in ppe38 block PE_PGRS secretion and increase virulence of Mycobacterium tuberculosis. Ates LS, Dippenaar A, Ummels R, Piersma SR, van der Woude AD, van der Kuij K, Le Chevalier F, Mata-Espinosa D, Barrios-Payán J, Marquina-Castillo B, Guapillo C, Jiménez CR, Pain A, Houben ENG, Warren RM, Brosch R, Hernández-Pando R, Bitter W. Nat Microbiol. 2018 3:181-188.

EspH is a hypervirulence factor for Mycobacterium marinum and essential for the secretion of the ESX-1 substrates EspE and EspF

Phan TH, van Leeuwen LM, Kuijl C, Ummels R, van Stempvoort G, Rubio-Canalejas A, Piersma SR, Jiménez CR, van der Sar AM, Houben ENG, Bitter W

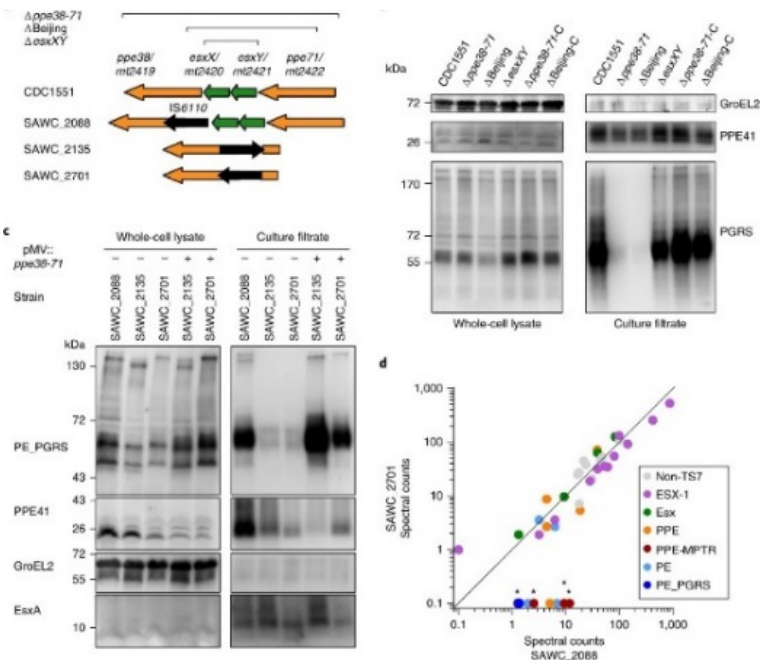
PLoS Pathog 2018 Aug 13;14(8):e1007247. PubMed PMID: 30102741.

The pathogen Mycobacterium tuberculosis employs a range of ESX-1 substrates to manipulate the host and build a successful infection. Although the importance of ESX-1 secretion in virulence is well established, the characterization of its individual components and the role of individual substrates is far from complete. Here, we describe the functional characterization of the Mycobacterium marinum accessory ESX-1 proteins EccA1, EspG1 and EspH, i.e. proteins that are neither substrates nor structural components. Proteomic analysis revealed that EspG1 is crucial for ESX-1 secretion, since all detectable ESX-1 substrates were absent from the cell surface and culture supernatant in an espG1 mutant. Deletion of eccA1 resulted in minor secretion defects, but interestingly, the severity of these secretion defects was dependent on the culture conditions. Finally, espH deletion showed a partial secretion defect; whereas several ESX-1 substrates were secreted in normal amounts, secretion of EsxA and EsxB was diminished and secretion of EspE and EspF was fully blocked. Interaction studies showed that EspH binds EspE and therefore could function as a specific chaperone for this substrate. Despite the observed differences in secretion, hemolytic activity was lost in all M. marinum mutants, implying that hemolytic activity is not strictly correlated with EsxA secretion. Surprisingly, while EspH is essential for successful infection of phagocytic host cells, deletion of espH resulted in a significantly increased virulence phenotype in zebrafish larvae, linked to poor granuloma formation and extracellular outgrowth. Together, these data show that different sets of ESX-1 substrates play different roles at various steps of the infection cycle of M. marinum.

Mutations in ppe38 block PE_PGRS secretion and increase virulence of Mycobacterium tuberculosis

Ates LS, Dippenaar A, Ummels R, Piersma SR, van der Woude AD, van der Kuij K, Le Chevalier F, Mata-Espinosa D, Barrios-Payán J, Marquina-Castillo B, Guapillo C, Jiménez CR, Pain A, Houben ENG, Warren RM, Brosch R, Hernández-Pando R, Bitter W

Nat Microbiol 2018 Feb;3(2):181-188. doi: 10.1038/s41564-017-0090-6. PubMed PMID: 29335553.



Mycobacterium tuberculosis requires a large number of secreted and exported proteins for its virulence, immune modulation and nutrient uptake. Most of these proteins are transported by the different type VII secretion systems. The most recently evolved type VII secretion system, ESX-5, secretes dozens of substrates belonging to the PE and PPE families, which are named for conserved proline and glutamic acid residues close to the amino terminus. However, the role of these proteins remains largely elusive. Here, we show that mutations of ppe38 completely block the secretion of two large subsets of ESX-5 substrates, that is, PPE-MPTR and PE_PGRS, together comprising >80 proteins. Importantly, hypervirulent clinical M. tuberculosis strains of the Beijing lineage have such a mutation and a concomitant loss of secretion. Restoration of PPE38-dependent secretion partially reverted the hypervirulence phenotype of a Beijing strain, and deletion of ppe38 in moderately virulent M. tuberculosis increased virulence. This indicates that these ESX-5 substrates have an

important role in virulence attenuation. Phylogenetic analysis revealed that deletion of ppe38 occurred at the branching point of the 'modern' Beijing sublineage and is shared by Beijing outbreak strains worldwide, suggesting that this deletion may have contributed to their success and global distribution.

Collaborative Cardiology/Physiology Research

Sex-related differences in protein expression in sarcomere mutation-positive hypertrophic cardiomyopathy

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INTRODUCTION - Sex-differences in clinical presentation contribute to the phenotypic heterogeneity of hypertrophic cardiomyopathy (HCM) patients. While disease prevalence is higher in men, women present with more severe diastolic dysfunction and worse survival. Until today, little is known about the cellular differences underlying sex-differences in clinical presentation.

AIM AND APPROACH - To define sex-differences at the protein level, we performed an unlabeled proteomic analysis (LC-MS/MS) in cardiac tissue obtained during myectomy surgery to relieve left ventricular outflow tract obstruction of age-matched female and male HCM patients harboring a sarcomere mutation (n=13 in both groups). Furthermore, these samples were compared to 8 non-failing controls. Women presented with more severe diastolic dysfunction.

RESULTS - Out of 2099 quantified proteins, direct comparison of male and female HCM samples revealed only 46 significantly differentially expressed proteins. Increased levels of tubulin and heat shock proteins were observed in female compared to male HCM patients. Western blot analyses confirmed higher levels of tubulin in female HCM samples. In addition, proteins involved in carbohydrate metabolism were significantly lower in female compared to male samples. Furthermore, we found lower levels of translational proteins specifically in male HCM samples. These sex-specific changes were confirmed by a second analysis in which we compared female and male samples separately to non-failing control samples. Transcription factor analysis showed that sex hormone-dependent transcription factors may contribute to differential protein expression, but do not explain the majority of protein changes observed between male and female HCM samples.

CONCLUSION - Based on our proteomics analyses we propose that increased levels of tubulin partly underlie more severe diastolic dysfunction in women compared to men. Since heat shock proteins have cardioprotective effects, elevated levels of heat shock proteins in females may contribute to later disease onset in woman, while reduced protein turnover in men may lead to the accumulation of damaged proteins which in turn affects proper cellular function.

Elevated Von Willebrand Factor expression in pulmonary artery endothelial cells of chronic thromboembolic pulmonary hypertension patients is driven by enhanced NFκB2 binding

Xue Manz[1], Robert Szulcek[1,2], Xiaoke Pan[1], Petr Symersky[2], Chris Dickhoff[2], Jisca Majolée[4], Veerle Kremer[4], Teodora Radonic[5], Frances de Man[1], Reinier Boon[4], Connie Jiminez[6], Jan Voorberg[7], Peter Hordijk[4], Jurjan Aman[1], Harm Jan Bogaard[1]

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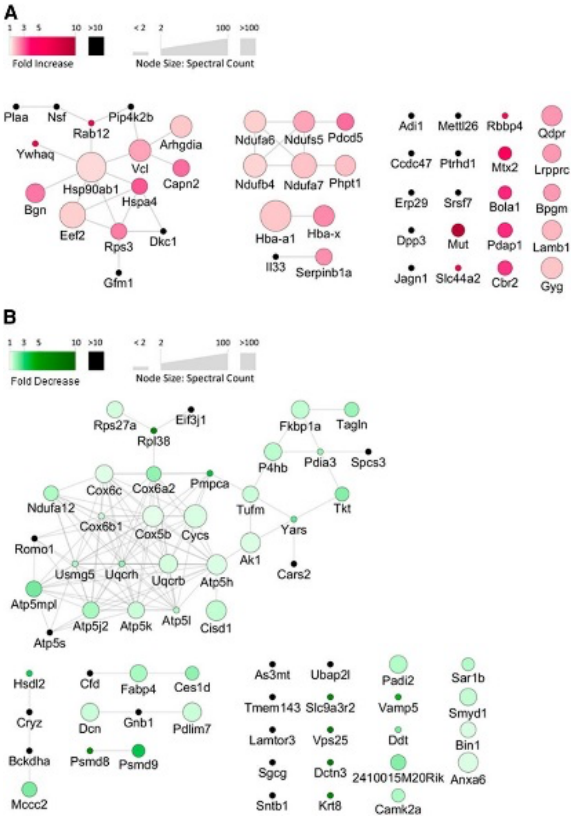
INTRODUCTION - Chronic thromboembolic pulmonary hypertension (CTEPH) is characterized by thromboembolic obstructions of the pulmonary circulation. Endothelial von Willebrand Factor (VWF) plays a key role in platelet recruitment during thrombosis at the vessel wall. Plasma VWF levels are elevated in patients with CTEPH. The underlying pathophysiological mechanism whether local endothelial VWF production and in situ thrombosis play a role in the development of CTEPH remain unknown.

AIM - We investigated how elevated endothelial VWF contributes to the development of CTEPH.

APPROACH - Pulmonary artery endothelial cells (PAEC) were isolated pulmonary endarterectomy material of CTEPH patients or control lung lobectomy tissue, and studied before and during perfusion of freshly isolated platelets of healthy subjects.

RESULTS - We demonstrated that histamine activated CTEPH-PAEC show increased platelet adhesion compared to control-PAEC. We found that this is caused by increased VWF secretion, that is predominantly regulated on gene-expression level. Mass spectometry analysis showed that ICAM-1 and NFκB2 were most significantly increased in the proteomes of CTEPH-PAEC compared to control. We demonstrated that epigenetic alterations in the VWF promotor in CTEPH-PAEC allows increased NFκB2 binding that stimulates VWF transcription and subsequently platelet adhesion.

CONCLUSIONS - We are the first study that show that epigenetic modifications of the VWF promotor in CTEPH endothelial cells, enhances NFκB2 binding and subsequently VWF transcription. This could be a possible mediator of in situ thrombosis in CTEPH.



Perivascular Adipose Tissue Controls Insulin-Stimulated Perfusion, Mitochondrial Protein Expression and Glucose Uptake in Muscle Through Adipomuscular Arterioles

Turaihi AH, Serné EH, Molthoff CF, Koning JJ, Knol J, Niessen HW, Jose Th Goumans M, van Poelgeest EM, Yudkin JS, Smulders YM, Jimenez CR, van Hinsbergh VW, Eringa EC

Diabetes 2020 Apr;69(4):603-613. doi: 10.2337/db18-1066. PubMed PMID: 32005705.

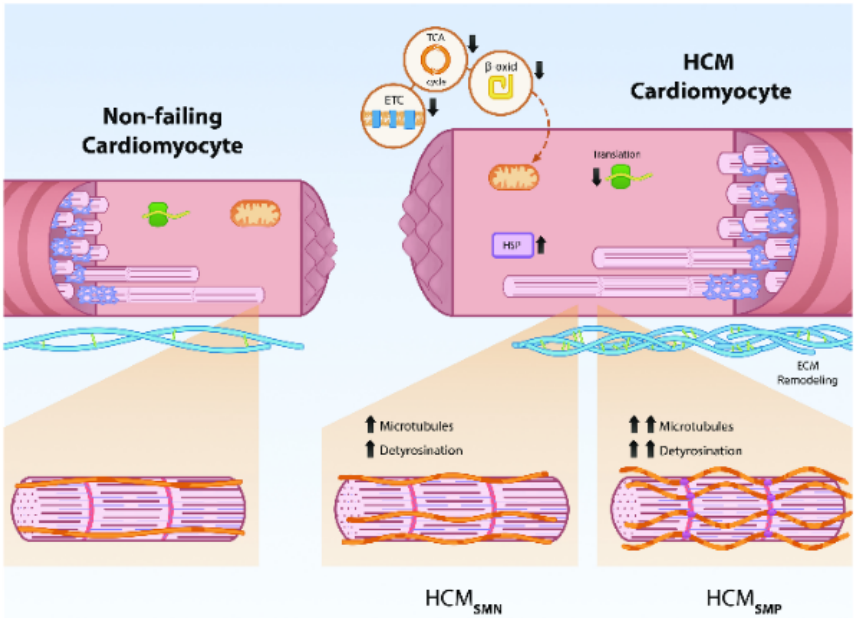
Insulin-mediated microvascular recruitment (IMVR) regulates delivery of insulin and glucose to insulin-sensitive tissues. We have previously proposed that perivascular adipose tissue (PVAT) controls vascular function through outside-to-inside communication and through vessel-to-vessel, or "vasocrine," signaling. However, direct experimental evidence supporting a role of local PVAT in regulating IMVR and insulin sensitivity in vivo is lacking. Here, we studied muscles with and without PVAT in mice using combined contrast-enhanced ultrasonography and intravital microscopy to measure IMVR and gracilis artery diameter at baseline and during the hyperinsulinemic-euglycemic clamp. We show, using microsurgical removal of PVAT from the muscle microcirculation, that local PVAT depots regulate insulin-stimulated muscle perfusion and glucose uptake in vivo. We discovered direct microvascular connections between PVAT and the distal muscle microcirculation, or adipomuscular arterioles, the removal of which abolished IMVR. Local removal of intramuscular PVAT altered protein clusters in the connected muscle, including upregulation of a cluster featuring Hsp90ab1 and Hsp70 and downregulation of a cluster of mitochondrial protein components of complexes III, IV, and V. These data highlight the importance of PVAT in vascular and metabolic physiology and are likely relevant for obesity and diabetes.

Proteomic and Functional Studies Reveal Detyrosinated Tubulin as Treatment Target in Sarcomere Mutation-Induced Hypertrophic Cardiomyopathy

Schuldt M, Pei J, Harakalova M, Dorsch LM, Schlossarek S, Mokry M, Knol JC, Pham TV, Schelfhorst T, Piersma SR, Dos Remedios C, Dalinghaus M, Michels M, Asselbergs FW, Moutin MJ, Carrier L, Jimenez CR, van der Velden J, Kuster DWD

Circ Heart Fail 2021. doi: 10.1161/CIRCHEARTFAILURE.120.007022. PubMed PMID: 33430602.

Background: Hypertrophic cardiomyopathy (HCM) is the most common genetic heart disease. While ≈50% of patients with HCM carry a sarcomere gene mutation (sarcomere mutation-positive, HCM_{SMP}), the genetic background is unknown in the other half of the patients (sarcomere mutation-negative, HCM_{SMN}). Genotype-specific differences have been reported in cardiac function. Moreover, HCM_{SMN} patients have later disease onset and a better prognosis than HCM_{SMP} patients. To define if genotype-specific derailments at the protein level may explain the heterogeneity in disease development, we performed a proteomic analysis in cardiac tissue from a clinically well-phenotyped HCM patient group.



Methods: A proteomics screen was performed in cardiac tissue from 39 HCM_{SMP} patients, 11 HCM_{SMN} patients, and 8 nonfailing controls. Patients with HCM had obstructive cardiomyopathy with left ventricular outflow tract obstruction and diastolic dysfunction. A novel *MYBPC3*$_{2373insG}$ mouse model was used to confirm functional relevance of our proteomic findings.

Results: In all HCM patient samples, we found lower levels of metabolic pathway proteins and higher levels of extracellular matrix proteins. Levels of total and detyrosinated α-tubulin were markedly higher in HCM_{SMP} than in HCM_{SMN} and controls. Higher tubulin detyrosination was also found in 2 unrelated *MYBPC3* mouse models and its inhibition with parthenolide normalized contraction and relaxation time of isolated cardiomyocytes.

Conclusions: Our findings indicate that microtubules and especially its detyrosination contribute to the pathomechanism of patients with HCM_{SMP}. This is of clinical importance since it represents a potential treatment target to improve cardiac function in patients with HCM_{SMP}, whereas a beneficial effect may be limited in patients with HCM_{SMN}.

REFERENCE - Schuldt et al. Proteomic and Functional Studies Reveal Detyrosinated Tubulin as Treatment Target in Sarcomere Mutation-Induced Hypertrophic Cardiomyopathy. Circ Heart Fail. 2021 Jan 12:CIRCHEARTFAILURE120007022. doi: 10.1161/CIRCHEARTFAILURE.120.007022. PMID: 33430602.

EDITORIAL COMMENTARY - Margulies KB, Prosser BL. Tubulin Detyrosination: An Emerging Therapeutic Target in Hypertrophic Cardiomyopathy. Circ Heart Fail. 2021 Jan 12:CIRCHEARTFAILURE120008006. doi: 10.1161/CIRCHEARTFAILURE.120.008006. PMID: 33430601.

REPORTS -

- Oral presentation: 4th Translational Cardiovascular Research meeting, Utrecht 2020 (online), “Detyrosinated Tubulin as treatment target in Hypertrophic Cardiomyopathy”
- Poster presentations: Advanced Proteomics summer school, Brixen 2017, “Differential protein expression in patients with Hypertrophic Cardiomyopathy”; ISHR world congress, Beijing 2019, “Proteomic profiling of a large cohort of HCM patients: genotype-specific protein changes”; Annual ACS symposium, Amsterdam, 2019, “Proteomic profiling of a large HCM patient cohort reveals detyrosinated tubulin as treatment target”.

Exploratory proteomics analysis and pro-fibrotic capacity of secretome derived from epicardial adipose tissue of patients with and without atrial fibrillation

Eva R. Meulendijks[1,2], Makiri Kawasaki[2], Nicoline W.E. van den Berg N[1,2], Jolien Neefs[1,2], Sarah W. Baalman[1,2], Robin Wesselink[1,3], Antoine H.G. Driessen[3], Tim R.A, Schelfhorst[4], Connie R. Jimenez[4], Joris R. de Groot[1,2]

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INTRODUCTION - Epicardial adipose tissue (EAT) is a metabolically active visceral adipose depot directly attached to the heart. EAT is known to secrete fibro-adipokines which is a subset of proteins with fibrotic, proinflammatory and anti-inflammatory capacities[1]. Since there is no anatomical barrier between the EAT and the myocardium, secreted adipokines potentially affect the function of adjacent cardiac cells in a paracrine manner. Interestingly, the secreted adipo-fibrokines from EAT promote fibrosis formation in the atria[2], which is an important substrate for atrial fibrillation (AF)[2]. Clinically, the patients with AF have a larger volume of EAT compared to the patients without AF[3]. These findings suggest that EAT activity and/or its volume around the heart play an important role in AF pathology. However, the pro-fibrotic components of EAT and its contribution to AF pathology remains largely unknown.

AIM - We aimed to identify the pro-fibrotic components of EAT secretome in the context of AF. We performed in-depth proteome analysis on the platform of high-resolution mass spectrometry, and compared the EAT secretome from patients with persistent AF (AF: n=3) to those without history of AF undergoing coronary artery bypass surgery (non-AF: n=3).

RESULTS - We found in total 312 proteins differentially expressed with significance (non-adjusted $p<0.05$) in the secretome from patients with AF compared to those without (fold-change>1.2). Gene ontology analyses based on these 312 proteins showed that the biological processes involved in coagulation were overrepresented in AF, and included fibrinogen subunits, kallikrein, and histidine rich glycoprotein (HRG). Coagulation factors are reported to have pro-fibrotic potentials. Extracellular matrix organization and biogenesis were also among the overrepresented processes in AF, and included type 15 collagen, metalloproteinase 2, and HRG. Myeloperoxidase, which has been associated with fibrosis in AF before, had the highest expression in AF (fold-change 18).

CONCLUSIONS - This small-scale study shows a substantial difference in the protein composition of EAT secretome between patients with and those without AF. AF secretome contains multiple proteins with pro-fibrotic potential. Our next research aim is to demonstrate the profibrotic potentials of those profibrotic components identified by proteomics analysis in human atrial fibroblasts.

REFERENCES

1. Hatem SN, Sanders P. Epicardial adipose tissue and atrial fibrillation. Cardiovasc Res 2014;102(2):205-13.
2. Venteclef N, Guglielmi V, Balse E et al. Human epicardial adipose tissue induces fibrosis of the atrial myocardium through the secretion of adipo-fibrokines. Eur Heart J 2015;36:795-805a.
3. Yorgun H, Canpolat U, Aytemir K et al. Association of epicardial and peri-atrial adiposity with the presence and severity of non-valvular atrial fibrillation. Int J Cardiovasc Imaging 2015;31(3):649-57.

Collaborative Other Research

Quantitative proteomic analysis reveals differential dendritic cell signaling after specific glycan binding to DC-SIGN

R.J. Eveline Li[1], J. Ernesto Rodriguez-Camejo[1], Hakan Kalay[1], Anouk Zaal[1], Sander R. Piersma[2], Thang V. Pham[2], Richard R. de Goeij- de Haas[2], Alex Henneman[2], Connie R. Jimenez[2], Sandra J.van Vliet[1], Yvette van Kooyk[1]*

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INTRODUCTION - Dendritic cells (DCs) are able to recognize a broad spectrum of pathogens through expression of Pattern Recognition Receptors (PRR), such as the C-type lectin receptors (CLRs). CLRs are involved in glycan

pattern recognition, endocytosis of bound antigens, and signaling that leads to tailored DC-mediated skewing of T cell responses. The immune modulatory properties of DC-SIGN is not completely understood, which is further more complicated by the fact that two different high affinity glycan ligands (high mannose (HM) and Lewis^{Y} (LeY)), trigger distinct cytokine pathways. Moreover many pathogens express a mixture of glycans, limiting the clarification of glycan specific induced signaling.

AIM – To elucidate the early events of signaling in monocyte-derived LPS-stimulated DCs through phosphoproteomic quantification.

APPROACH – We here conjugated HM and LeY to dendrimers and allowing triggering of the different immune modulatory properties of DC CLR DC-SIGN.

RESULTS – LeY dendrimer stimulated moDCs increased the IL-10:IL-12 secretion profile after overnight stimulation, while HM dendrimers did not. Phosphoproteomic analysis was associated with a significant downregulation in phosphoproteins that were shared between the HM and LeY dendrimer conditions after 30 minutes of moDC stimulation. The significantly altered proteins were annotated to multiple biological processes, including the IL-12 signaling pathway and phagosomal routing. The high mannose and LeY dendrimers were both able to affect cell activation in the immune response, however through differentially altered proteins within the signaling pathways. Binding of the HM dendrimer to DC-SIGN specifically affected proteins involved in the endolysosomal trafficking. Both RAB7A and DYNC1H1, that are known to exist in a complex, were significantly dephosphorylated after HM stimulation with LPS. Furthermore, HM dendrimer binding dephosphorylated LYN kinase, which promotes DC maturation and cytokine release. No correlation was found between the proteins within the same biological processes that were affected by LeY binding.

CONCLUSION – The early biological pathways affected here by DC-SIGN triggering by two of its glycan ligands HM and LeY are promising leads for further investigation. The ligand-specific effects of DC-SIGN on antigen routing, processing, maturation and cytokine secretion implies a prominent role in DC reprogramming to elicit specific T cell response upon glycan pattern recognition.

Quantitative phosphoproteomic analysis reveals dendritic cell-specific STAT signaling after α2-3-linked sialic acid ligand binding

R.J. Eveline Li[1], Aram de Haas[1], J. Ernesto Rodriguez-Camejo[1], Hakan Kalay[1], Anouk Zaal[1], Connie R. Jimenez[2], Sander R. Piersma[2], Thang V. Pham[2], Alex Henneman[2], Richard R. de Goeij- de Haas[2], Sandra J.van Vliet[1], Yvette van Kooyk[1]*

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INTRODUCTION – Dendritic cells (DCs) are key initiators of the adaptive immunity, and are able to skew T cell differentiation by cytokines to elicit appropriate responses. DCs possess the extraordinary capacity to discern external signals from self through recognition of pathogen-associated molecular patterns, such as glycan signatures.

AIM - This study explores the early signaling events in DC upon binding of α2-3 sialic acid (α2-3sia) via Immune inhibitory Siglec receptors.

APPROACH – We conjugated α2-3sia to a dendrimeric core, as α2-3sias are commonly found on bacteria, e.g. Group B *Streptococcus*, or tumor cells. Through phosphoproteomic analysis, we found differential signaling profiles in DCs after α2-3sia binding alone or in combination with LPS co-stimulation. α2-3sia was able to modulate the TLR-4 signaling cascade, resulting in 109 altered phosphoproteins. The altered phosphoproteins were annotated to seven biological processes, including the regulation of IL-12. Indeed, secretion of IL-12 by DCs was also downregulated after overnight stimulation with the α2-3sia dendrimer. Analysis of kinase activity revealed altered signatures in the MAPK/ERK and the JAK-STAT signaling pathways. PhosphoSTAT3 (Ser727) and phosphoSTAT5A (Ser780) involved in the IL-12 regulation pathway were downregulated. Flow cytometric quantification indeed revealed a decrease in phosphorylation over time upon stimulation with α2-3sia. De-phosphorylation of the STATs was furthermore specific to α2-3sia stimulation and could not be triggered through α2-6sia binding.

CONCLUSION - This study revealed a specific alteration of the JAK-STAT pathway in DCs upon α2-3sia and LPS stimulation, resulting in increased IL10:IL-12 cytokine secretion. Targeted control of the STAT phosphorylation status is therefore an interesting lead for the abrogation of immune escape that bacteria or tumors impose on the host.

Protein identification in the varnish of seventeenth-century Rembrandt paintings

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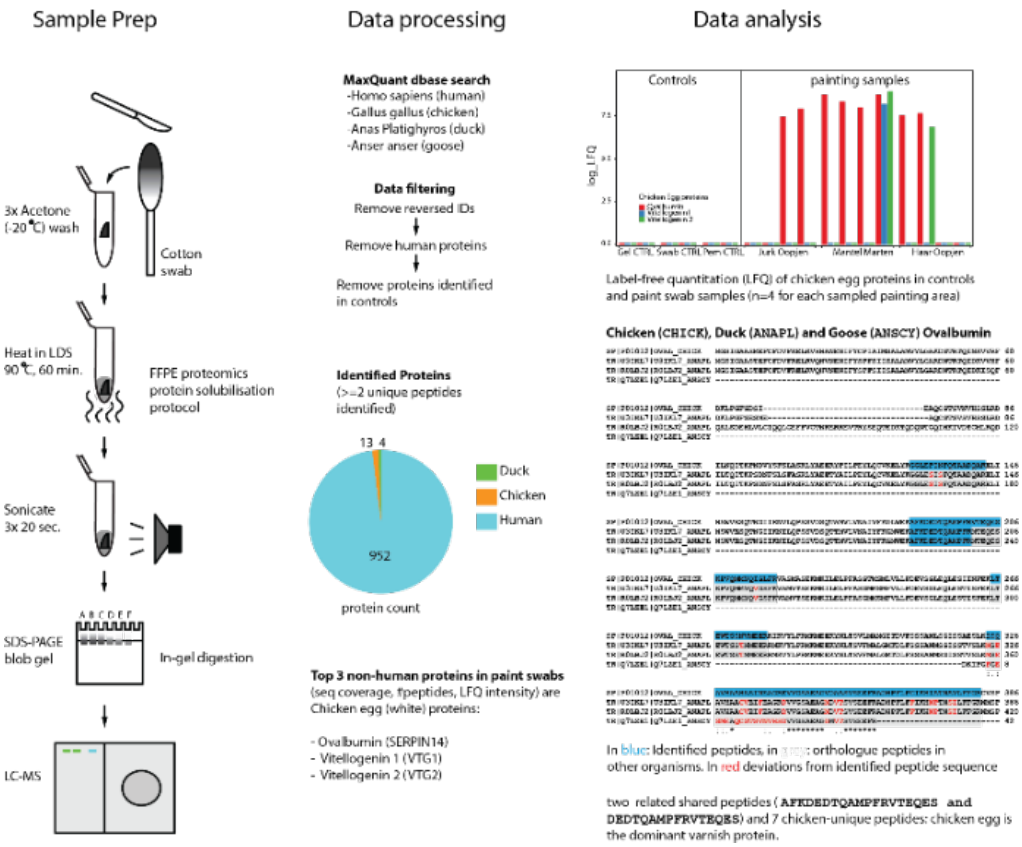
INTRODUCTION - In 2016, VUmc and Rijksmuseum teamed up to explore application of state-of-the-art biomedical analysis techniques to cultural heritage research in a joint collaboration. In 1634, Rembrandt van Rijn painted the portaits of Marten Soolmans en Oopjen Coppit. In 2016, the paintings were purchased together by the Dutch state and the republic of France. The paintings are now being conserved and analyzed in the conservation studio of the Rijksmuseum. After conservation both paintings will be exhibited alternatingly in Amsterdam and in Paris. During conservation and detailed analysis, issues were found in the 19th century varnish layer of both paintings. One of the questions was if there was a protein ingredient present in the varnish and if yes, which protein(s) and from which origin. The Marten and Oopjen varnish analysis using LC-MS based proteomics is one of the collaborative VUmc-Rijksmuseum projects.

AIM - Identification and analysis of protein in the varnish layer of Rembrandt's paintings of Marten and Oopjen.

APPROACH - Since the aged paint and varnish layer constitute a highly insoluble matrix we applied protein extraction protocols that are used in proteomics analysis of formalin-fixed paraffin embedded (FFPE) tissue material. During the conservation process of the paintings, surface samples were taken using cotton swabs. These cotton swabs were collected and washed in cold acetone to remove hydrophobic compounds and to precipitate protein. Subsequently, swabs were heated in lithium dodecyl sulfate detergent solution for 1 hour at 90°C. After cooling-down the swabs were sonicated in an ultrasonic cup for three cycles and resulting protein extract was applied to SDS-PAGE and in-gel digestion. Extracted peptides were analyzed by LC-MS on a Q Exactive HF mass spectrometer.



RESULTS - LC-MS raw files were searched against FASTA sequences of human, chicken, duck and goose, the latter three species representing the most likely origin of egg protein commonly used in 19th century varnishes. Mainly human contaminant proteins were identified, consistent with the conservation process and the history of the paintings. The most dominant non-human proteins were egg-white proteins ovalbumin and vitellogenin 1 and 2. Peptide sequences identified were consistent with the chicken protein sequences and a minority was shared between chicken and duck. None of the egg proteins were found in the control samples (cotton swabs, permulen cleaning gel, saliva and SDS PAGE gel).

CONCLUSIONS - Taken together, we conclude that chicken egg-white was used in the varnish layer of Rembrandt's paintings of Marten and Oopjen. State-of-the-art proteomics methods can successfully be applied to protein-related questions in cultural heritage research projects.

Changes in the urinary extracellular vesicle proteome are associated with nephronophthisis-related ciliopathies

Stokman MF, Bijnsdorp IV, Schelfhorst T, Pham TV, Piersma SR, Knol JC, Giles RH, Bongers EMHF, Knoers NVAM, Lilien MR, Jiménez CR, Renkema KY

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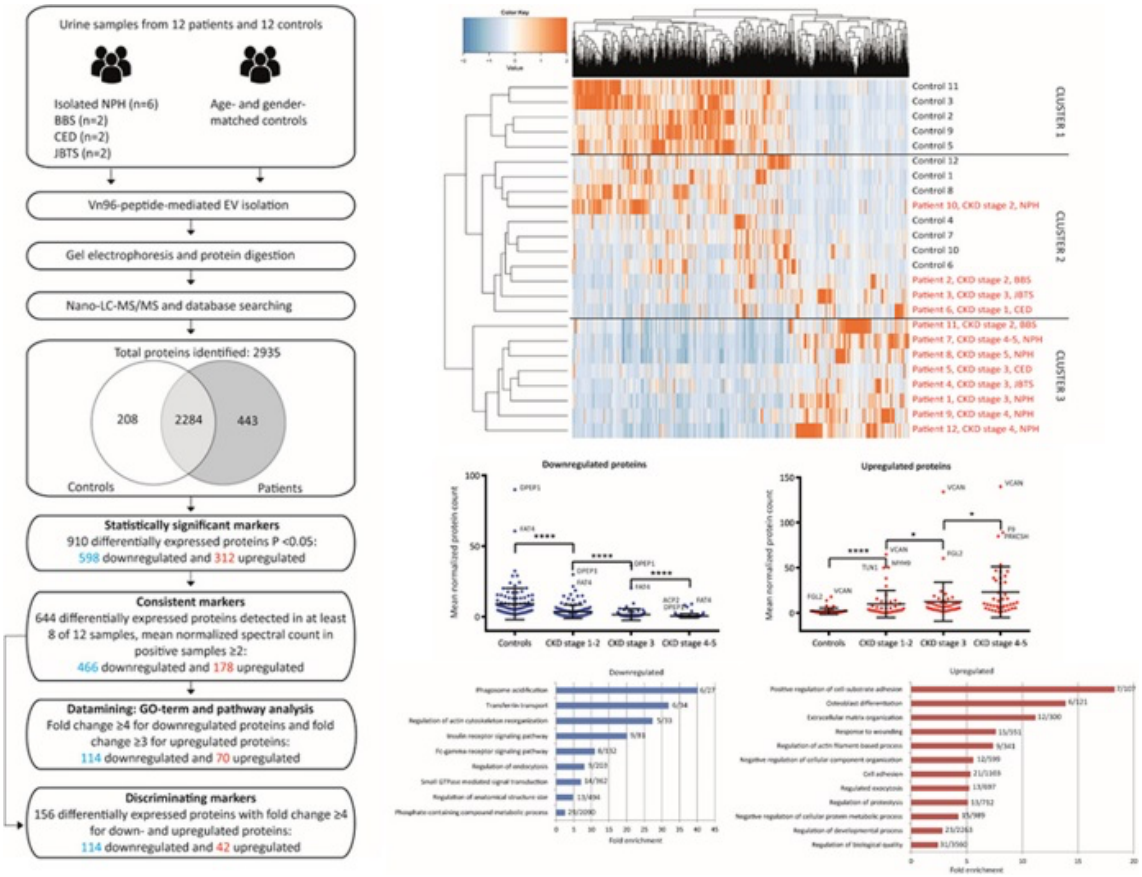

Nephronophthisis is one of the leading genetic causes of end-stage renal disease in childhood. Early diagnostics and prognostics for nephronophthisis are currently limited. We aimed to identify non-invasive protein biomarkers for nephronophthisis in urinary extracellular vesicles. Extracellular vesicles were isolated from urine of 12 patients with a nephronophthisis-related ciliopathy and 12 age- and gender-matched controls, followed by in-depth label-free LC-MS/MS proteomics analysis of gel fractionated extracellular vesicle proteins. Supervised cluster analysis of proteomic profiles separated patients from controls. We identified 156 differentially expressed proteins with fold change ≥4 in patients compared to controls ($P < .05$). Importantly, expression levels of discriminating proteins were correlated with chronic kidney disease stage, suggesting possible applications for urinary extracellular vesicle biomarkers in prognostics for nephronophthisis. Enrichment analysis of gene ontology terms revealed GO terms including signaling, actin cytoskeleton and endocytosis among the downregulated proteins in patients, whereas terms related to response to wounding and extracellular matrix organization were enriched among upregulated proteins. Our findings represent the first step towards a non-invasive diagnostic test for nephronophthisis. Further research is needed to determine specificity of the candidate biomarkers. In conclusion, proteomic profiles of urinary extracellular vesicles differentiate nephronophthisis-related ciliopathy patients from healthy controls.

SIGNIFICANCE: Nephronophthisis is an important cause of end-stage renal disease in children and is associated with an average diagnostic delay of 3.5 years. This is the first study investigating candidate biomarkers for nephronophthisis using global proteomics analysis of urinary extracellular vesicles in patients with

nephronophthisis compared to control individuals. We show that measuring protein markers in urinary extracellular vesicles is a promising approach for non-invasive early diagnostics of nephronophthisis.

Exploratory proteomics analysis and pro-fibrotic capacity of secretome derived from epicardial adipose tissue of patients with and without atrial fibrillation

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INTRODUCTION - Epicardial adipose tissue (EAT) is a metabolically active visceral adipose depot directly attached to the heart. EAT is known to secrete fibro-adipokines which is a subset of proteins with fibrotic, proinflammatory and anti-inflammatory capacities[1]. Since there is no anatomical barrier between the EAT and the myocardium, secreted adipokines potentially affect the function of adjacent cardiac cells in a paracrine manner. Interestingly, the secreted adipo-fibrokines from EAT promote fibrosis formation in the atria[2], which is an important substrate for atrial fibrillation (AF)[2]. Clinically, the patients with AF have a larger volume of EAT compared to the patients without AF[3]. These findings suggest that EAT activity and/or its volume around the heart play an important role in AF pathology. However, the pro-fibrotic components of EAT and its contribution to AF pathology remains largely unknown.

AIM - We aimed to identify the pro-fibrotic components of EAT secretome in the context of AF. We performed in-depth proteome analysis on the platform of high-resolution mass spectrometry, and compared the EAT secretome from patients with persistent AF (AF: n=3) to those without history of AF undergoing coronary artery bypass surgery (non-AF: n=3).

RESULTS - We found in total 312 proteins differentially expressed with significance (non-adjusted $p<0.05$) in the secretome from patients with AF compared to those without (fold-change>1.2). Gene ontology analyses based on these 312 proteins showed that the biological processes involved in coagulation were overrepresented in AF, and included fibrinogen subunits, kallikrein, and histidine rich glycoprotein (HRG). Coagulation factors are reported to have pro-fibrotic potentials. Extracellular matrix organization and biogenesis were also among the overrepresented processes in AF, and included type 15 collagen, metalloproteinase 2, and HRG. Myeloperoxidase, which has been associated with fibrosis in AF before, had the highest expression in AF (fold-change 18).

CONCLUSIONS - This small-scale study shows a substantial difference in the protein composition of EAT secretome between patients with and those without AF. AF secretome contains multiple proteins with pro-fibrotic potential. Our next research aim is to demonstrate the profibrotic potentials of those profibrotic components identified by proteomics analysis in human atrial fibroblasts.

REFERENCES

1. Hatem SN, Sanders P. Epicardial adipose tissue and atrial fibrillation. Cardiovasc Res 2014;102(2):205-13.
2. Venteclef N, Guglielmi V, Balse E et al. Human epicardial adipose tissue induces fibrosis of the atrial myocardium through the secretion of adipo-fibrokines. Eur Heart J 2015;36:795-805a.
3. Yorgun H, Canpolat U, Aytemir K et al. Association of epicardial and peri-atrial adiposity with the presence and severity of non-valvular atrial fibrillation. Int J Cardiovasc Imaging 2015;31(3):649-57.

Specific T Cell Responses against Minor Histocompatibility Antigens Cannot Generally Be Explained by Absence of Their Allelic Counterparts on the Cell Surface

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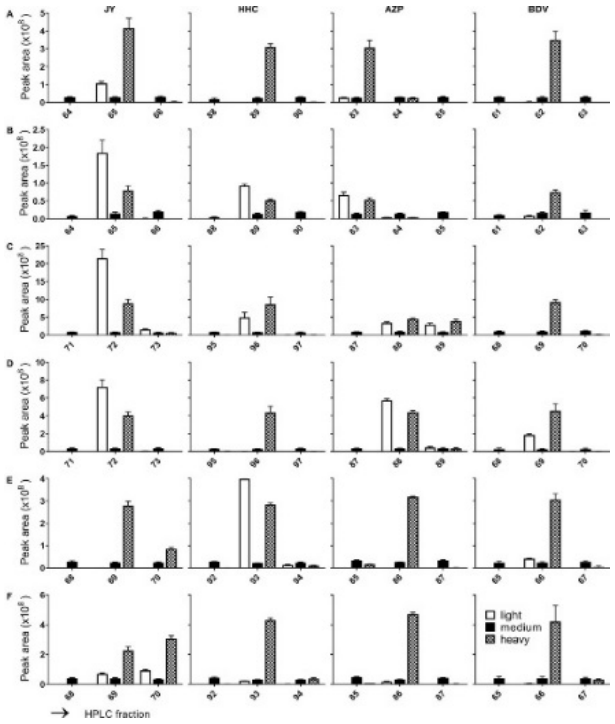

Allogeneic stem cell transplantation has emerged as immunotherapy in the treatment of a variety of hematological malignancies. Its efficacy depends on induction of graft versus leukemia by donor lymphocytes. Both graft versus leukemia and graft versus host disease are induced by T cells reactive against polymorphic peptides, called minor histocompatibility antigens (MiHA), which differ between patient and donor and are presented in the context of self-HLA (where HLA is human leukocyte antigen). The allelic counterpart (AC) of the MiHA is generally considered to be absent at the cell surface, based on the absence of immune responses directed against the AC. To study this in detail, we evaluate the recognition, HLA-binding affinity, and cell surface expression of three selected MiHA. By quantitative MS, we demonstrate the similarly abundant expression of both MiHA and AC at the cell surface. We conclude that the absent recognition of the AC cannot generally be explained by insufficient processing and presentation at the cell surface of the AC.

Alternative splice variant of the thiazide-sensitive NaCl cotransporter: a novel player in renal salt handling

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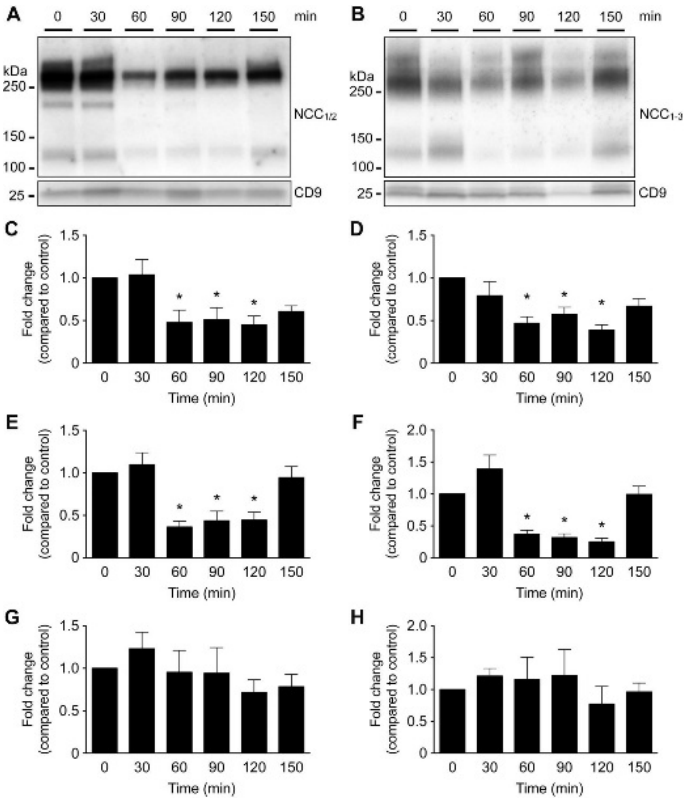

The thiazide-sensitive NaCl cotransporter (NCC) is an important pharmacological target in the treatment of hypertension. The human SLC12A3 gene, encoding NCC, gives rise to three isoforms. Only the third isoform has been extensively investigated. The aim of the present study was, therefore, to establish the abundance and localization of the almost identical isoforms 1 and 2 (NCC1/2) in the human kidney and to determine their functional properties and regulation in physiological conditions. Immunohistochemical analysis of NCC1/2 in the human kidney revealed that NCC1/2 localizes to the apical plasma membrane of the distal convoluted tubule. Importantly, NCC1/2 mRNA constitutes ~ 44% of all NCC isoforms in the human kidney. Functional analysis performed in the Xenopus laevis oocyte revealed that thiazide-sensitive (22)Na(+) transport of NCC1 was significantly increased compared with NCC3. Mimicking a constitutively active phosphorylation site at residue 811 (S811D) in NCC1 further augmented Na(+) transport, while a

nonphosphorylatable variant (S811A) of NCC1 prevented this enhanced response. Analysis of human urinary exosomes demonstrated that water loading in human subjects significantly reduces the abundance of NCC1/2 in urinary exosomes. The present study highlights that previously underrepresented NCC1/2 is a fully functional thiazide-sensitive NaCl-transporting protein. Being significantly expressed in the kidney, it may constitute a unique route of renal NaCl reabsorption and could, therefore, play an important role in blood pressure regulation.