OncoProteomics Laboratory

Progress Report

2015-2017

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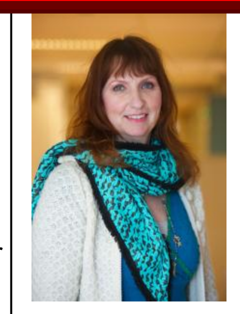


Foreword

This progress report 2015-2017 covers cancer proteomics research of the OncoProteomics Laboratory (OPL) and collaborators, as well as proteomics activities performed in the context of our function as Enabling Technology Center / 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 1**).

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

- Last year was special, since we obtained an instrumentation grant from NWO and generous funding from CCA (Amsterdam Marathon) that allowed us to expand the mass spectrometry infrastructure. The new platform is very fast (20 MS/MS /sec) and enables a new way of proteome data acquisition that will be important for large-scale clinical sample profiling.
- 2016 was also a special year, because I became full Professor of Translational OncoProteomics! The day of the inaugural lecture Dec.14, was a very special day and included a great symposium with keynote speakers Prof. Ruedi Aebersold and Prof. Bing Zhang.
- In total 29 peer-reviewed research papers were published, including papers in Annals of Internal Medicine (results from our longest running project on stool biomarkers for improved colorectal cancer screening), PNAS, Nature Microbiology, Molecular and Cellular Proteomics, Bioinformatics and the Journal of Extracellular Vesicles.
- In total 5 grants were obtained as PI or coPI: 1. NWO (see above); 2. KWF (on pancreatic cancer with Elisa Giovanetti and Maarten Bijlsma); 3. KWF/Alped'Hu6 (on prostate cancer, Immprove consortium with PI Guido Jenster, EMC, and Irene Bijnsdorp); 4. KWF (on t-ALL, with PI Jules Meijerink, Prinses Maxima) 5. CCA (to develop a kinome acitivity assay).
- The number of phosphoproteomics projects has grown a lot and they have also gone large-scale with profiling of > 100 samples per project now becoming routine. Importantly the feasibility of needle biopsy phosphoproteomics in the clinical trial setting was shown. Read more in appendix 1.
- An integrative analysis pipeline to infer kinase activities from 4 types of phosphoproteomics data has been developed to predict oncogene addiction as well as drug resistance and response. The resulting manuscript is a great OPL co-production and has been submitted for publication.
- The ability to interrogate cancer at the proteome level and integrate acquired knowledge with genome data is called proteogenomics. With support of KWF, a data analysis pipeline was developed to pinpoint cancer specific alternative protein splicing and it has been applied to study colorectal adenoma to carcinoma progression in a large series of samples
- In the context of the KWF Alpes d'HuZ6 Connection consortium, we have identified and validated protein biomarkers for CRC subtypes. A protein signature is being developed for application in a clinical trial that aims to link CRC subtypes to response to neoadjvant chemotherapy.
- The KWF projects on cisplatin response prediction in NSCLC and on homologous recombination deficiency in triple negative breast cancer are running smoothly, with lots of xenograft and clinical tumor samples collected and profiled.
- This year we attended the international meeting of the Human Proteome Organisation (HUPO) in Dublin with almost the whole team and we had a record-braking number of 6 abstracts as orals, next to 12 posters (See in appendix 1).
- At the HUPO2017 Dublin meeting, as co-chair of the Cancer-Human Proteome Project, I announced TCPA (a la TCGA) to create a cancer proteomics landscape covering a broad range of tumor types (~20) and make the data available via an interactive, queriable portal. OPL is kick-starting this international community effort with the generation of ~400 cancer proteomes representing ~15 tumor types in the coming year(s).
- Last year was our 10-year anniversary. For this purpose, we quantified our mass spec runs and output in relation to our users. I also collected collaborator support quotes. The overall results were very positive. If you want to learn more, see **appendix 2 and 3** on pages 93 and 95.
- Last but not least, OPL will join the International Proteogenomics Cancer Consortium, a spin-off of the Cancer Moonshot initiative, which operates under the umbrella of the NCI Clinical Proteomics Office.

Finally, I hope you will enjoy reading on our OncoProteomics endaveour and that this report triggers new ideas and collaborative projects.

Connie Jimenez
Head of the OPL, Professor of Translational OncoProteomics, Dept. Medical Oncology, VUmc

Members of the Research Group:

OncoProteomics Laboratory, Sept.2017 (missing Tessa, Gosia, Ayse, Giulia, Meike, Mariette, Henk who were on the next flight)

Head: Prof. dr. Connie R Jimenez

Mass Spectrometry:
- Dr. Sander Piersma (OPL-core: nanoLC-MS/MS, DDA)
- Dr. Davide Chiasserini (OPL core: nanoLC-MS/MS, DIA)

Dry lab support:
- Dr. Thang V. Pham (OPL core: (bio)informatics)
- Dr. Alex Henneman (OPL core: (bio)informatics, ICT)
- Dr. Jaco C. Knol (OPL core: down-stream data mining, back-up nanoLC-MS/MS)

Wet lab research technicians
- Ing. Richard Goeij-de Haas (OPL core: phosphoproteomics)
- Ing. Tim Schelfhorst (OPL core: expression and exosome proteomics)

Post-doctoral fellows:
- Dr. Franziska Böttger (NSCLC predictive biomarkers)
- Dr. Frank Rolfs (HR deficiency predictive biomarkers)
- Dr. Sanne Martens de Kemp (CRC oncogenesis kinase drivers)
- Dr. Meike de Wit (CRC screening biomarkers)

PhD students:
- Carolien van Alphen (AML phosphoproteomics)
- Tessa Le Large (Pancreatic cancer phosphoproteomics)
- Robin Beekhof (CRC phosphoproteomics)
- Malgorzata Komor (CRC oncogenesis splice variants)
- 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, Prof. Henk M Verheul MD, PhD

Visiting scientists
- Beatriz Escudero Paniagua (CRC secretome proteomics, 2016)
- Dr. Shuang Wu (CRC secretome proteomics, 2017)
- Paula Gonzalez Alonso (Breast cancer phosphoproteomics 2017)
- Laura Lorenzo Sanz (Phosphoproteomics of melanoma progression model, 2017)
- Dr. Mitra Rezaee (gastric cancer, 2017)

Internship students 2015-2017: 8 students of the VU masters Biomedical Sci, Bioinformatics & HLO

Introduction

Most cellular functions are executed by proteins (Fig. 1). Therefore, large-scale protein analysis through proteomics can provide an unprecedented and comprehensive source of information that uncovers the state and activity of cancer cells and, on a higher level, diverse aspects of tumor biology. Importantly, proteomic information may be mined to initiate development of new diagnostic applications and/or therapeutic treatments. Mass spectrometry (MS) is the core technology in any modern proteomics toolbox. Both instruments and bioinformatics strategies have undergone huge developments in the past decade, now turning proteomics into a mature technology.

In recent years, the Clinical Proteomic Tumor Analysis Consortium of the National Cancer Institute applied has performed large scale tumor proteome profiling for 3 selected tumor types to complement the TCGA genome data. These comprehensive MS-based proteome analyses included ~100-150 genomically (TCGA) characterized cancers per tumor type and the results were published in Nature (2014, CRC; 2016, breast cancer) and Cell (2016, ovarian cancer). In these studies, the biochemical impact of cancer-related genomic abnormalities was determined, including expression of variant proteins encoded by mutated genes, changed protein levels driven by altered DNA copy number, chromosomal amplification and deletion events, epigenetic regulation, and changes in microRNA expression. Furthermore, analysis of post-translational protein modifications, in particular reversible protein phosphorylation, enabled the detection of signaling network adaptations driven by genomic as well as micro-environmental changes. Importantly, cancer proteomics could also uncover protein aberrations not readily detected by analysis of DNA/RNA.

A recent comparison of RNA co-expression versus protein co-expression analysis of the TCGA colorectal, breast, and ovarian cancer proteome datasets to infer co-functionality of gene products, demonstrated that proteome profiling outperforms transcriptome profiling for co-expression based gene function prediction (Wang, Zhang et al., Mol. Cell. Proteomics 2017). The authors concluded that proteomics should be integrated, if not preferred, in gene function and human disease studies.

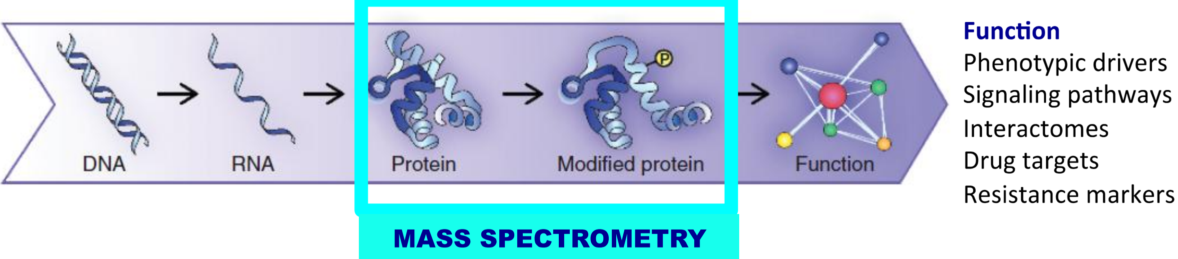


Figure 1. Genes (DNA) and transcripts (RNA) execute most of their functions through the proteins they encode and their modifications (*eg.*, phosphorylation) (modified after Ellis et al., Cancer Discovery 2013).

Translational OncoProteomics Research of the OncoProteomics Laboratorium

Comprehensive proteome analysis by MS 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. The goal of OPL's proteomics research is to identify tumor-associated changes in the proteome using a mass spectrometry-based strategies, and to translate information gleaned from these studies – preferably insights relating to tumor biology - into protein-based clinical tests and new treatment options. To this end, the activities of OPL are focused on **1**) identifying protein markers for non-invasive (early) detection of cancer and for monitoring disease; **2**) identifying new predictive markers/targets for targeted therapy of cancer and for tailored therapy for individual patients; and **3**) developing/implementing innovative proteomics and data analysis strategies to enable the above.

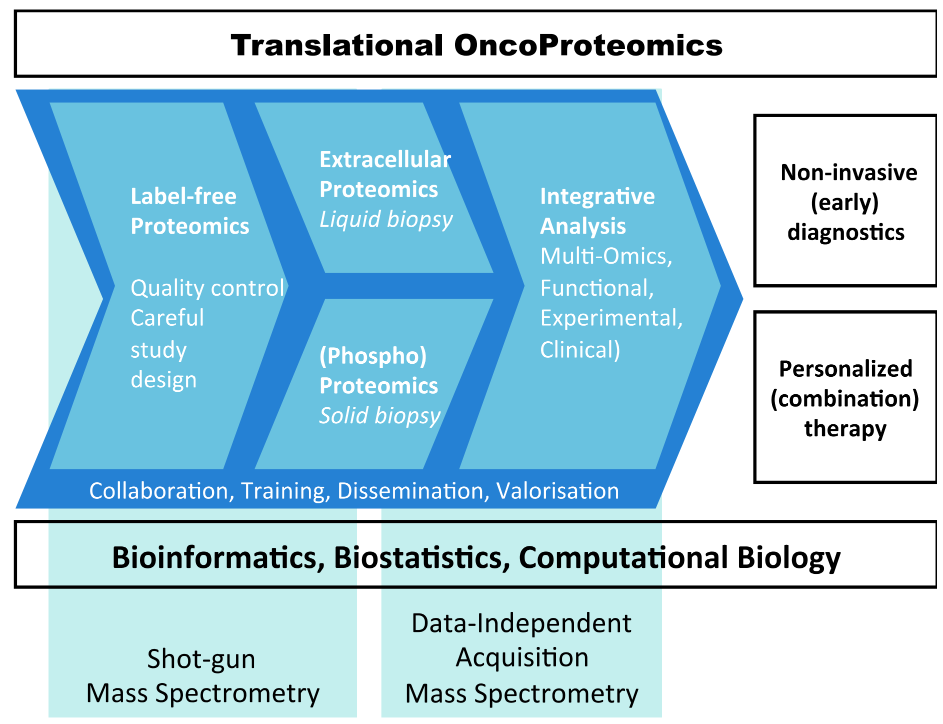


Figure 2. Schematic representation of translational OncoProteomics research at OPL.

The OPL performs its cancer research in diverse collaborations, both within Cancer Center Amsterdam (CCA) and outside the research institute (see Figure 3). OPL's infrastructure and (label-free) research strategies are state-of-the-art. The laboratory's embedding in the VUmc Department of Medical Oncology and the CCA institute (with exquisitely short lines to clinical samples and expertise), provides a unique setting, enabling a research path for identification and validation of biomarkers and therapeutic targets. This innovative, translational oncoproteomics approach has received national and international recognition as evidenced by awarded funding, the number of scientific publications (> 100 since foundation of OPL in 2006), and a large number of invitated lectures at scientific meetings with a focus on proteomics and/or cancer research.

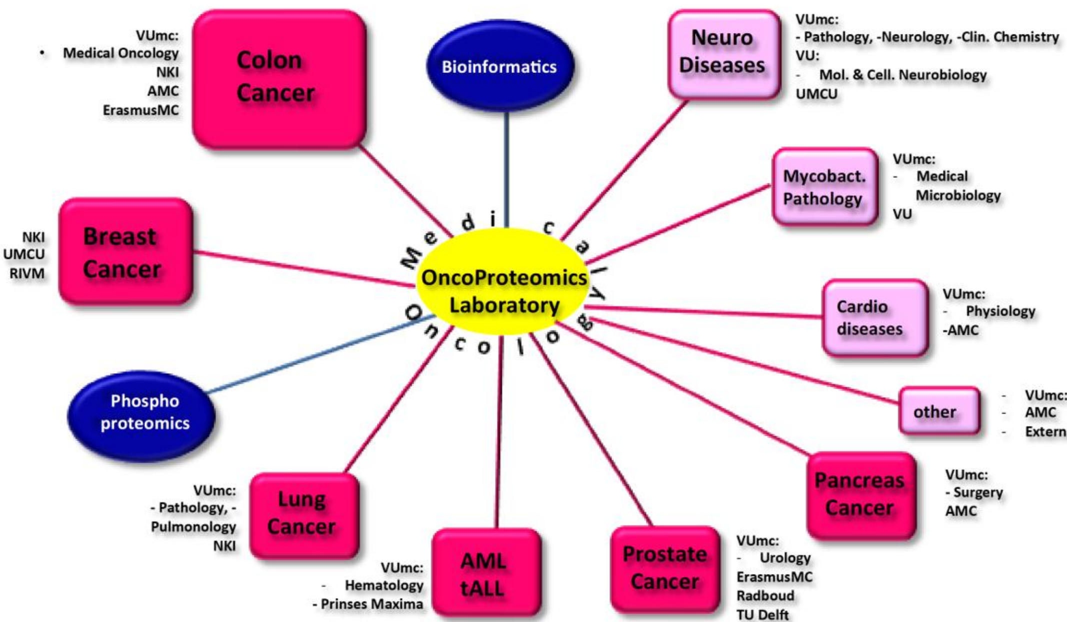


Figure 3. Diagram of OPL research and collaborations in 2017. Indicated are the disease areas and most important collaboration partners within VUmc and with other academical centers (NKI, AMC, UMCU, ErasmusMC, RadboudMC, RIVM, Hubrecht Institute, Prinses Maxima Center).

The OncoProteomics Laboratory as Enabling Technology Center

OPL functions since 2006 as a CCA enabling technology center and core facility for (cancer) proteomics, usually within the framework of collaborative projects (see also www.oncoproteomics.nl). In addition, OPL also supports other disease proteomics, ie., neuroproteomics (focus on CSF) with researchers of the VUmc, AMC and EMC, as well as cardio proteomics with VUmc and AMC and microbial proteomics with the VUmc and VU. Altogether, OPL currently performs MS-based proteomics in >50 core and collaborative projects (research hotel function) with 16 different local departments, 9 national institutes, and 9 international institutes, which has resulted in over 100 peer-reviewed publications to date. See appendix 2 for OPL facts. In the past 11 years, the OPL has gained a national and international reputation in the area of clinical proteomics and cancer proteomics.

OPL proteomics infrastructure consists of nano-liquid chromatography systems on-line coupled to tandem mass spectrometry (MS/MS) platforms that are maintained by OPL scientists who supervise study design and wet-lab sample preparation, generate and process MS/MS data, and perform statistical/ bioinformatics analyses. Current infrastructure anno 2017 includes two first generation QExactive platforms (installed in 2011 with various private funding sources, CCA instrumentation funding and (grant) money from the Medical Oncology department) and one QExactive HF (installed in 2016 with funding from NWO-Middelgroot and CCA) (Fig. 4).

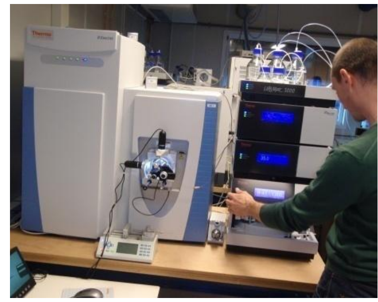
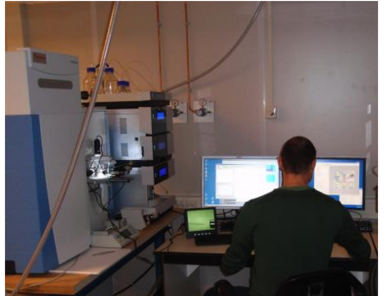



Figure 4. OPL infrastructure consists of 3 nanoLC-MS/MS set-ups (two first generation QExactives and one QExactive HF platform).

The OPL core team consists of highly qualified personel, anno 2017:
Wet lab. One PhD level research technician specialized in phosphoproteomics (Richard De Haas) and one research technician (Ing. Tim Schelfhorst) specialized in expression workflows and exosome isolation.
Mass spectrometry. Two PhD level research associates: one highly experienced mass spectrometrist (Dr. Sander Piersma), one biologist and additional trained operator (Dr. Davide Chiasserini). Dr. Jaco Knol is back-up operator.
Data analysis (dry lab). Three PhD level research associates: one computer scientist (Dr. Thang Pham), one theoretical physicist /informatician (Dr. Alex Henneman) and one molecular biologist (Dr. Jaco Knol) specialized in bioinformatics for biological interpretation.
All these people are relevant for the OPL core process and depend on 4e geldstroom of the Dept. of Medical Oncology (Piersma) and 3e geldstroom of the OPL (Pham, Knol, Schelfhorst, Henneman). Only De Haas is 1e geldstroom (as is head OPL). Of course this is a vulnerable situation.

As core facility, OPL operates as a research hotel, with OPL providing expertise, individual training sessions, (help with) dedicated sample preparation protocols, expert-operated nanoLC-MS/MS infrastructure, data analysis support. Relevant information, workflow and data analysis expertise is available via website- and intranet-shared protocols (see also www.oncoproteomics.nl). Collaborators are invited to the weekly OPL meetings to stimulate exchange of experiences and to increase the overall quality of the proteomics experiments. Proteome analyses are offered to users at material and running cost price.
The OPL core group has been able to (just about) handle the ever-increasing work-load due to (more) efficient organization and streamlining of processes as well as temporary employment of extra personnel. However, since data analysis is steadily becoming a larger and more and more complex and specialized component of the whole research effort, more steady funding for data science personel is essential but wanting.

Expertise of the OncoProteomics Laboratory

In the past decade, the field of proteomics has matured due to rapid developments in MS technology and bioinformatic tools. Both discovery ("shot-gun")- and, more recently, targeted MS-based proteomics approaches are revolutionizing comprehensive protein analysis in biological and clinical samples, which are all operational at OPL. Moreover, the OPL has expertise in label-free proteomics and phosphoproteomics in a translational setting, for which we have obtained an (inter)national reputation and a leading role in the national Center for Personalized Cancer Treatment. The recent development of proteogenomic approaches and clinical application of emerging high-throughput, targeted (data-independent acquisition) approaches on the newly acquired high-end MS platform are strengthening the leading position of the OPL and CCA in translational cancer proteomics.

In-depth proteomics pipelines for biomarker/ target discovery

To enable large-scale protein identification and quantification, discovery proteomics by shot-gun mass spectrometry is performed on a fast-scanning high-resolution (tandem) instrument. Such platforms can not only be used to measure protein levels, but also to obtain information about primary protein structure (splice variants, isoforms, mutant variants) and post-translational modifications (such as phosphorylation) that govern the activity and fate of key cellular proteins. This type of information is crucial to gain molecular insight into the different states of cancer cells and their activaties, and to identify drug targets and biomarkers for clinical needs. To assist analysis of differences in protein abundance (as determined through label-free quantification based on spectral count data), the OPL has devised a dedicated statistical test.

Protein expression profiling

Discovery proteomics for protein expression profiling typically makes use of our optimized "GeLC"-MS/MS workflow (Fig. 5). This workflow allows for routine fractionation at the protein and peptide level, to enable in-depth analysis of complex biological and clinical samples at medium throughput (~8 hours of instrument time per sample). In case sample complexity is low or intermediate depth of analysis is sufficient, fractionation may be omitted and only short-stack electrophoresis is performed preparing a "blob" gel band for single shot nanoLC-MS/MS analysis (2-4 hours of instrument time per sample). To this end, after instruction, collaborators/users fractionate their own samples by gel electrophoresis and perform in-gel tryptic digestion in a keratin-free laminar flow set-up at the OPL. Subsequent nanoLC-MS/MS analysis of extracted peptides is performed full-service with high resolution and scanning speed on an Orbitrap type of mass spectrometer (e.g., QExactive series ThermoFisher), which is a must for in-depth proteomics of complex biological samples. Data supplemented with dedicated statistics and bioinformatics for data overviews, is returned to investigators in user-friendly Excel files.

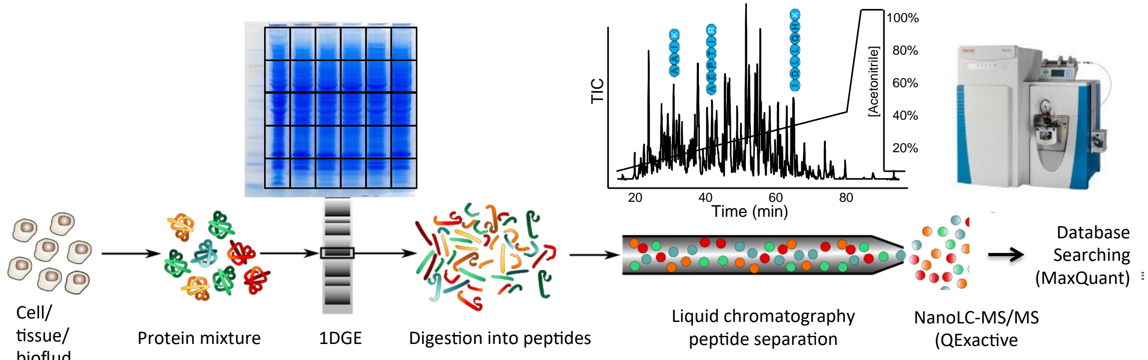


Figure 5. Proteomics workflow.

Protein phosphorylation profiling

For successful phosphoproteome analyses, phosphopeptides need to be enriched prior to analysis by nanoLC-MS/MS (~2 hours of instrument time per sample). The OPL has implemented and benchmarked two robust and reproducible modalities for phosphopeptide capture: (i) titanium oxide for global capture of phosphopeptides with pSer, pThr or pTyr residues, and (ii) a pTyr-specific antibody for selective capture of pTyr residues (Fig. 6). In recent years, successful downscaling of these methods has enabled phosphoproteomic analyses of tumor biopsies in clinical studies. Moreover, a dedicated integrative data analysis strategy has been developed to identify hyperactive phosphokinases in individual tumor samples. We foresee that this approach will be used in the future to complement genomic information in the setting of patient selection for targeted therapies and personalized medicine.

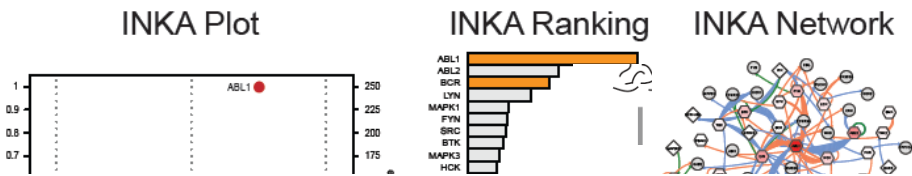


Figure 6. Phosphoproteomics workflow. Phosphopeptides are enriched using TiOx or a phosphotyrosine (pTyr)-specific antibody to enrich for the global or pTyr phosphoproteome respectively.

Extracellular and organelle proteomics

To obtain insight into the tumor microenvironment (Fig. 7), and to develop non-invasive biomarker applications, workflows for the global analysis of secreted proteins (secretome), proximal biofluids (e.g., urine, sputum, cerebrospinal fluid and stool), as well as extracellular vesicles isolated from such matrices have been implemented and benchmarked at the OPL. More specifically, protocols have been benchmarked and applied to the analysis of proteins on the cell surface, proteins that are secreted or released in the extracellular compartment (forming a 'secretome'), proteins enclosed in extracellular vesicles (including 'exosomes') that are actively secreted by cells, and proteins in proximal (tumor-contacting) body fluids/excrements such as sputum and faeces. Researchers may be trained in workflows of interest to prepare samples that can be subjected to in-depth discovery proteomics (as described above under protein expression profiling). For extracellular vesicle (exosome) proteomics, a novel isolation method has been implemented, and its use is being optimized for the capture of vesicles from various biofluids.

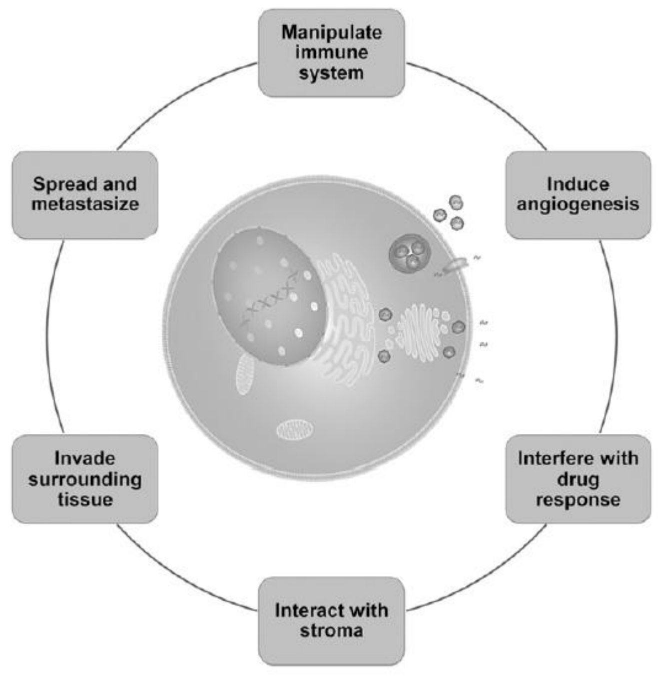


Fig. 7. Hallmarks of cancer secreted proteins and extracellular vesicles (modified after Schaaij-Visser, Jimenez et al., BBA 2013)

Multiplex protein validation pipeline: Data Independent Acquisition (DIA) MS

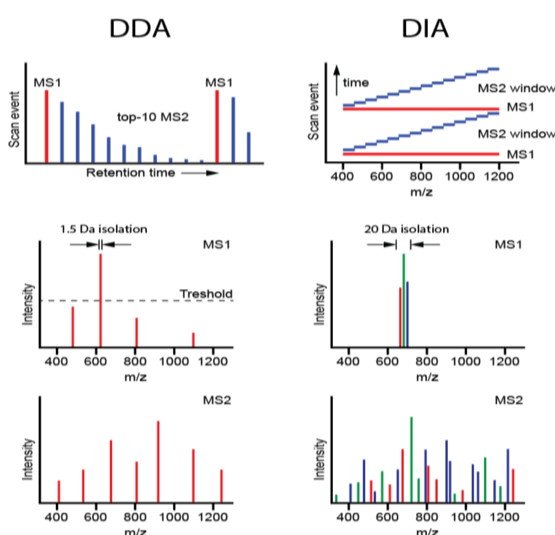


Figure 8. Data dependent acquisition (DDA< shot gun proteomics) *vs.* data independent acquisiton (DIA) MS

Identification of specific biomarkers and the development of robust multi-parameter assays is essential for the realization of personalized medicine. A prerequisite for successful clinical validation of protein biomarker candidates is reproducible quantification.

To get the best of discovery proteomics (in-depth protein inventories) and targeted MS (highly reproducible quantitation of selected proteins), recently a revolutionary MS approach has been developed that was implemented at the OPL in 2017. Whereas both global and targeted approaches entail the use of survey MS scans for final analysis in MS/MS mode ('data-dependent acquisition', DDA), in the novel DIA-MS strategy, consecutive mass windows are immediately subjected to MS/MS (data-independent acquisition, DIA), followed by targeted data extraction using a spectral library (Fig. 8). The resulting 'digital proteome maps' (files of MS/MS

data) can be (re-)analyzed and compared across multiple samples. Such innovative analyses with promising clinical potential are now performed on the high-resolution, fast-scanning MS that was installed end of 2016 at the OPL. Besides DIA-MS for large-scale tumor profiling, we are also exploring phospho-DIA-MS with dedicated support of a scientist. First results of our own hands show that DIA-MS has high precision quantitation (CVs < 5-6%) and low numbers of missing values, and therefore it may ultimately find its way to the clinical chemistry labs for routine diagnostics.

Method development: wet and dry lab

Proteomics technology is constantly under development, therefore, innovation of research methodologies and analysis strategies is an ever on-going effort at the OPL, often as a spin-off of specific questions or problems addressed in our research projects.

Wet lab methods studies

Expression profiling: For protein expression profiling, we performed pioneering work in the area of label-free proteomics (Piersma et al., J Proteome Res. 2010; reviewed by Pham et al. in Expert Rev Mol Diagn. 2012) and improved the throughput of the GeLC workflow (Piersma et al., Proteome Sci. 2013).

Phosphoproteomics: For phosphoproteomics, we bench-marked label-free single shot global phosphoproteomics using titanium-oxide (Piersma et al., J Proteomics. 2015), and compared different phosphotyrosine antibodies for phosphopeptide capture (Van der Mijn et al., J Proteomics. 2015). Recently, we bench-marked the performance of a miniaturized pTyr-specific phosphoproteomics workflow to allow for the analysis of small(er) clinical samples (Labots et al., J Proteomics. 2017).

Exosome proteomics: For analysis of liquid biopsies, we benchmarked and optimized a novel high-throughput extracellular vesicle (enriched for exosomes) isolation protocol using cancer cell secretome (Knol et al, EuPA Open Proteomics 2016) and using urine (Bijnsdorp et al., J. Extracelular Vesicles 2017). Further implementation for of this promising highthroughput, clinically compatible EV capture method to cerebrospinal fluid (CSF) is on-going (Chiasserini in prep).

Biofluid/ CSF proteomics: Through our neuroproteomics projects, we obtained our expertise with biofluid proteomics, most notably CSF. To this end, we bench-marked two different abundant protein depletion filters for in-depth proteome analysis of CSF (Fratantoni et al., Proteomics. Clin. Appl. 2010).

Dry lab studies

Data analysis for biological interpretation

After protein identificatioin and quantification by sequence database searching using the freely web-based tool MaxQuant, for subsequent data interpretation, other web-based software (Cytoscape, String, Webgestalt) is used to perform functional data mining and network-based analyses (Fig. 9). The latter involve visualization of protein-protein association networks which can aid in gaining insights into (the role of candidates in) tumor (cell) biology. Moreover, information gleaned from such networks may be used to prioritize candidate markers/targets for follow-up. Furthermore, for the analysis of phosphoproteomics data, OPL is actively engaged in the development of algorithms that enable prediction of hyperactive/driver kinases and visualize the data as kinase-substrate interaction networks (manuscript in prep).

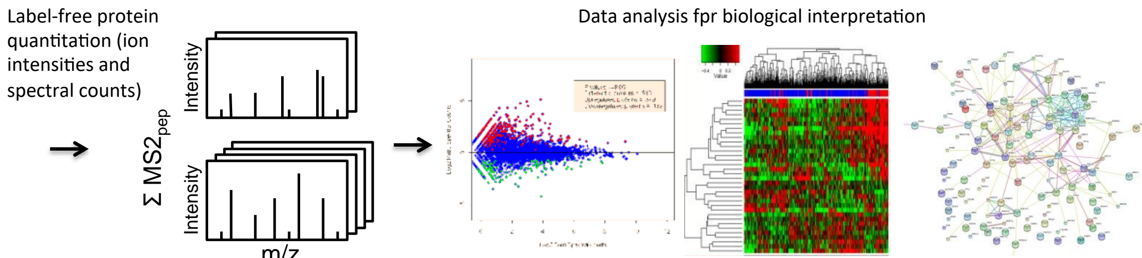


Figure 9. Data analysis workflow. Sequence database searching of combined MS and MS/MS data enables protein identification and quantification. Differential expression analysis and dedicated statistics can uncover proteins with altered abundance. Volcano plots, heat maps and protein interaction networks are complementary ways to get data overviews and biological information.

Proteogenomics

In the coming years, our research focus will include tumor-specific protein variants in pre-clinical and clinical samples (e.g., splice variants and mutated proteins) in order to develop specific protein markers. To allow for such research, OPL has set up a “proteogenomics” data analysis pipeline that

can identify aberrant protein variants in cancer. In this approach, the MS/MS data are searched against genomics-based (sample/patient-specific) sequence databases that include disease variants. Moreover, in a KWF-funded project, we developed “Splicify”, a proteogenomic pipeline for identification of differentially expressed splice variants (Komor, Fijneman, Jimenez et al., MCP 2017).

Database to enable cross-experiment queries

Our ambition is to implement a smart searchable database for all (phospho)proteome data generated in the different projects. The database may also include relevant external datasets to enable protein validation in independent cohorts. Importantly, we will make use of existing national collaborations (follow-up projects from CTMM-TraIT, SurfSara, DTL) and connect to new international efforts (cBioPortal). In this way we will build up a repertoire of external data, medical informatics tools and computing infrastructure appropriate for advanced analysis. To this end, funding is being requested.

Summary of Translational OncoProteomics research

Below, a summary is given of OPL research activities and multi-disciplinary collaborations involving different tumor types in each research line, with listing of publications of the last 3 years and the on-going projects with Jimenez as PI or coPI. In the **appendix 1** abstracts can be found of each research project and **appendix 2** contains pie chart overviews of OPL production in terms of mass spec runs and publication quantified over the last 10 years.

1. Identification of (phospho)protein markers/targets for tumor subtyping, targeted treatment of cancer and tailored therapy for individual patients.

In 2014, research was started to uncover protein biomarkers for CRC subtype classification linked to prognosis (collaboration with Prof. Dr. Jan Paul Medema (AMC) and Prof.Jan Ijzermans (EMC) and partners KWF Alpesd’hu6 Connection consortium). CRC subtype markers have been identified in a set of 40 tumors and 25 cell lines and validated in an independent series of CRCs from Rotterdam as well as in external TCGA CRC tumor proteome data (Nature 2014). Currently a DIA-MS-based protein classifier is developed for profiling a third CRC biopsy series to be obtained in a clinical trial that aims to link CRC subtypes to (lack of) response to chemotherapy.

Large scale protein profiling is also done on a FFPE series of lung cancers (NSCLC) of patients that received adjuvant platina-based therapy, with the goal of identifying biomarkers for cisplatin response prediction (KWF project with Sjaak Burgers and Egbert Smit, NKI).

Protein phosphorylation by kinases plays a key role in the crucial change of the activity and/or wiring of intracellular signaling pathways and in the action of machinery that transforms cells into cancer cells. Recent analysie of AML cell lines and patient blasts (collaboration Dr. Jeroen Janssen, Dr. Jacqueline Cloos), colorectal adenomas and carcinomas (collaboration Beatriz Carvalho, Gerrit Meijer, NKI), patient-derived xenograft (PDX) models of colorectal cancer (collaboration with Prof. Livio Trusolini, IRCCS, Italy) and pancreatic cancer (collaboration dr. Elisa Giovanetti, Dr. Maarten Bijlsma), have shown the power of phosphoproteomics to uncover hyperactive kinases and identify known and novel “driver kinases” that are thought to play a central role in oncological transformation/progression. Phosphoproteomics of PDX models is also successfully being applied in the context of Homologous Recombination Deficiency in triple negative breast cancer (KWF project with Jos Jonkers).

Successful downscaling of the phosphotyrosine workflow and application to tumor biopsies has uncovered drug-specific signatures in a clinical trial (collaboration Mariette Labots and Henk Verheul). Currently, tumor needle biopsies are being collected in multiple clinical trials of the Dept. Medical Oncology (IMPACT-CRC, Soprano, Reposit) of the national Center for Personalized Cancer Treatment. In the coming years, we aim to perform phosphoproteomics of these tumor biopsies to establish its value for patient selection for targeted therapy.

Publications

- Johannes C. van der Mijn, Henk J. Broxterman, Jaco C. Knol, Sander R. Piersma, Richard R. De Haas, Henk Dekker, Thang V. Pham, Victor W. Van Beusechem, Balazs Halmos, James W. Mier, Connie R. Jiménez*, Henk M.W. Verheul*. Sunitinib activates Axl signaling in renal cell cancer. **Int. J. Cancer 2016**; **Shared senior authors*; Accepted manuscript
- Le Large TYS, Bijlsma MF, Kazemier G, van Laarhoven HWM, Giovannetti E, Jimenez CR. Key biological processes driving metastatic spread of pancreatic cancer as identified by multi-omics studies. ***Semin Cancer Biol.*** **2017** Mar 30. pii:S1044-579X(17)30066-4.

On-going projects

- Discovery and clinical validation of novel protein biomarkers for homologous recombination deficient breast cancer (KWF VU2013-6020 Dutch Cancer Society, PIs Jimenez, Jonkers (NKI), Van Diest (UMCU))
- Tumor-specific protein biomarkers for early detection of colorectal cancer (KWF NKI2014-6025, PIs Fijneman (NKI), Jimenez, Meijer (NKI))
- The Molecular Signalling Pathways of Folliculin (FLCN): a Tumor Suppressor in Birt-Hogg Dubé Hereditary Kidney Cancer (VUmc PhD student Dept. Clin. Genetics with Wolthuis).
- Improving clinical management of colon cancer through CONNECTION, a nation-wide Colon Cancer Registry and Stratification effort. (KWF/Alpe d'Huzes UvA2013-6331, PIs Medema (AMC), Van Krieken (RUMC), IJzermans (EMC), Jimenez, Koopman (UMCU)).
- Response prediction for cisplatin-based treatment regimens in non-small cell lung cancer using a protein-based assay (KWF VU2014-6816, PIs Jimenez (VUmc), Grunberg (VUmc/RUMC), Burgers (NKI))
- Phosphoproteomics for therapy response prediction in solid tumors with a focus on colorectal cancer (Vitromics-sponsored PhD project, PIs Verheul and Jimenez)
- AML phosphoproteomics for personalized therapy (CCA-sponsored PhD project, VUmc PIs Janssen, Jimenez, Verheul)
- Signaling pathways involved in colorectal adenoma-to-carcinoma progression (KWF NKI2014-6813 Dutch Cancer Society, PIs Carvalho (NKI), Jimenez, Meijer (NKI))
- Phosphoproteomics for therapy response prediction in pancreatic cancer (VUmc-AMC alliance project 2014, PIs Giovanetti (VUmc), Jimenez, Bijlsma (AMC))
- How KRAS mutations in colon cells are linked to a new cancer hallmark: cohesion weakness (CCA 2015, PIs De Lange, Jimenez, Wolthuis)
- Interrogating the phosphoproteome for targets and markers in pancreatic cancer (KWF VU2016-1012, PIs Jimenez (VUmc), Giovannetti (VUmc), Bijlsma (AMC))
- Identification of biomarkers by whole-genome sequencing and phospho-proteomics to predict responses to high-precision cancer medicines in T-cell acute lymphoblastic leukemia (KWF Maxima 2016-10355, PIs Meijerink (Prinses Maxima), Jimenez (VUmc))

2. Identification of protein markers for non-invasive (early) detection and monitoring of cancer

To gain insight into the tumor microenvironment and identify protein biomarkers for non-invasive applications, OPL has built expertise in the measurement of extracellular proteins. Due to their extracellular localization, these proteins are more prone to end up in the blood circulation and therefore they are interesting biomarker candidates. In past years, proteomic analyses of secretomes and extracellular vesicles have been performed in multiple tumor types and have indicated that non-classical protein secretion forms an important characteristic of (aggressive) cancer cells. This makes this characteristic an interesting feature to explore for biomarker development and possibly drug targeting. Especially our published work on patient AML secretomes and exosomes (collaboration Dr. Jacqueline Cloos) that implicates transfer of RNA splice complexes has received media attention and numerous orals at meetings.
Most prominent progress in the area of non-invasive markers has been achieved with the identification and validation of protein markers for colorectal cancer screening. Over the past years, tens of candidate protein markers for screening for colorectal cancer have been identified in stool and recently validated in faecal samples of ~300 individuals (colorectal cancer patients, people harboring colorectal adenomas, and healthy controls) (De Wit et al., Annals of Internal Medicine, 2017). The most promising, validated biomarker candidates emerging from the proteomics screens are being used for further development of an antibody assay that is complementary to the immunological hemoglobin test of the national screening program for colorectal cancer in the Netherlands. This research is one of the mainstays of OPL research, showcasing OPL's expertise (and that of their collaborating partners (former VUmc pathology, now at NKI)). It has culminated in several publications, several awarded research grants, media coverage, and patents.
Finally, proteomics of platelets is another promising non-invasive strategy for disease detection, for which proof-of-concept has already been generated in years past.

Publications

- Wojtuszkiewicz, G.J. Schuurhuis, F.L. Kessler, S. Piersma, J. Knol, T.V. Pham, G. Jansen, R.J.P. Musters, J. van Meerloo, Y.G. Assaraf, G.J.L. Kaspers, S. Zweegman, J. Cloos*, C.R. Jimenez*. Exosomes secreted by apoptosis-resistant AML blasts harbor regulatory network proteins potentially involved in antagonism of apoptosis. ***Mol Cell Proteomics.*** **2016** **Shared senior authors.*

- Warmoes M, Lam SW, der Groep PV, Jaspers JE, Smolders YH, de Boer L, Pham TV, Piersma SR, Rottenberg S, Boven E, Jonkers J, van Diest PJ, Jimenez CR. Secretome proteomics reveals candidate non-invasive biomarkers of BRCA1 deficiency in breast cancer. ***Oncotarget***. **2016** Aug 23.
- van Linde ME, van der Mijn JC, Pham TV, Knol JC, Wedekind LE, Hovinga KE, Aliaga ES, Buter J, Jimenez CR, Reijneveld JC, Verheul HM. Evaluation of potential circulating biomarkers for prediction of response to chemoradiation in patients with glioblastoma. ***J Neurooncol***. 2016 Sep;129(2):221-30.
- Rovithi M, Lind JS, Pham TV, Voortman J, Knol JC, Verheul HM, Smit EF, Jimenez CR. Response and toxicity prediction by MALDI-TOF-MS serum peptide profiling in patients with non-small cell lung cancer. ***Proteomics Clin Appl***. 2016 Jul;10(7):743-9.
- Knol, J.C., de Reus, I., Schelfhorst, T., Beekhof, R., de Wit, M., Piersma, S.R., Pham, T.V., Smit, E.F., Verheul, H.M.W., Jiménez. C.R. Peptide-mediated 'miniprep' isolation of extracellular vesicles is suitable for high-throughput proteomics. **EuPA Open Proteomics** Volume 11, June 2016, Pages 11–15.
- Bijnsdorp IV, Maxouri O, Kardar A, Schelfhorst T, Piersma SR, Pham TV, Vis A, van Moorselaar RJ, Jimenez CR. Feasibility of urinary extracellular vesicle proteome profiling using a robust and simple, clinically applicable isolation method**. *J Extracell Vesicles*. 2017** Apr 28;6(1):1313091.
- De Wit, M*., Bosch, L*.,….RJA Fijneman, B Carvalho, CR. Jimenez#, GA Meijer#. Novel Stool-Based Protein Biomarkers for Improved Colorectal Cancer Screening, A Case–Control Study; **Shared first authors*; #*Shared senior authors*. **Annals of Internal Medicine, 2017** Accepted manuscript

On-going projects

- Molecular Early Detection of Colorectal Cancer (SU2C MEDOCC, Dream team leaders Meijer and Velculescu)
- Urinary extracellular vesicles and their content as novel markers for minimally invasive diagnosis and prognosis of prostate cancer (KWF/Alpe d'Huzes EMCR 2015-8022 (PIs Jenster (ErasmusMC), Jimenez (VUmc), Schalken (RadboudMC))

3. Development/ implementation of innovative proteomics and data analysis strategies

Innovation of research methodologies and analysis strategies is an on-going effort, often as a spin-off of specific questions or problems addressed in regular projects. OPL's basic, label-free proteomics workflow is based on GeLC-MS/MS and spectral counting. This workflow for global protein identification and quantification has proved very robust and reliable, and the pertinent publication where this procedure was compared to other approaches is one of OPL's most cited papers (Piersma et al., JPR 2010). For the analysis of spectral count data, OPL has devised a dedicated statistical test (Pham et al., Bioinformatics 2010; 2012). To speed up the gel band processing step in the GeLC workflow, we introduced the "whole gel" protocol. To enable analysis of tumor biopsies, we scaled-down the pTyr phosphoproteomics workflow. Furthermore, OPL is actively engaged in the development of algorithms that make prediction of driver kinases from phosphoproteome data possible (unpublished data). Finally a proteogenomics pipeline to enable analysis of alternative splicing was recently developed (Komor et al., Mol.Cell.Proteomics 2017).

Publications

- Piersma, SR., Knol, JC., de Reus, I., Labots, M., Sampadi, BK., Pham, TV., Ishihama, Y., Verheul, HMW., Jimenez, CR. Feasibility of label-free phosphoproteomics and application to base-line signaling of colorectal cancer cell lines. **J. Proteomics 2015**, doi:10.1016/j.jprot.2015.03.019.
- Van der Mijn, J. C., Labots, M., Piersma, S. R., Pham, T. V, Knol, J. C., Broxterman, H. J., Verheul, HM., Jiménez, CR. Evaluation of different phospho-tyrosine antibodies for label-free phosphoproteomics. **J. Proteomics 2015**, doi:10.1016/ j.jprot.2015.04.006.
- Labots M, van der Mijn JC, Beekhof R, Piersma SR, de Goeij-de Haas RR, Pham TV, Knol JC, Dekker H, van Grieken NCT, Verheul HMW, Jimenez CR. Phosphotyrosine-based-phosphoproteomics scaled-down to biopsy level for analysis of individual tumor biology and treatment selection. **J Proteomics. 2017** Apr 23. pii: S1874-3919(17)30140-9.
- Komor MA, Pham TV, Hiemstra AC, Piersma SR, Bolijn AS, Schelfhorst T,Delis-van Diemen PM, Tijssen M, Han SK, Sebra RP, Ashby M, Meijer GA, Jimenez CR, Fijneman RJA. Identification of differentially expressed splice variants by the proteogenomic pipeline SPLICIFY. **Molecular Cellular Prot. 2017** Accepted Manuscript.

On-going projects

- Kinome pathway activity assay by targeted mass spectrometry-based phosphoproteomics to enable personalized treatment of cancer with kinase inhibitors (CCA 2016-2017, PIs Jimenez, Grieken, Verheul)
- Next-generation proteomics to create digital cancer proteome maps for translational research and personalized medicine (NWO Middelgroot, PIs Jimenez, Verheul)

Dissemination and education

Dissemination OPL methods development and research is published in scientific papers. Futhermore, the OPL website (www.oncoproteomics.nl) provides information on proteomics workflows and protocols, and offers dedicated R analysis tools for download. All raw and preprocessed data are deposited in public repositories (primarily ProteomeXchange) after optional protection of intellectual property and publication.

Education OPL contributes to oncology-related and bioinformatics education of VU/VUmc. This includes a two-week elective master's course "Biomedical Proteomics" that includes a theory and lab part as well as a data analysis workshop. This course is also available to OPL collaborators who are furthermore trained during the year on an individual basis.
FInally, in the past 3 years, 8 VU bioinformatics master students and Biomedical Sciences students performed their internship at the OPL.

Indicators of Esteem

Obtained grants

Grants PI	**Amount** *k€*	**Year award**	**Status**
CCA Kinome pathway activity assay by targeted mass spectrometry-based phosphoproteomics to enable personalized treatment of cancer with kinase inhibitors (Jimenez, Van Grieken, Verheul)	150	2015	on-going
NWO Middelgroot: Next-generation proteomics to create digital cancer proteome maps for translational research and personalized medicine (Jimenez, Verheul)	500	2016	on-going
KWF VU2016-1012: Interrogating the phosphoproteome for drug targets in pancreatic cancer (Jimenez, Giovanetti, Bijlsma)	835	2016	on-going
Grants Co-PI	**Amount** *k€ (proteomics)*	**Year award**	**Status**
KWF/Alpe d'Huzes EMCR 2015-8022: Urinary extracellular vesicles and their content as novel markers for minimally invasive diagnosis and prognosis of prostate cancer (Jenster, Jimenez,)	750	2016	on-going
KWF Prinses Maxima 2016-10355: Identification of biomarkers by whole-genome sequencing and phospho-proteomics to predict responses to high-precision cancer medicines in T-cell acute lymphoblastic leukemia (Meijerink, Jimenez)	50+AIO	2016	started

Invited lectures at (inter)national conferences

International invited lectures Connie R Jimenez

2017 25 Oct. Clinical Proteomics ProteoRed A Coruna, Spain. "Clinical proteomics with a focus on cancer"

2016 Conference Cancer Stem Cells: Impact on Treatments, Obergurgl, Austria (9 dec. 2016); "(Phospho)Proteomics to uncover tumor biology, biomarkers and drug targets"

2016 30 nov. VII Reunión Científica sobre Proteómica Clínica, Valencia, Spain. "Clinical proteomics in translational colorectal cancer research"

2015 14th Human Proteome Organisation Worl Congress, Vancouver (28 sept. 2015); "Integrative Analysis of the Colorectal Cancer Proteome and Its Subtypes with Differential Prognosis"

National invited lectures

2017 Thermo User meeting, Benelux, 10 & 12 Oct. Data-independent MS for comprehensive tumor proteome profiling: classification of colorectal cancer subtypes (Sander Piersma)

2017 SMRT, Leiden, 2-3 May. Proteogenomic analysis of alternative splicing: the search for novel biomarkers for colorectal cancer (Malgorzata A Komor)

2016 Thermo User meeting, Benelux, 31 June. Phosphoproteomics, towards clinical application (Sander Piersma)

2015 Symposium of the Center for Personalized Cancer Treatment (29 0kt 2014, Utrecht); Title: (Phospho)Proteomics complementing the CPCT genomics-based personalized treatment effort with the functionally relevant –OME (Connie Jimenez)

2015 NPP2015, Leiden; Peptide-mediated 'miniprep' isolation of extracellular vesicles is suitable for high-throughput proteomics; method evaluation and application in colon cancer (Meike de Wit)

Oral presentations OPL members

2017 HUPO2017, Dublin. MD1-06S Platelet proteomics: towards non-invasive cancer detection (Connie Jimenez)

2017 HUPO2017, Dublin. MD1-04S Phosphoproteome networks identify focal adhesion kinase as new target in pancreatic ductal adenocarcinoma (Tessa Le Large)

2017 HUPO2017, Dublin. MD2-04S Proteogenomic analysis of alternative splicing: protein isoforms as biomarkers for early detection of colorectal cancer (Malgorzata Komor)

2017 HUPO2017, Dublin. TF2-05S pTyr-phosphoproteomics of serial tumor biopsies from patients with advanced cancer treated with protein kinase inhibitors (Mariette Labots)

2017 HUPO2017, Dublin. TF2-06S Phosphoproteomics in metastatic colorectal cancer for predicting response to anti- EGFR therapy (Robin Beekhof)

2017 HUPO2017, Dublin. WD2-03S Phosphoproteomics of an AML cell line panel pinpoints hyperactive tyrosine kinases as targets for treatment (Carolien Van Alphen)

2016 AACR2016, New Orleans. Phosphoproteomics for target discovery and therapy response prediction in colorectal cancer (Robin Beekhof)

2016 AACR2016, New Orleans. Proteogenomic analysis of alternative splicing: the search for novel biomarkers for colorectal cancer (Malgorzata A Komor)

2016 DHC2015, Arnhem..The phosphoproteomic landscape of AML cell lines reveals oncogenic signaling and candidate kinase drivers as targets for treatment (Carolien Van Alphen)

2016 EORTC-PAMM, Antwerpen. "Phosphoproteomics identifies new drug targets in pancreatic cancer" (Tessa Le Large)

2016 Chirurgendagen, Veldhoven. "De strijd tegen pancreascarcinoom; predictie van de sensitiviteit voor therapieën" (Tessa Le Large)

2016 ECCB2016, The Hague. Simulated linear test applied to quantitative proteomics (Thang V. Pham)

2015 BioSB, Lunteren, Nederland Identification of protein biomarkers for colorectal cancer by proteogenomic analysis (Malgorzata A Komor)

2015 DHC2015, Arnhem. Phosphoproteomics of a panel of AML cell lines reveals oncogenic signaling and candidate drivers (Carolien Van Alphen)

(Inter)national functions:

- Steering committee — Netherlands Proteomics Platform
- General Council Member — European Proteomics Association (EuPA)
- Co-Chair — Cancer initiative of the Human Proteome Organisation
- Advisory Board — Dutch Techcentre for Life Sciences
- Theme leader Biomarkers — CCA research institute VUmc-AMC

Memberships of editorial boards:

- Journal of Proteomics
- Molecular and Cellular Proteomics
- Proteomics
- Proteomics Clinical Applications
- Clinical Proteomics

Organisation of congresses:

2016 Chair and local organizer, 16th Fall Meeting of the Netherlands Proteomics Platform 'Next-generation proteomics approaches, 14 Dec. 2016, Amsterdam

2015 Co-organizer, 15th Fall Meeting of the Netherlands Proteomics Platform 'Current state-of-the-art of Proteomics in the Netherlands", 13 Nov. 2015, Leiden

Media attention/ Societal Impact

2017 29 Okt. Interview with Connie Jimenez in Spanish newspaper La Voz de Galicia, Title article "Con el proteoma cada paciente tendrá un tratamiento personalizado"

2017 juli. Interview with Connie Jimenez door Rob Buiter voor VUmc CCA jaaroverzicht 2016. Ttile

“De vingerafdruk van een kankercel”

2017 30 maart. Interview with Connie Jimenez in Dutch newspaper De Telegraaf. Title article “Lopen tegen Kanker”

2017 maart. Column Connie Jimenez in VUmc donateurskrant Eigenwijs “Topprestatie voor volgende stap in kankeronderzoek”

2016 May. ASBMB today, Highlight of our MCP paper entitled “Leukemia cells tell other cells not to self-destruct” By Bree Yanagisawa.

2016 Jan. Interview met Connie Jimenez in Zorgkrant VUmc ZH Amstelland entitleld “Wetenscahppers kijken in de toekomst”

2015 April. Interview met oa Connie Jimenez in C2W Chemisch Weekblad, Title “Oplossingen in het Proteoom” by Arno van ’t Hoog.

Concluding remarks and future developments

In the past 3 years, clinical cancer proteomics has gone lare-scale, with profiling of > 100 patient samples now becoming routine. Our oncoproteomics research in multiple tumor types has yielded novel insights into cancer biology and a multitude of candidate biomarkers, many of which are currently undergoing validation. Now that we know that phosphoproteomics of tumor biopsies obtained in clinical trials is feasible, in the coming years we will focus on applying phosphoproteomics and explore its (added) value (as compared to genomics) for patient selection for targeted therapy. Our recently developed integrative data analysis pipeline for kinase activity scoring will be instrumental in this respect.

The number of OPL core and collaborative projects is still growing and sample sizes per experiment are growing to cope with disease heterogeneity and subtypes. Parallelization of sample preparation, and/or automation, will become more important with the increase of sample number per experiment seen in recent years and also expected for future studies. Sofar, all samples have been manually processed. Therefore, next to the 5-yearly investment in MS equipment (the 2 QEs from 2011 are running in grace time!), new investment is needed to automate selected applications, most notably the phosphoproteomics procedure, to enable large scale profiling of longitudinal tumor solid and liquid biopsies in clinical trials.

Data analysis is increasingly becoming a bottle-neck and yet crucial, not only to obtain a better understanding of underlying (tumor) biology, but also for target/biomarker prioritization. Therefore, consolidated investment in our dedicated, PhD-level data mining scientists is quintessential to enable advanced integrative data analysis and visualization in the future. Furthermore, for many (pre)clinical samples, multiple OMICs data types are available offering new opportunities for data mining as well as challenges. Integrative analysis and computational biology and big-data strategies are needed to identify statistical associations and biological relationships in these diverse large datasets. This will require a concerted, multi-disciplinary effort that can translate the enormous amounts of ever-increasing genomic and proteomic information into novel clinical knowledge and tools with a favorable impact for cancer patients.

Finally, despite the wealth of cancer genome data and cancer proteome data for selected tumor types (most notably colorectal cancer, breast cancer, and ovarian cancer), currently comprehensive proteome data across multiple tumor types are missing. Also a cancer proteome data portal is lacking. To this end, we are kick-starting The Cancer Proteome Atlas (TCPA) project for which OPL in collaboration with 9 CCA departments (VUmc and AMC) will profile 20-50 tumors for ~18 different tumor types by DIA-MS in the next year(s). To recruit proteome researchers world-wide, we recently announced TCPA in a viewpoint paper by the chairs of the Cancer Project of the Human Proteome Organisation (HUPO) (Jimenez et al., submitted). In view of the relevance of cancer proteomes for insight into cancer phenotypes and the complementarity to cancer genome information, we anticipate this to be an important resource to mine for cancer core and cancer specific markers and a high profile project that will increase the visibility of CCA at the (inter)national and level.

In summary, the ability to interrogate cancer at the proteome level and integrate acquired knowledge with genome data is expected to improve clinical decision-making and catalyze new clinical and translational cancer research. I believe the future is bright, especially in view of the advent of novel mass spectrometry approaches that combine the best of discovery and targeted mass spectrometry, which will facilitate the accurate analysis of global disease related expression and post-translational

modifications in large series of clinical samples. I expect that cancer proteogenomics powered by precise measurements will realize the full potential of multi-parameter diagnostics and personalized medicine.

In **appendix 1** of the OPL progress report you can learn about on-going core and collaborative research projects, in **appendix 2** you can get an overview of our production in terms of nanoLCMSMS and papers in the past 10 years and in **appendix 3** quotes from our collaborators. I hope this report will trigger new ideas and collaboration.

Appendix 1. Abstracts & poster summaries of OPL core research

OncoProteomics: Oncogenes, drug targets and predictive biomarkers

Identification of differentially expressed splice variants by the proteogenomic pipeline Splicify.

Komor MA, Pham TV, Hiemstra AC, Piersma SR, Bolijn AS, Schelfhorst T,Delis-van Diemen PM, Tijssen M, Han SK, Sebra RP, Ashby M, Meijer GA, Jimenez CR, Fijneman RJA.

[1]Pathology, Netherlands Cancer Institute, Netherlands; [2]OncoProteomics Laboratory, Medical Oncology, VU University Medical Center, Netherlands.

Background Proteogenomics, i.e. comprehensive integration of genomics and proteomics data, is a powerful approach identifying novel protein biomarkers. This is especially the case for proteins that differ structurally between disease and control conditions. As tumor development is associated with aberrant splicing, we focus on this rich source of cancer specific biomarkers.

Aim To develop a proteogenomic pipeline, which is able to detect differentially expressed protein isoforms.

Results Our proteogenomic pipeline called Splicify is based on integrating RNA massive parallel sequencing data and tandem mass spectrometry proteomics data to identify protein isoforms resulting from differential splicing between two conditions.
Proof of concept was obtained by applying Splicify to RNA sequencing and mass spectrometry data obtained from colorectal cancer cell line SW480, before and after siRNA-mediated down-modulation of the splicing factors SF3B1 and SRSF1. These analyses revealed 2172 and 149 differentially expressed isoforms, respectively, with peptide confirmation upon knock-down of SF3B1 and SRSF1 compared to their controls. Splice variants identified included RAC1, OSBPL3, MKI67 and SYK. One additional sample was analyzed by PacBio Iso-Seq full-length transcript sequencing after SF3B1 down-modulation. This analysis verified the alternative splicing identified by Splicify and in addition identified novel splicing events that were not represented in the human reference genome annotation.

Conclusion Splicify offers a validated proteogenomic data analysis pipeline for identification of disease specific protein biomarkers resulting from mRNA alternative splicing.
Splicify is publicly available on GitHub (https://github.com/NKI-TGO/SPLICIFY) and suitable to address basic research questions using pre-clinical model systems as well as translational research questions using patient-derived samples, e.g. allowing to identify clinically relevant biomarkers.

Reference Komor MA, Pham TV, Hiemstra AC, Piersma SR, Bolijn AS, Schelfhorst T,Delis-van Diemen PM, Tijssen M, Han SK, Sebra RP, Ashby M, Meijer GA, Jimenez CR, Fijneman RJA. Identification of differentially expressed splice variants by the proteogenomic pipeline SPLICIFY. *Molecular Cellular Prot.* Accepted Manuscript.

Acknowledgements: This project is supported by the Dutch Cancer Society (KWF VU2014-6025)

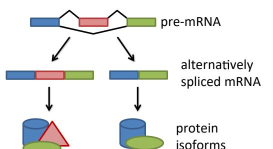
Proteogenomic pipeline for identification of novel biomarkers for colorectal cancer

Malgorzata A Komor [1], Annemieke C Hiemstra[1], Thang V Pham[2], Sander R Piersma[2], Anne S Bolijn[1], Pien M Delis-van Diemen[1], Marianne Tijssen[1], Robert P Sebra[3], Bo Han[4], Meredith Ashby[4], Beatriz Carvalho[1], Gerrit A Meijer[1], Connie R Jimenez[2], Remond JA Fijneman[1]

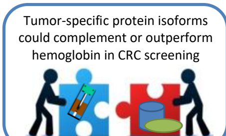
1 Department of Pathology, Netherlands Cancer Institute, 2 Oncoproteomics Laboratory, Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands, 3 Icahn School of Medicine at Mount Sinai, New York, NY, United States, 4 Pacific Biosciences, Menlo Park, CA, United States

Background

- **Colorectal cancer (CRC) has high cure rate when diagnosed early.**
- **The fecal immunochemical test** (FIT) is a CRC screening test detecting protein hemoglobin
  - FIT **sensitivity is suboptimal**
  - CRC screening test needs to be **improved**
- **RNA alternative splicing (AS)** accompanies tumor progression from benign to malignant lesions



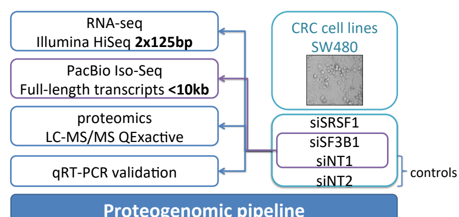
Tumor-specific protein isoforms could complement or outperform hemoglobin in CRC screening



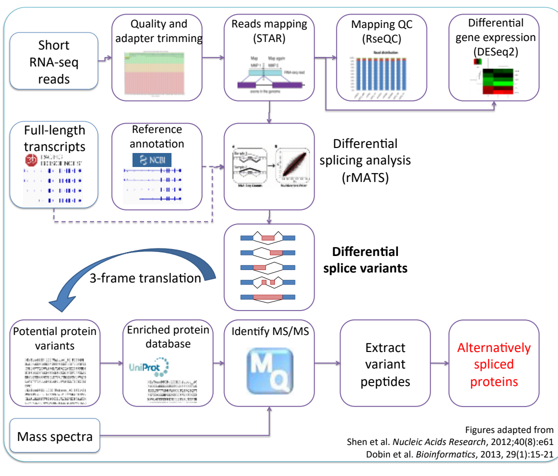
AIM: Design an approach to identify tumour specific protein variants

Approach

siRNA mediated down-modulation of splicing machinery (SF3B1, SRSF1) allows to investigate alternative splicing in a **controlled setting**



Proteogenomic pipeline

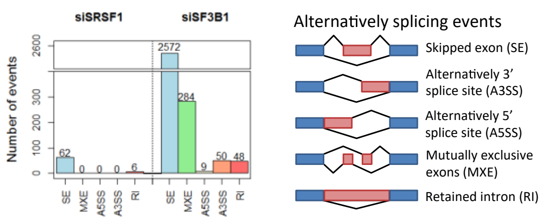


Figures adapted from
Shen et al. *Nucleic Acids Research*, 2012;40(8):e61
Dobin et al. *Bioinformatics*, 2013, 29(1):15-21

Results

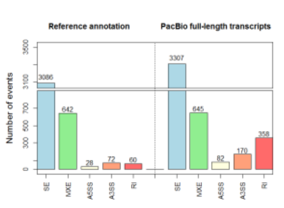
Down-modulation of splicing machinery causes differential splicing

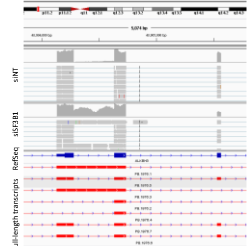
The number of differential splice variants identified on RNA level



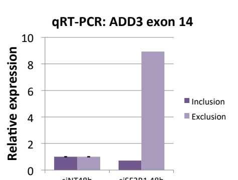
PacBio Iso-Seq provides a number of novel AS events

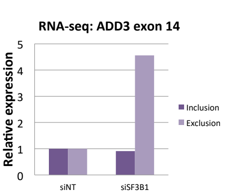
A number of retained intron events are missing in the reference annotation.





In silico findings are validated by qRT-PCR

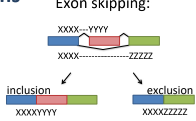




Mass spectrometry reveales that a subset of the isoforms are translated into proteins

Experiment	Isoforms for which peptides where detected
siSF3B1	36 + 4 (PacBio)
siSRSF1	7

Exon skipping:



For a subset of proteins both isoform-specific and canonical peptides detected

Conclusions and Future plans

- Established proteogenomic pipeline by combining RNA-seq and LC-MS/MS data
- Obtained proof of concept
- Application of the proteogenomic pipeline for discovery and validation of CRC related splice variant proteins
  - human colorectal (tumour) tissues
  - patient derived organoids
- Novel candidates will be evaluated for the performance as screening markers

This project is supported by KWF project nr: NKI 2013-6025

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Identification and validation of protein biomarkers for homologous recombination deficiency in breast cancer

F. Rolfs[1, 2], S. Piersma[1], T. Pham[1], J. Knol[1], P. ter Brugge[2], J. Wesseling[2,3], E. Marangoni[4], V. Serra[5], J. Jonkers[2] & C. R. Jimenez[1]

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3 Division of Pathology, The Netherlands Cancer Institute, Amsterdam, The Netherlands
4 Translational Research Department, Institut Curie, Paris, France
5 Experimental Therapeutics Group, Vall d'Hebron Institute of Oncology, Barcelona, Spain

Background Triple negative breast cancers show very poor prognosis, with chemotherapy as the only treatment option. Because of a BRCA1 mutation and thus deficiency in homologous recombination (HR), a subgroup of these tumors is sensitive to DNA damaging drugs or blocking non-homologous end joining via PARP inhibition. Therefore, the identification of HR deficient breast cancer via biomarkers is of importance for personalized therapy.

Methods In a pilot experiment we used three biopsies each from HR-proficient or HR–deficient patient-derived xenograft (PDX) models, either untreated or 24h after cisplatin treatment. Global protein expression was profiled via label-free GeLC-MS/MS and changes in phosphorylation were examined using TiOx based phosphopeptide enrichment and single-shot LC-MS/MS (label-free).

Results In total, we measured 8764 proteins and 6613 phosphopeptides with 6266 phosphosites. Samples cluster according to origin and treatment and depending on the status of HR, we could identify differential regulated (phospho)proteins. GO-term analysis of candidates upregulated in HR deficient samples revealed processes that are shared for both cisplatin treated and untreated tumors. These candidates are involved in processes such as DNA replication, DNA duplex unwinding and geometric change. They seem to be important in the context of HR deficiency and are possible biomarkers.

Conclusions Our pilot data point to the feasibility of detecting biomarkers for HR deficiency in breast cancer, also in the untreated situation. We will now expand our sample set of PDX models with known status of HR. Moreover, predictive potential of candidate markers will be validated with in vivo intervention studies using PARP inhibitors.

Acknowledgements: This project is supported by the Dutch Cancer Society (KWF VU2013-6020)

IDENTIFICATION & VALIDATION OF PROTEIN BIOMARKERS FOR HOMOLOGOUS RECOMBINATION DEFICIENCY IN BREAST CANCER

F. Rolfs[1,2], S. Piersma[1], T. Pham[1], J. Knol[1], P. ter Brugge[2], J. Wesseling[2,3], E. Marangoni[4], V. Serra[5], J. Jonkers[2] & C. R. Jimenez[1]

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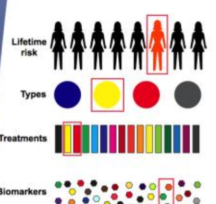
INTRODUCTION & AIM

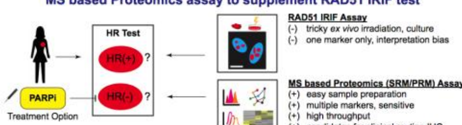


Triple negative breast cancers (TNBCs) show very poor prognosis, with chemotherapy as the only treatment option. Because of a BRCA1 mutation and thus deficiency in homologous recombination (HR) repair, a subgroup of these tumors is sensitive to DNA damaging drugs or inhibition of non-homologous end joining (NHEJ) via PARP inhibition.
Therefore, the identification of HR deficient breast cancer via biomarkers is of importance for personalized therapy. The RAD51 IRIF assay, a current test for HR-deficiency on freshly isolated primary biopsies is complex and based on a single marker.

We would like to complement this situation and identify as well as validate additional protein biomarkers for HR deficient breast cancer using (phospho)proteomics and patient derived xenograft models of HR deficiency.
The identified biomarkers will likely support patient selection for breast cancer therapy in the future.

BACKGROUND

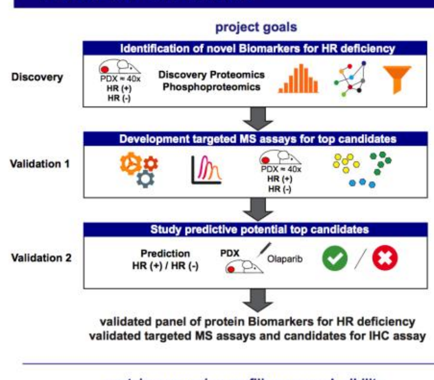
MS based Proteomics assay to supplement RAD51 IRIF test

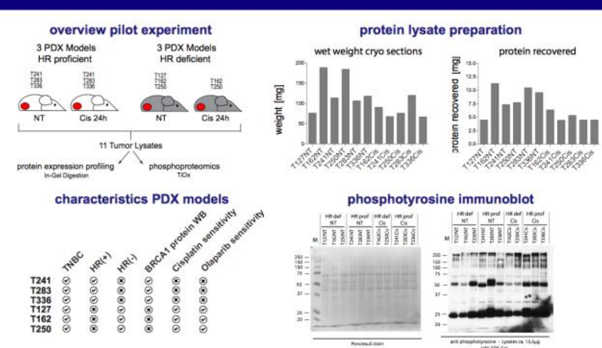


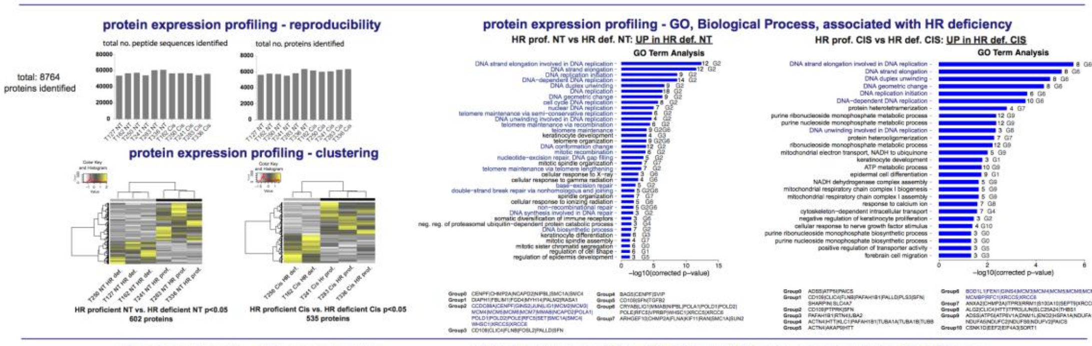
Patient Derived Xenograft (PDX) models of breast cancer

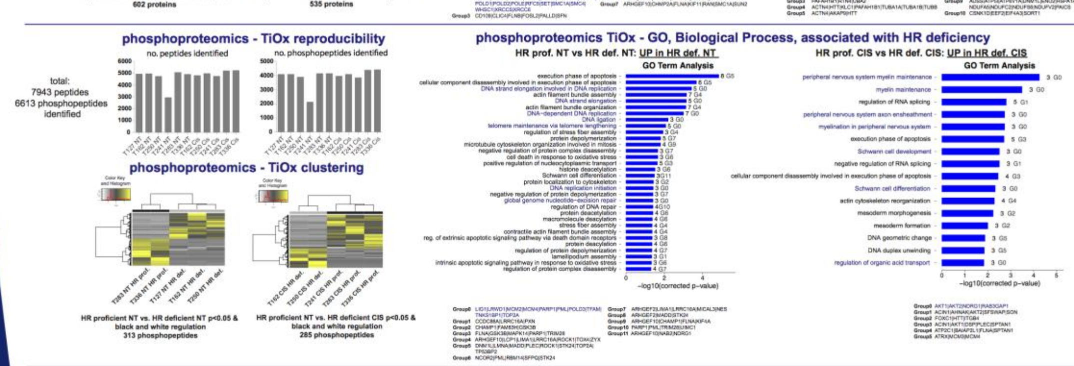
Tumor Biopsy; Passages; 6-12m; 6m; 6m; p0; p1; p2; • 4th mammary fat pad • Swiss nude mice; Tumor Characterization • Histology • aCGH • WB, mRNA

PROJECT OVERVIEW









Pilot study data are very promising and point to feasibility of the planned project. Both, protein expression profiling and phosphoproteomics data point to the importance of processes such as DNA replication, DNA initiation and DNA strand elongation in the context for HR deficiency in breast cancer.

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

Franziska Böttger[1], Tieneke B.M. Schaaij-Visser[1], Inge de Reus[1], Sander R. Piersma[1], Thang V. Pham[1], Jaco C. Knol[1], Erik Thunnissen[2], Kim Monkhorst[3], Egbert F. Smit[4], Sjaak A. Burgers[4], Connie R. Jimenez[1]

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Background Platinum-based drugs are the most common constituents of first-line treatment after surgery (adjuvant platinum-based chemotherapy) for non-small cell lung cancer (NSCLC), and the majority of patients are treated in a one-size-fits-all approach. However, efficacy is limited by occurrence of innate and acquired drug resistance; consequently, a significant number of patients do not benefit in terms of survival and, moreover, quality of life deteriorates due to side effects of this treatment.

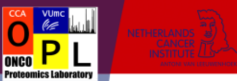
The **aim** of the project is to pinpoint which NSCLC patients who are more likely to derive clinical benefit from platinum-based chemotherapies using molecular markers will improve clinical outcome and reduce both toxicity and health care costs.

Approach Using label-free GeLC-MS/MS-based proteomics, we profiled archived tumor resection material of NSCLC patients (n=20) who received adjuvant platinum-based chemotherapy, and subsequently correlated protein expression profiles to clinical outcome in order to identify platinum response prediction biomarkers.

Results Preliminary results indicate increased hemostasis and immune system activity, as well as elevated expression of cell adhesion proteins as potential predictors of good response to platinum-based chemotherapy. Additionally, elevated ribosomal processes and antioxidant mechanisms correlate with poor response to platinum-based treatment. Furthermore, proteins involved in known platinum-response and -resistance pathways (apoptosis/DNA damage) were found to be significantly differentially expressed between good and poor responding patient groups.

Outlook Following extension of sample number size and analysis depths for the current biomarker discovery set, clinical proteomics data will be integrated with pre-clinical cell line proteomics data and public transcriptomics datasets to generate an annotated ranked list of the most promising platinum-response prediction biomarkers for further validation.

Acknowledgements: This project is supported by the Dutch Cancer Society (KWF VU2014-6816)



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

Franziska Böttger[1], Tieneke B.M. Schaaij-Visser[1], Inge de Reus[1], Sander R. Piersma[1], Thang V. Pham[1], Jaco C. Knol[1], Erik Thunnissen[2], Kim Monkhorst[3], Egbert F. Smit[4], Sjaak A. Burgers[4], Connie R. Jimenez[1]

[1]OncoProteomics Laboratory, Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands; [2]Department of Pathology, VU University Medical Center; [3]Division of Pathology, The Netherlands Cancer Institute, Amsterdam; [4]Division of Thoracic Oncology, The Netherlands Cancer Institute

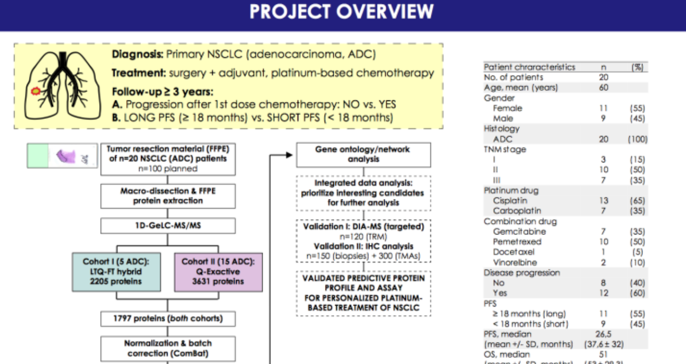
INTRODUCTION

- Platinum-based drugs are the most common constituents of first-line treatment after surgery (**adjuvant platinum-based chemotherapy**) for non-small cell lung cancer (**NSCLC**)
- Efficacy is limited by occurence of innate and acquired **drug resistance**; consequently, a significant number of patients do not benefit in terms of survival and, moreover, quality of life deteriorates due to side effects of this treament

AIM

Identification of a predictive protein profile (platinum-response **biomarkers**) for **personalized platinum-based treatment of NSCLC patients**

PROJECT OVERVIEW

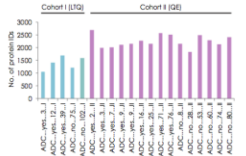


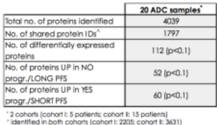
Patient chraracteristics	n	(%)
No. of patients	20	
Age, mean (years)	60	
Gender		
Female	11	(55)
Male	9	(45)
Histology		
ADC	20	(100)
TNM stage		
I	3	(15)
II	10	(50)
III	7	(35)
Platinum drug		
Cisplatin	13	(65)
Carboplatin	7	(35)
Combination drug		
Gemcitabine	7	(35)
Pemetrexed	10	(50)
Docetaxel	1	(5)
Vinorelbine	2	(10)
Disease progression		
No	8	(40)
Yes	12	(60)
PFS		
≥ 18 months (long)	11	(55)
< 18 months (short)	9	(45)
PFS, median (mean +/- SD, months)	26,5 (37,6 ± 32)	
OS, median (mean +/- SD, months)	51 (53 ± 29,3)	

TNM, tumor-node-metastasis; PFS, progression-free survival; OS, overall survival

RESULTS

Summary MS Results

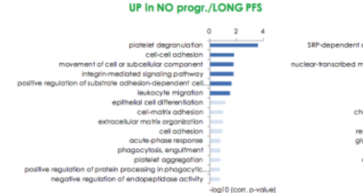


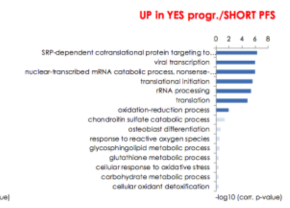


	20 ADC samples*
Total no. of proteins identified	4039
No. of shared protein IDs^	1797
No. of differentially expressed proteins	112 (p<0.1)
No. of proteins UP in NO progr./LONG PFS	52 (p<0.1)
No. of proteins UP in YES progr./SHORT PFS	60 (p<0.1)

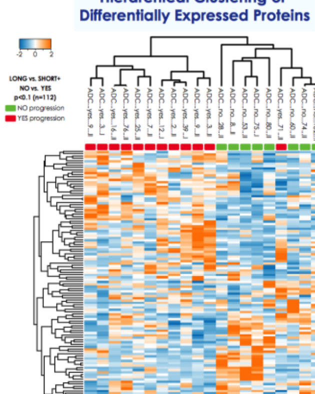
* 2 cohorts (cohort I: 5 patients; cohort II: 15 patients)
^ identified in both cohorts (cohort I: 2205; cohort II: 3631)

Enriched Gene Ontology Biological Process





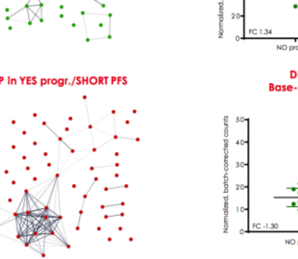
Hierarchical Clustering of Differentially Expressed Proteins



Functional Protein Association Networks

UP in NO progr./LONG PFS

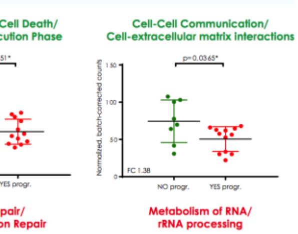
UP in YES progr./SHORT PFS



Examples of Candidate Platinum-Response Predictors

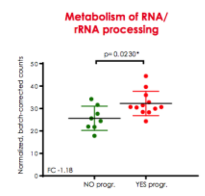
Programmed Cell Death/ Apoptotic Execution Phase

Cell-Cell Communication/ Cell-extracellular matrix interactions



DNA Repair/ Base-Excision Repair

Metabolism of RNA/ rRNA processing



CONCLUSIONS AND PERSPECTIVES

- Proteomic profiling of lung adenocarcinoma indicates increased **hemostasis/immune system activity** as well as elevated expression of **cell-cell and cell-ECM interaction** proteins as potential predictors of **good response** to platinum-based chemotherapy
- Elevated **ribosomal components/processes** and **antioxidant mechanisms** correlate with **poor response** to platinum-based chemotherapy
- **Proteins involved in known platinum-response and -resistance pathways (Apoptosis/DNA damage) found to be differentially expressed between good and poor responding patient groups**
- Following extension of sample number size and analysis depths for the current biomarker discovery set, clinical proteomics data will be **integrated** with our **pre-clinical cell line proteomics data** and **public transcriptomics datasets** to generate an annotated ranked list of the **most promising platinum-response prediction biomarkers for further validation**

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FUNDED BY DUTCH CANCER SOCIETY

Phosphotyrosine-based phosphoproteomics of a panel AML cell lines reveals oncogenic signaling and hyperactive tyrosine kinases as targets for treatment.

Carolien van Alphen[1,2], Jacqueline Cloos[2,3], Sander R. Piersma[1], Jaco C. Knol[1], Thang V. Pham[1], Johan van Meerloo[2], Gert J. Ossenkoppele[2], Henk MW. Verheul[1], Jeroen JWM. Janssen[2*] and Connie R. Jimenez[1*] *Shared senior/corresponding authors

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Background Acute myeloid leukemia (AML) is a clonal disorder arising from hematopoietic progenitors developing in the myeloid pathway characterized by deregulated differentiation and maturation programs. In AML, tyrosine kinases have been implicated in leukemogenesis, and are associated with poor treatment outcome. Kinase inhibitor treatment has shown promise in improving patient outcome in AML, however patients may benefit from better patient selection.

Aim Exlplore phosphotyrosine (pY) based phosphoproteomics to identify hyperphosphorylated, active kinases as targets and predictive biomarkers in AML cell lines to select KIs for treatment.

Results Kinase activation was determined based on kinase phosphorylation and, to strengthen the analysis, inferred kinase activity was assessed based on known kinase-substrate relations. In total, we identified 3605 class I phosphorylation sites in 16 AML cell lines that exhibited large variation in the number and level of phosphopeptides per cell line (241-2764), implying differences in signaling. Ranking analyses successfully pinpointed the hyperactive kinases PDGFRA, FGFR1, KIT and FLT3 in eight cell lines with a corresponding kinase mutation. These cell lines also were highly responsive to treatment with their respective kinase inhibitor.
Additionally, we identified unexpected drivers in two more cell lines (PDGFRA in Kasumi-3 and FLT3 in MM6) that also proved highly sensitive to specific kinase inhibitors. Six cell lines without a clear receptor tyrosine kinase (RTK) driver showed evidence of MAPK1/3 activation, consistent with the presence of activating RAS mutations. Importantly, and in line with cell line results, Flt3 phosphorylation and activity could also be demonstrated in two AML patients with a *FLT3* internal tandem duplication mutation.

Conclusions Our data show the potential of pY phosphoproteomics to identify key drivers in AML cells, and the predictive value of the phosphoproteome profiles for selecting for targeted treatment. These results warrant future investigation in clinical samples to improve our understanding of individual tumor biology and enable pY-phosphoproteomics-based personalized medicine.

Acknowledgements: This research is supported by a VUmc-CCA

Department of Medical Oncology
Department of Hematology
VUmc Cancer Center Amsterdam

Phosphoproteomics of a panel of AML cell lines reveals oncogenic signaling and candidate drivers

Carolien van Alphen[1,2], Jacqueline Cloos[2], Sander R. Piersma[1], Jaco C. Knol[1], Thang V. Pham[1], Gert J. Ossenkoppele[2], Henk Verheul[1], Jeroen JWM. Janssen[2*] and Connie R. Jimenez[1*]
[1]OncoProteomics Laboratory, Department of Medical Oncology, and [2]Department of Hematology, VU University Medical Center, Amsterdam, the Netherlands
*Shared senior authorship

Background

- Acute Myeloid Leukemia (AML) is a bone marrow malignancy.
- Patients receive standard induction therapy consisting of intensive chemotherapy.
- Long-term survival in AML is low (around 30%) due to relapse.
- Clinical successes have been achieved using kinase inhibitors and novel targets are needed to expand the treatment of AML.

Aim: **To characterize the phosphoproteome of 16 AML cell lines to gain insight into kinase signaling and identify hyperactive signal transduction pathways and key associated kinases that may be suitable for targeted treatment with kinase inhibitors .**

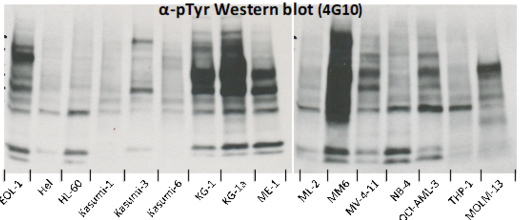
Methods

P-Tyr antibody-based based phosphoproteomics workflow

- AML cell lines harvested during growth phase.
- 10mg Protein input.
- PTMScan® Phospho-Tyrosine Motif (Y*) (P-Tyr-1000) Immunoaffinity Beads (Cell signaling)
- nanoLC: 50 or 75µm 20cm column, 2 hour gradient.
- QExactive: Top 10MS/MS per sec.
- Database searching: MaxQuant version 1.4.1.2

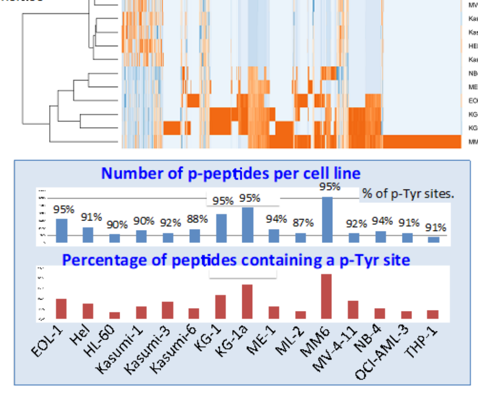
Results

1. Differential tyrosine phosphorylation patterns in AML cell lines implicate differential signaling activity



2. Unsupervised cluster analysis using all pTyr peptides (4220) reveals AML clusters with high and low tyrosine peptide phosphorylation patterns

Unsupervised clustering using phosphopeptide intensities



Number of p-peptides per cell line

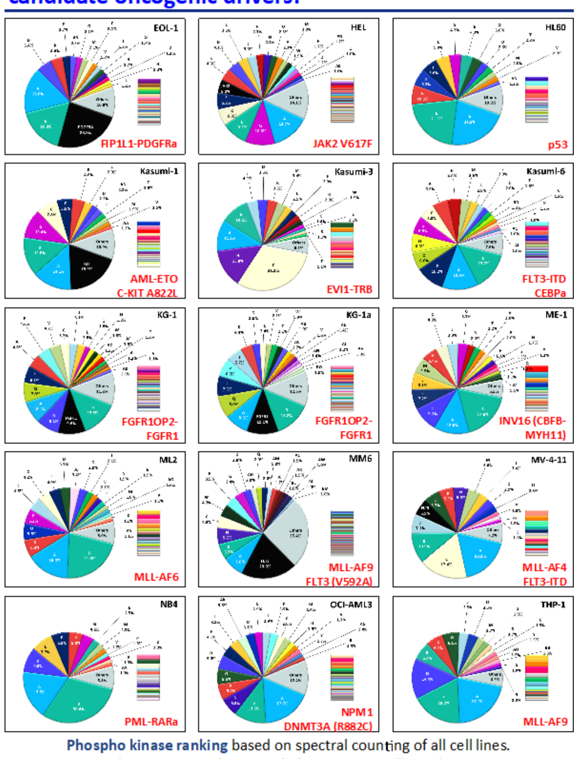
% of p-Tyr sites.

95% 91% 90% 90% 92% 88% 95% 95% 94% 87% 95% 92% 94% 91% 91%

Percentage of peptides containing a p-Tyr site

EOL-1, Hel, HL-60, Kasumi-1, Kasumi-3, Kasumi-6, KG-1, KG-1a, ME-1, ML-2, MM6, MV-4-11, NB-4, OCI-AML-3, THP-1

3. Phosphokinase ranking reveals known and candidate oncogenic drivers.



Phospho kinase ranking based on spectral counting of all cell lines.
Colors correspond to a single kinase across all pie charts.

4. Phosphoprotein network using kinases and direct interactors display multiple cellular processes

1. Translation
2. Proteasome
3. Transcription
4. Signal transduction

(String conf. 0.700)

EOL-1 kinase network of detected kinases and their direct interactors

Conclusion

- P-Tyr phosphoproteomics uncovers cellular protein phosphorylation patterns
- Analysis of 16 AML cell lines reveals know kinase drivers and new potential candidate oncogenic drivers not previously associated with AML
- Predicted drivers with matched kinase inhibitors (KIs) will be tested for their ability to induce cell kill

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Phosphoproteome networks display consistent hyperactive kinase activity in pancreatic cancer: evidence for new therapeutic options

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Introduction Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal disease due to its aggressive nature. Patients typically present with distant metastases, at which point cytotoxic agents can extend life expectancy by several months at most. Large-scale phosphoproteomics complements our knowledge obtained from genomics and transcriptomics as it provides information on which proteins and kinases are phosphorylated, thereby implicating pathways that are activated. This approach in cancer research may lead to improved patient selection for treatment with tyrosine kinase inhibitors (TKI). This study is the first to employ phosphotyrosine-based phosphoproteomics on three different preclinical PDAC models as well as patient tumor tissues to understand the aggressive nature of this disease and identify new drug targets.

Approach We performed phosphoproteomics on a panel of 11 PDAC cell lines, 7 primary cell cultures, 10 patient-derived xenografts (PDX) and 16 fresh frozen human tumor tissues. Tyrosine phosphopeptides were enriched via immunoprecipitation and phosphopeptides were analyzed by high-resolution nano-LC mass spectrometry.

Results Using phosphotyrosine-based phosphoproteomics, we identified a total of 1723 tyrosine phosphorylated proteins and 138 phosphorylated kinases, representing 27% of the kinome. The reproducibility of our workflow was very high, with Pearson correlation coefficients of $r = 0.937$ for technical replicates of cell lines and $r = 0.876$ for biological replicates of tumors. In our cell line panels, multiple kinases were commonly highly phosphorylated (e.g. PTK2, EPHA2, EGFR and MET). Functional testing of PTK2 by using the TKI defactinib in primary cell lines with high phosphorylation resulted in inhibition of proliferation and migration *in vitro*. Inhibition of EPHA2 by shRNAs resulted in reduced proliferation *in vitro*. To validate the relevance of these candidate target proteins *in vivo*, the tyrosine phosphoproteome of PDXs and human tumors was analyzed. In these tumors, kinase activity analysis based on phosphorylation levels and kinase-substrate networks validated these common active nodes in the majority of these tumors.

Conclusion Our extensive tyrosine phosphoproteome analysis spanning a wide range of PDAC models revealed high phosphorylation levels of multiple kinases. Interestingly, the phosphorylated kinase profiles of tumors and cell lines did not show as much heterogeneity as expected, taken into account the existence of biological subtypes in PDAC identified by others via transcriptomics. The aggressive biology of this disease may be correlated with the consistent activation of multiple pathways, some of which we have shown to be targetable *in vitro*. This study prompts further validation and prognostic evaluation of the identified active kinases to improve treatment of PDAC.

Le Large TYS, Bijlsma MF, Kazemier G, van Laarhoven HWM, Giovannetti E, Jimenez CR. Key biological processes driving metastatic spread of pancreatic cancer as identified by multi-omics studies. *Semin Cancer Biol.* 2017 Mar 30. pii:S1044-579X(17)30066-4.

Acknowledgements: This research is supported by a VUmc-AMC alliance project

Gemcitabine resistant pancreatic cancer cells are sensitive to paclitaxel treatment

T.Y.S. Le Large[1,2,3]. B. El Hassouni[1]. G. Kazemier[3].H.W.M. van Laarhoven[2,4]. C.R. Jimenez[1*]. M.F. Bijlsma[2*]. E. Giovannetti[1,5*]
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Cancer Pharmacology Lab (5). AIRC Start Up Unit. University of Pisa. Pisa. Italy.* These authors contributed equally.

Summary of Key Findings

1. Gemcitabine resistant cells of pancreatic ductal adenocarcinoma (PDA) show different protein expression and phosphorylation
2. Cytoskeleton and microtubule biology are differentially active in resistant cells
3. Resistant PDA cells show increased tumorigenic capacity
4. Resistant cells are sensitive to (nab-)paclitaxel treatment

Introduction

Patient suffering from PDA have a very poor prognosis.
The expected 5-year survival is currently 7.7%.

Adjuvant chemotherapy with gemcitabine results in a disease-free survival benefit of 7 months. However, treatment is hampered by intrinsic and acquired resistance.

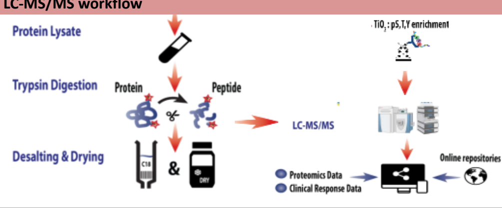
In this study, we report results of our label-free global and phosphoproteomic approach to identify the mechanism of gemcitabine resistance in PDA

Aim of the study:
Understand resistance mechanism in PDA against gemcitabine

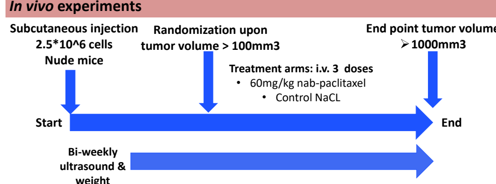
Material & Methods

PANC1 cell line & gemcitabine resistant cells of PANC1

LC-MS/MS workflow



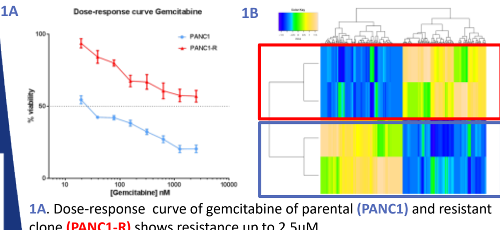
In vivo experiments



Example tumor volume measurement by ultrasound

Results

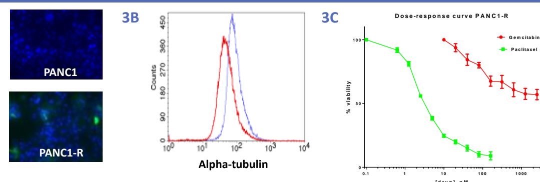
1. Gemcitabine resistant cells show differential protein expression



1A. Dose-response curve of gemcitabine of parental (PANC1) and resistant clone (PANC1-R) shows resistance up to 2.5uM
1B. Differential proteins clusters PANC1 and PANC1-R separately

Results

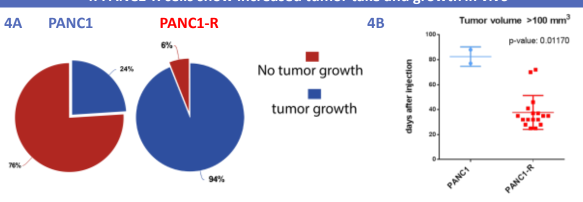
2. PANC1-R displays different biological processes



Gene name	Fold change	P-value
MAP2	10.2	0.000244
ANK3	22.8	0.000463
NES	6.3	0.000619
STAT6	5.9	0.001935
GSTM2	5.4	0.001971
CSPG4	13.4	0.004707
APLP2	6.2	0.013629
ARAP1	13.3	0.015568
SUN1;UNC84A	6.2	0.015885
SCARB1	6.2	0.015885

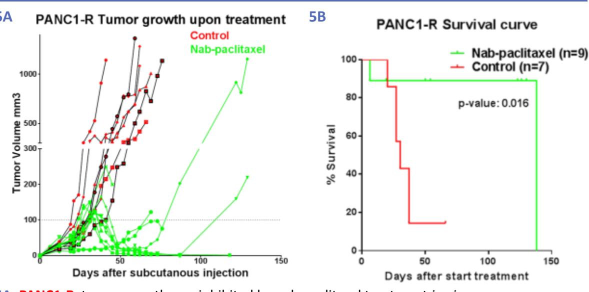
2A. Biological GO terms of upregulated proteins in resistant cells. Network was created with Cytoskape and ClueGO plugin. Filtering of GO terms p-value < 0.01
2B. List of top upregulated genes in PANC1-R. Microtubule-associated protein 2 (MAP2) was most significantly upregulated by protein expression and phosphorylation

3. MAP2 is upregulated and results in less stable microtubules in PANC1-R PANC1-R cells are sensitive to paclitaxel



3A. MAP2 expression is upregulated in PANC1-R compared to the PANC1, visualized with IF
3B. PANC1-R cells have less stable microtubules than PANC1 as measured by flow cytometry
3C. Dose-response curve of paclitaxel; PANC1-R cells remain sensitive to taxane treatment

4. PANC1-R cells show increased tumor take and growth *in vivo*



4A. Subcutaneous injection of PANC1 and PANC1-R resulted in more tumor establishment in the PANC1-R group. The low tumor take of the parental cells might be due to microbioma and nude mice strain, while the more agressive resistant cells can adapt more easily
4B. PANC1-R tumors grow significant faster than the parental sensitive cell line

5. PANC1-R tumors are sensitive to nab-paclitaxel treatment *in vivo*

5A. PANC1-R tumor growth was inhibited by nab-paclitaxel treatment *in vivo*
5B. Mice harboring PANC1-R tumors survive significantly longer than the control group

Conclusion and Discussion

Through our global proteome analyses, we identified that gemcitabine resistant PDA cells have different protein expression and activated biological processes. MAP2 is one of the proteins differentially upregulated and phosphorylated in gemcitabine resistant cells. Resistant PDA cells were sensitive to taxane treatment *in vitro* & *in vivo*. Interestingly, resistant cells showed increased potential of tumor initiation. Possibly, this indicates a more stem cell-like property of some resistant subclones.

These results underline the potential of current clinical treatment with combination of gemcitabine and nab-paclitaxel. Differential proteins, like MAP2, might be predictive biomarkers for patient selection of combination treatment, also in the adjuvant setting.

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Identification of signaling pathways involved in colorectal adenoma-to-carcinoma progression using phosphoproteomics

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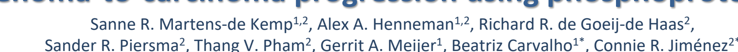
Background Colorectal cancer (CRC) develops in a multi-step-process from normal epithelium, through a pre-malignant lesion (adenoma), into a malignant lesion (adenocarcinoma). A minority of about 5% of adenomas will ultimately progress into cancer. To improve clinical practice it is important to identify the subgroup of colorectal adenomas that is at risk of progressing to cancer.
By extensive genomic analysis we have shown that specific copy number alterations are associated with risk of progression. An example of such an alteration is the gain of chromosome arm 20q and we identified AURKA and TPX2 as major drivers of this amplicon. Yet, the activation status of these and other genes during colorectal carcinogenesis and the downstream signaling pathways affected with gene (in)activation are not fully known.
In order to better understand the biology of adenoma to carcinoma transition, we performed a comprehensive analysis of phosphoproteomes at different stages of colorectal carcinogenesis.

Methods Phosphotyrosine containing peptides were immunoprecipitated from 5 mg of colorectal adenoma (n=81) and colorectal carcinoma tissues (n=50) using agarose bead-coupled phosphotyrosine antibody P-Tyr-1000. A Q Exactive HF mass spectrometer was used to perform NanoLC-MS/MS. Spectral counts of phosphoproteins and ion intensities of phosphopeptides were defined by MaxQuant for relative quantitation of protein phosphorylation.

Results Phosphotyrosine-based phosphoproteomics of the 131 colorectal tissue samples yielded 6056 phosphopeptides, corresponding to 2745 unique phosphoproteins including 183 phosphokinases. Data analysis to identify regulated phosphorylation states and inference of kinase activity is ongoing.

Conclusions and future plans This is the first large phosphoproteomics dataset of colorectal adenomas and carcinomas. Preliminary data analysis shows promising differences in kinase activities between adenomas and carcinomas. After further analysis, we will manipulate the activity of the driver kinases in adenoma-derived organoids and monitor outgrowth in mice.

Acknowledgements: This project is supported by the Dutch Cancer Society (KWF VU2014-6813)



Identification of signaling pathways associated with colorectal adenoma-to-carcinoma progression using phosphoproteomics

Sanne R. Martens-de Kemp[1,2], Alex A. Henneman[1,2], Richard R. de Goeij-de Haas[2], Sander R. Piersma[2], Thang V. Pham[2], Gerrit A. Meijer[1], Beatriz Carvalho[1*], Connie R. Jiménez[2*]

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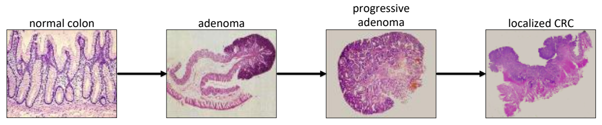
2 Oncoproteomics Laboratory, Medical Oncology, VU University Medical Center; *shared senior authors

INTRODUCTION

- Colorectal cancer (CRC) develops in a **multistep process**
- **Adenoma** is a CRC premalignant lesion
- Approximately **5% of adenomas progresses** to carcinoma



BACKGROUND

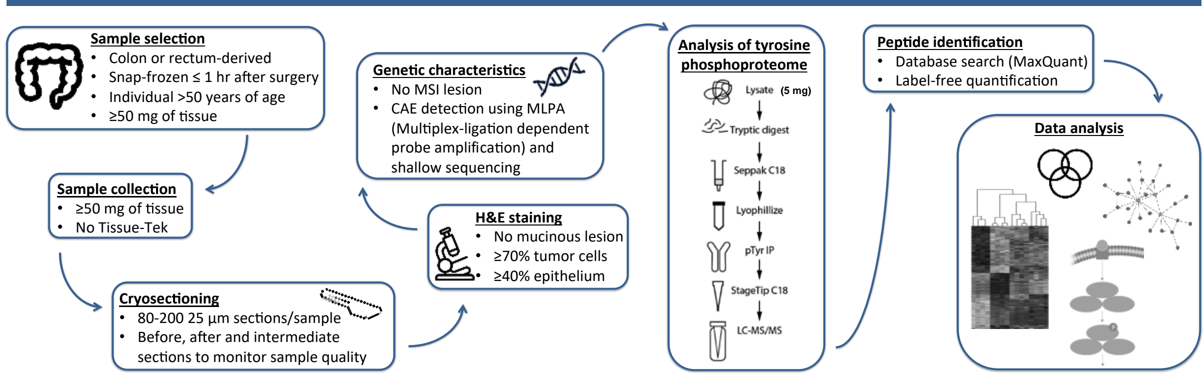
- **CRC development is extensively studied** on DNA, RNA and protein level
- Specific DNA copy numbers alterations are associated with **risk of progression (CAEs – Cancer Associated Events)**[1]
- Unknown which **biochemical signaling pathways** are involved in progression from adenoma to carcinoma

[1] Hermsen et al., Gastroenterology, 2002

AIM

Identify which signaling pathways are active at different stages of colorectal adenoma-to-carcinoma progression using phosphoproteomics.

STUDY DESIGN



RESULTS

Sample collection

Sample	Selected from database	Retrieved from biobank[a]	After H&E inspection[b]
Adenoma	136	119	81
Carcinoma	126	107	59

[a] Some tissues were not available, too small or clearly defrosted
[b] Some tissues had too low tumor cells and/or epithelium

Genetic characteristics

Sample	MSI detected	Samples included
Adenoma	0	81
Carcinoma	9	50

Sample	CAEs detected	Risk of progression	Samples included
Adenoma	<2	Low-risk	69
	≥2	High-risk	9
	ND	Unknown	3

Samples in pTyr

Sample	CAEs detected	Risk of progression	Succesfull pTyr capture
Adenoma	<2	Low-risk	67
	≥2	High-risk	9
	ND	Unknown	3
Carcinoma	ND	-	46

Peptide identification

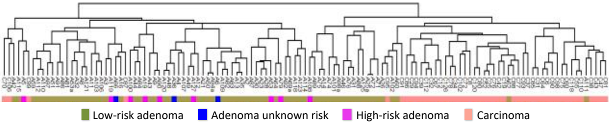
	79 adenomas and 46 carcinomas
Peptides identified	34923
Phosphopeptides identified	6056
Shared phospopeptides[ǂ]	3841
Up in adenomas (p<0.01)*	66
Up in carcinomas (p<0.01)*	95

ǂ Shared between adenomas (n=79; 5850 peptides) and carciomas (n=46; 4022 peptides)
* All adenomas vs carcinomas, phosphopeptides ≥40% valid values across all samples (n=697), student's t-test

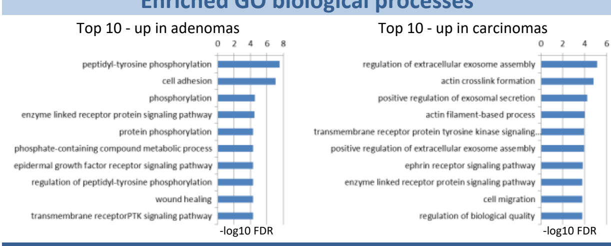
Peptides Phosphopeptides
Median no. of Ids
14000 12000 10000 8000 6000 4000 2000 0
Low-risk Unknown High-risk CRC
Adenoma

Adenomas versus carcinomas

Hierarchical clustering of significantly differential detected phosphopeptides in adenomas versus carcinomas (Student's t-test, p<0.01, Pearson correlation distance)



Enriched GO biological processes



Protein interaction network

Up in adenomas (p<0.01)

Up in carcinomas (p<0.01)

CONCLUSIONS AND FUTURE PLANS

- **First large clinical phosphoproteomics dataset** of colorectal adenomas and carcinomas
- Preliminary data analysis shows **promising differences in kinase activities** between adenomas and carcinomas
- After **further data analysis**, we will **manipulate the activity** of the driver kinases in adenoma-derived organoids and **monitor outgrowth** in mice

This project is supported by KWF project: NKI 2014-6813

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Phosphoproteomics in metastatic colorectal cancer for predicting response to anti-EGFR therapy

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Background: The discovery of the key role of the epidermal growth factor receptor (EGFR) and its downstream signalling effectors in the pathophysiology of colorectal cancer (CRC) has resulted in the clinical use of targeted therapies in the treatment of metastatic CRC (mCRC). However, clinical benefit to EGFR blockade is observed in only a subgroup of CRC patients wild type for *KRAS*, *NRAS* and *BRAF*. In this study we performed phosphoproteomics of patient-derived xenograft models to shed new insights into cellular signaling of mCRC, identify of alternative drug targets and predictive markers.

Methods: Pharmacologically and genomically characterized mCRC-PDX models[1,2] were analyzed. Phosphopeptides where enriched by (p)-tyrosine immunoprecipitation and titanium dioxide. Dedicated bioinformatics was used to identify hyperactive kinases and predictive biomarkers. Identified alternative drug targets where functionally validated in mCRC-PDX models and PDX derived organoids.

Results: In total 15.095 phosphorylated peptides derived from 4236 proteins where detected, including 255 phosphorylated kinases. Among other kinase ERBB2, EGFR, INSR and MET where identified as hyperactive driver kinases in cetuximab-resistant models. Sensitivity to inhibition of driver kinases alone or in combination with EGFR was confirmed in corresponding PDX-derived organoids. Group-based analysis identified potential biomarkers for response to cetuximab

Conclusion: Our findings improve the understanding of signaling pathways in mCRC and provide proof-of-principle of using phosphoproteomics for identification of driver kinases and patient stratification for individualized treatment.

Acknowledgements: This work was supported by VHS Health Services

Phosphoproteomics for Cetuximab Response Prediction in Colorectal Cancer

Robin Beekhof[1], Mariette Labots[1], Richard de Haas[1], Sander Piersma[1], Thang V. Pham[1], Jaco Knol[1], Tim Schelfhorst[1], Andrea Bertotti[3], Nicole van Grieken[2], Livio Trusolino[3], Henk Verheul[1], Connie R Jimenez[1]

[1]Dept. Medical Oncology, [2]Dept. of Pathology, VU University Medical Centre, Amsterdam, The Netherlands
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Introduction

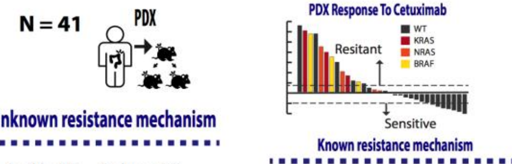
There is an urgent need for systemic treatment options in patients with metastatic colorectal cancer (mCRC). Multiple tyrosine kinase inhibitors have been approved for clinical use. However, only a subset of patients benefits from kinase inhibition. Despite current knowledge of cancer genomes it is still a challenge to predict response to therapy. Typically only 50% of selected patients benifid from current molecular profiles. Phosphoproteomics allows for comprehensive protein phosphorylation profiling and may be a valuable complement to current genomic profiling. Patient derived xenografts (PDX) are a valuable model to mimic clinical response to therapy. Thus far, PDX models have extensively been used to characterize the genomic background of tumors.

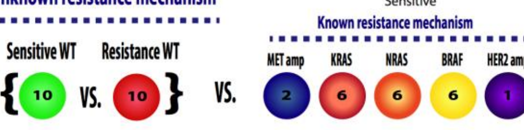
Aim: Provide novel predictive biomarkers and alternative kinase targets in CRC

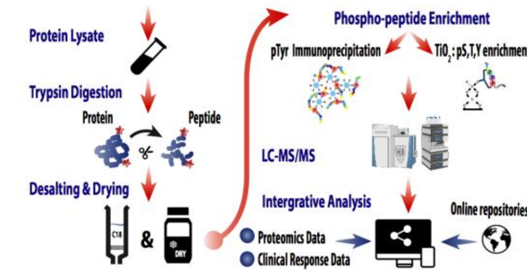
Phosphoproteome profiling in mCRC PDX models (known Cetuximab resp.)

- To Identified resistance mechanisms that cannot be explained by current genomic profiling
- Predictive Biomarkers, Alternative Drug Targets

Approach

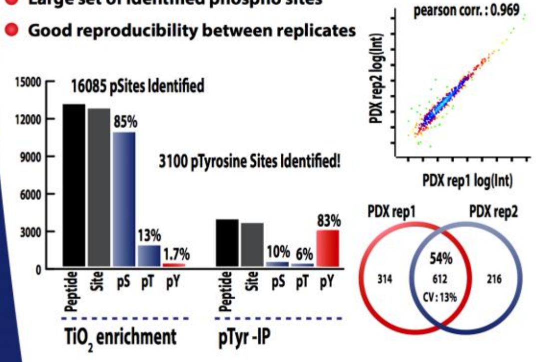




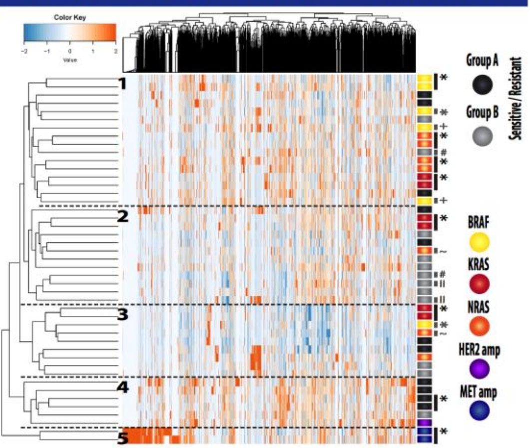


Results

- **Large set of identified phospho sites**
- **Good reproducibility between replicates**



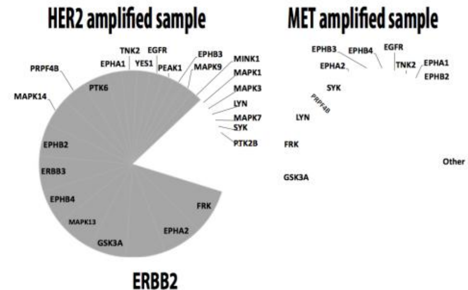
Unsupervised clustering of pTyr IP phosphopeptides



- Most replicates cluster togeter
- Cluster 1 contains mostly BRAF, NRAS, KRAS mutatad samples
- Cluster 2 contains mostly Group B
- Cluster 4/5 seems to represent RTK amplified samples

Summarized pPetide counts per Kinase

Clear hyper phosporylation of MET /HER2



Conclusion and Future Plans

* Currently largest pTyr-proteomics data set on Patient-derived xenografts
* Correlation to Cetuximab response data
* 45% of Kinome covered

Good reproducibelity between replicates

Some logic is visible in Unsupervised clustering

Clear hyper phosporylation of RTK amplified samples

- **Continue Data Analysis**
- Correlation to response data, Network Analysis
- Biomarker selection, Validation of drug targets

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The Signaling Pathways of Folliculin: a Tumor Suppressor in Birt-Hogg-Dubé Syndrome

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Background Birt-Hogg-Dubé syndrome (BHD) is an autosomal dominant syndrome caused by a loss-of-function germline mutation of the Folliculin (FLCN) gene. Upon LOH or a second somatic mutation patients suffer from benign skin lesions, spontaneous pneumothoraces, lung cysts and, most importantly, bilateral kidney cancer. Until now the function and molecular pathways that are controlled by FLCN are insufficiently clear.
Using a proteomic approach we wish to elucidate FLCN's biological role and uncover how FLCN loss leads to kidney cancer.

Methods As a first step we have performed label-free GeLC-MS/MS-based proteomics on a BHD tumor derived cell line UOK257 ($FLCN^{-}$) and the FLCN restored cell line UOK257-2 (n=2 per condition). For future proteomic analyses we will use the CRISPR/Cas9 technology to develop a new unique BHD model system *in vitro*.

Results Proteomic analyses of UOK257 vs. UOK257-2 cell line lysates identified 5112 proteins, of which 451 were significantly different. Using our stringent EdgeR filtering script, 94 proteins were selected to be the most differential expressed between UOK257 and UOK257-2 cells. GO analysis revealed involvement of multiple biological processes such as MAPK signaling, cytoskeleton organization, cell migration and metabolism.

Conclusions Proteomics of the BHD cancer cell line system has shown wide impact of FLCN status on the proteome, with multiple biological processes affected including MAPK signaling, cytoskeleton organization, cell migration and metabolism. On-going analyses of our newly created isogenic FLCN $^{-/-}$ isogenic cell lines will reveal how FLCN influences gene and protein expression in an oncogenic model setting. Future studies will focus on functional and clinical validation of the most important proteins.

CRISPR & proteomic studies of Folliculin:
a kidney-specific tumor suppressor in Birt-Hogg-Dubé syndrome

Iris E. Glykofridis[1,2], Jaco C. Knol[1], Irsan E. Kooi[2], Thang V. Pham[1], Tim R.A. Schelfhorst[1], Sander R. Piersma[1], Rob M.F. Wolthuis[2,*] & Connie R. Jimenez[1,*]

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[2] Oncogenetics Laboratory, Department of Clinical Genetics, VUmc Cancer Center Amsterdam, The Netherlands
[*] Shared senior authors

Introduction

Birt-Hogg-Dubé syndrome (BHD) is an autosomal dominant syndrome caused by a loss-of-function germline mutation of the Folliculin gene (FLCN). Upon LOH or a second somatic mutation, patients develop benign skin lesions, spontaneous pneumothoraces associated with lung cysts and, most importantly, kidney cancer. For mutation carriers the risk for pneumothorax is ~29% and the risk for developing kidney cancer is ~16%.[1] Although some patients exhibit all three components of the syndrome, pneumothorax-only and kidney-cancer-only families have also been described.

Until now the function and molecular pathways that are controlled by FLCN are insufficiently clear. In this study we wish to identify new FLCN pathways and uncover how FLCN loss leads to kidney cancer, which might be conducive to therapy development.

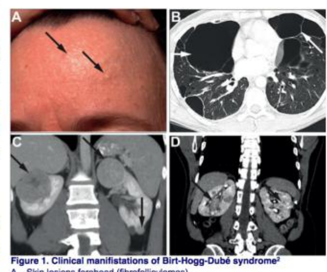

Figure 1. Clinical manifestations of Birt-Hogg-Dubé syndrome[2]
A – Skin lesions forehead (fibrofolliculomas)
B – Chest CT scan showing bilateral multiple pulmonary cysts
C & D – Abdominal CT scans of BHD renal tumors demonstrating bilateral multifocal tumors

Use CRISPR/Cas9 to develop BHD model system *in vitro* → Proteomic & RNAseq analyses to assess expression differences → Functional analyses of newly identified pathways → Evaluation clinical importance BHD patients

Results

I – BHD cell line models

Figure 2. Targeting the FLCN gene using CRISPR/Cas9 system.
A – Western blot validation of αFLCN antibody at BHD tumor model cell line UOK257 ($FLCN^{neg}$) and UOK257-2 ($FLCN^{pos}$) lysates. To check antibody specificity a siFLCN experiment was performed in RPE/hTERT cells.
B – Design of different gRNAs against multiple exons of FLCN gene. Specific FLCN isoforms were taken into account.
C – CRISPR/Cas9 mediated KO of FLCN in Renal Proximal Tubule Epithelial Cells (RPTEC/hTERT). In tet-on Cas9 RPTEC cells TP53 was knocked out, followed by transfection of different gRNAs against FLCN. FLCN KO clones were confirmed by western blot and sequencing.

II – Proteomic workflow & analyses

Data summary UOK257 vs. UOK257-2
proteins identified: 5112
significant differential proteins: 94

Figure 3. Proteomic workflow, cell line lysate details & data summary.
A – Overview of label-free GeLC-MS/MS workflow[3]
B – Coomassie staining of 1DGE gel with UOK257 & UOK257-2 lysates used for mass spectrometry. As depicted by arrows, two replicates per cell line were used.
C – Proteomic analyses identified 5112 protein IDs in total. Using EdgeR[4], peptide counts were TMM normalized and a 2-group comparision of unpaired data was performed; $FLCN^{pos}$ (n=2) vs. $FLCN^{neg}$ (n=2). 94 proteins were selected to be most differential (FDR<0.05).

III – Differentially expressed FLCN dependent proteins

Figure 4. FLCN dependent differentially expressed proteins in BHD tumor model system.
A – 94 most differential proteins between $FLCN^{pos}$ (UOK257-2) and $FLCN^{neg}$ (UOK257) cell lines (FDR<0.05).
B – Networks of significantly different proteins[5]. Top shows proteins up in $FLCN^{neg}$, bottom shows proteins down in $FLCN^{neg}$

Summary & future plans

- Proteomics of the BHD tumor model system has shown wide impact of FLCN status on the proteome, with multiple biological processes affected including MAPK signaling, cytoskeleton organization, cell migration and metabolism
- Successfully created multiple FLCN and FLCN/TP53 knockout cell line pairs using CRISPR/Cas9
- Future proteomic & RNAseq analyses of our newly developed BHD model system will reveal how FLCN influences gene and protein expression even further

References
[1] Houweling *et al.*, (2011)
[2] Schmidt & Linehan (2015)
[3] Piersma, Jimenez *et al.*, (2013)
[4] Robinson *et al.*, (2010)
[5] Snel *et al.*, (2000)

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Oncogenetics

Phosphoproteomic analysis of acquired resistance to trastuzumab and dual HER2-blockade in HER2-positive breast cancer cells

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

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*Shared senior authors.

Background While HER2-overexpressing breast cancers targeted by trastuzumab frequently acquire resistance to this therapy, dual HER2-blockade with trastuzumab and pertuzumab significantly improves the prognosis of this disease. In this study, we used an integrative approach to compare global protein expression and tyrosine phosphorylation events involved in both acquired trastuzumab-resistance and response to dual HER2-targeted therapy with trastuzumab and pertuzumab.

Methods We performed phosphotyrosine peptide immunoprecipitation coupled to quantitative label-free mass spectrometry and database searching in an established *in vitro* model of acquired trastuzumab-resistance to investigate initial sensitivity to trastuzumab, eventual reprogramming in resistance and inhibitor response to dual therapy. Dedicated computational approaches were combined to identify altered signalling networks and molecular reprogramming upon trastuzumab-resistance.

Results In total, our study yielded 2308 phosphopeptides on 1134 proteins, including 1855 class I phosphopeptides and 94 protein kinases. Compared to baseline, incubation with trastuzumab and dual therapy of trastuzumab-resistant cells resulted in downregulation of 218 and 356 phosphopeptides, respectively, while 294 and 218 phosphopeptides were upregulated (Fc> 2). Interestingly, exposure of trastuzumab-resistant cells to dual therapy resulted in 24 phosphoproteins significantly more downregulated than compared to exposure to trastuzumab. We further prioritized certain signalling nodes by overlapping: (i) differential expression in untreated resistant and parental cells, (ii) regulation relevant for sensitivity in response to both trastuzumab and dual therapy of parental cells, (iii) opposite/deficient regulation in response to trastuzumab of resistant cells, and (iv) same response to dual therapy of resistant and sensitive cells.

Conclusions Our data confirmed previously known roles for Ras/MAPK and PI3-kinase signalling in trastuzumab-resistance, as tyrosine-phosphorylated FGFR1, FGFR3-4, MET, MAPK3, and PIK3R1-3 were upregulated in trastuzumab-resistant cells. Altogether, our phosphoproteomics study provides network-level insights into molecular alterations associated with cancer drug resistance and suggests potential biomarkers and treatment options for trastuzumab-resistant tumors.

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Phosphoproteomic analysis of acquired resistance to trastuzumab and dual HER2-blockade in HER2-positive breast cancer cells

Paula González-Alonso[1,2], Sander R Piersma[2], Jaco C Knol[2], Alex Henneman[2], Thang V Pham[2], Richard R de Haas[2], Ester Martín-Aparicio[1], Sandra Zazo[1], Juan Madoz-Gúrpide[1], Ana Rovira[3], Joan Albanell[3], Pilar Eroles[4], Ana Lluch[4], Connie R Jiménez[2,*], and Federico Rojo[1,*] (paula.galonso@fjd.es).

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INTRODUCTION

- HER2-positive (HER2+) breast cancers targeted by trastuzumab frequently acquire resistance to the therapy.
- Dual therapy with trastuzumab and pertuzumab significantly improves the prognosis of trastuzumab-resistant cancer.

AIM

1. To identify predictive biomarkers of resistance to trastuzumab comparing BT-474 trastuzumab-sensitive and acquired trastuzumab-resistant phenotypes.
2. To identify HER2-targeted drug response biomarkers in trastuzumab-sensitive and acquired trastuzumab-resistant BT-474 cell lines.

MATERIALS AND METHODS

Experimental design

2h treatments →	Control	Trastuzumab	Trastuzumab Pertuzumab
Sensitive			
Resistant*			

*Trastuzumab-resistant, Dual treatment-sensitive

Cell Harvest,
In-solution Trypsin Digestion,
SCX,
Phosphopeptide Enrichment by pTyrIP,
LC-MS/MS (Q Exactive)
Class I Phosphosites Quantification (MaxQuant)
Analysis of Protein Phosphorylation Profiles (Perseus and R),
PPI Networks (Phosphopath, Cytoscape)

RESULTS

Data filtering strategy

Priority to signalling nodes by overlapping all tyrosine phosphorylation profiles:

Aim 2 — HER2-targeted drug response biomarkers:

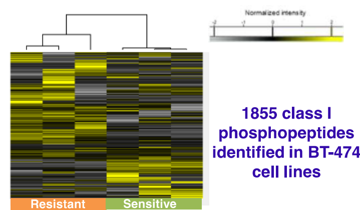
A. Regulation relevant for sensitivity in response to trastuzumab of sensitive cells
B. Regulation relevant for sensitivity in response to dual therapy of sensitive cells.
C. Same response to dual therapy of resistant cells and sensitive cells.
D. Non-response or opposite regulation of trastuzumab-treated resistant cells.

Aim 1 — Trastuzumab-resistance biomarkers:

E. Differential expression in untreated trastuzumab-resistant and -sensitive cells.
E'. Differential expression in trastuzumab-resistant and -sensitive cells upon trastuzumab treatment.

HER2+ breast cancer trastuzumab-resistance biomarker identification

Unsupervised clustering separates cell groups



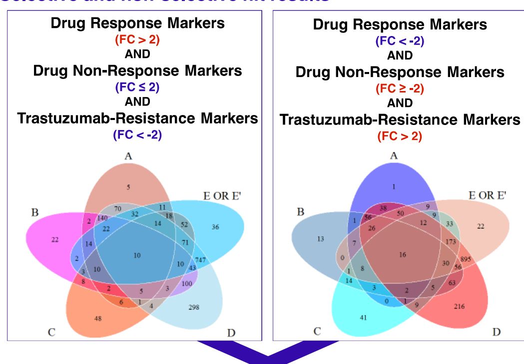
1855 class I phosphopeptides identified in BT-474 cell lines

CONCLUSIONS

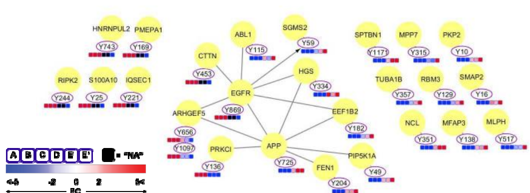
- We identified functional differences in tyrosine phosphoprotein profiles between trastuzumab-resistant and -sensitive BT-474 cells.
- Out of 2308 phosphorylation sites, 10 and 16 promising biomarker candidates were identified that showed a highly significant up- and down-regulation, respectively, upon trastuzumab-resistance and treatment effect filtering.
- Most of drug response biomarkers belonged to cell adhesion proteins and were involved in regulating immune processes.
- Our phosphoproteomics study provides network-level insights into molecular alterations associated with HER2+ breast cancer drug resistance and suggests potential biomarkers for trastuzumab-resistant tumors.
- These results are currently being confirmed in an independent experiment with bio-replicates

RESULTS

Selective and non-selective hit results

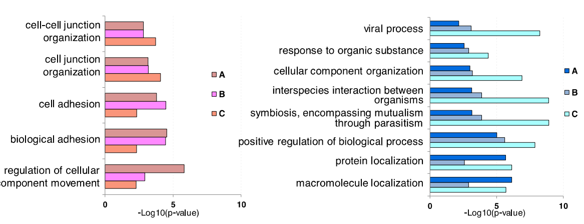


PPI network: potential biomarkers of trastuzumab-resistance and sensitivity to anti-HER2 therapy



GO Biological Processes up in Drug Response

GO Biological Processes down in Drug Response



Information:
www.oncoproteomics.nl
http://www.fjd.es/iis_fjd/en/areas-research-groups/cancer/histopathology

Stromal and epithelial proteomic landscapes of pancreatic cancer predict poor prognosis and recurrence

Authors: T.Y.S. Le Large, G. Mantini, L.L. Meijer, T.V. Pham, N. Funel, N.C.T. van Grieken, S.R. Piersma, H.W.M. van Laarhoven, M.F. Bijlsma#, E. Giovannetti#, C.R. Jimenez*, G. Kazemier*
corresponding authors, * shared senior authorship

Introduction Large scale efforts to identify key mediators of tumor biology have focused on the tumor compartment as an entity by itself. However, the composition of the tumor can be reflected inadequately with the measurement of bulk tumor. The relevance of elucidating tumor biology with their surrounding is specifically of importance in tumors consisting of high stromal content. One of the most characteristic cancers embedded in a large desmoplastic microenvironment is pancreatic ductal adenocarcinoma (PDA). Their tumor volume can consist of small tumor islands engulfed by up to 90% of extracellular matrix and stromal cells. Their stroma is known to influence multiple pro-tumorigenic features of the PDA cells, stimulating proliferation and metastatic growth, creating an immune suppressive environment and activate signaling via mechanobiology. Laser capture micro dissection (LCM) can select compartment specific areas, increasing our knowledge of the complete tumor composition. Up to date, no large scale proteomic studies have been performed to identify the tumor and stromal composition in PDA. In this study, we hypothesize that tumor and stromal proteomic landscapes can yield additive molecular information and have different prognostic impact.

Methods Consecutive snap frozen tumor samples from January 2014 until November 2015 of the HPB Biobank from the VU University Medical Center were evaluated for their quality and tumor percentage. 16 selected samples were prepared on PEN foil slides (Leica). Sections were stained with Mayers Haematoxylin and subsequently dehydrated. LCM was performed for a total surface of 3.000.000um2 per compartment. Selected areas were captured in 0.1% rapigest and underwent in solution digestion after which they were measured by nano-LC-MS/MS. Spectra were matched *in silico* against a human protein database. Match-between-run was allowed for analysis of low abundant proteins. Data was analyzed with R, paired Limma statistics and corrected for multiple testing by the Benjamini Hochberg equation. Correlation to survival was performed to identify prognostic proteins. Unsupervised clustering was used to identify subtypes. Gene set enrichment (GSEA) was performed to understand biological differences.

Preliminary results Out of 39 tumor samples, 16 were selected based on their quality to establish adequate LCM enrichment of both compartments. We identified over 6000 proteins in our PDA samples, of which over 2000 were differentially expressed between the two compartments. GSEA showed matrisome, coagulation, hypoxia-related proteins and epithelial-to-mesenchymal transition enriched in stroma. Interestingly, spliceosome and fatty acid metabolism were highly enriched in tumor sections, guiding possible new treatment targets. Clustering does not show obvious proteomic subtypes, however, further analysis and correlation to known subtypes will need to be evaluated. Several patients had no recurrence and differential expression of these long surviving patients compared to patients with a very quick disease progression identified prognostic markers in each compartment. Many of these genes are known prognostic poor markers, however, several new prognostic genes are unknown in PDA, highlighting the added importance for proteome analysis.

Conclusion This is the first large-scale compartment specific proteomic landscape of PDA. With this dataset, bulk protein analysis can possibly be deconvolved to their compartment. Moreover, we have shown that prognostic genes can be in either tumor or its microenvironment. This underlines the need to understand the heterocellular biology of PDA.

Future perspectives This dataset will be the basis for future *in vitro* analysis of new targets of tumor signaling and bidirectional stimulatory communication between PDA and stromal cells. Elucidation of immune signatures can guide us to understand and modulate the immune

evasive microenvironment of PDA. Moreover, prognostic signatures will be validated on a larger cohort by immunohistochemistry.

Acknowledgements: This research is supported by a VUmc-AMC alliance project

Loss of NARFL function deregulates iron regulatory and cohesin complex cleavage proteins under hyperoxia-induced oxidative stress

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Background NARFL is a component of the essential CIA pathway, which is responsible for the assembly of Fe-S proteins. In a previous study we showed, using a unique set of hyperoxia-resistant HeLa cells, that NARFL is a key component in cellular survival against hyperoxia-induced oxidative stress,with a potential role for this protein in sister-chromatid cohesion and maintenance of genomic integrity.

Aim In this study, we set out to further unravel the role of NARFL using a proteomics analysis.

Results First, cell cycle analysis results showed an apparent accumulation of cells in the G2 phase as a result of NARFL depletion combined with hyperoxic stress. To gain further insight, we compared the proteomes of a hyperoxia-resistant cell lines placed under hyperoxic stress, in the absence or presence of NARFL depletion. Loss of NARFL combined with hyperoxic stress resulted in the deregulation of 222 proteins out of a total of 5645 identified proteins. Regulated proteins included proteins of the CIA pathway and the proteins IRP1 and IRP2 that form the cells iron regulatory system. We also identified three AKR1C (AKR1C1, AKR1C2, and AKR1C3) proteins which have previously been associated with drug resistance in tumor cells, as well as ionizing-radiation. The protein ESPL1 was also deregulated and has been shown to be involved in cleavage of centromeric cohesin complexes.

Conclusion The loss of NARFL function in combination with hyperoxic stress might jeopardize the function of these proteins resulting in the disruption of cellular mechanisms such as iron homeostasis and centromeric cohesion.

Appendix 1. Abstracts & poster summaries of OPL core research

OncoProteomics: Liquid biopsy-based non-invasive biomarkers

Novel stool-based protein biomarkers for improved colorectal cancer screening: a case-control study

Linda JW Bosch[1#], Meike de Wit[1,2#], Thang V Pham[2], Veerle HM Coupé[3], Annemieke C Hiemstra[1], Sander R Piersma[2], Gideon Oudgenoeg[2], George L Scheffer[4], Sandra Mongera[4], Jochim Terhaar Sive Droste[5], Frank A Oort[5], Sietze T van Turenhout[5], Ilhame Ben Larbi[5], Joost Louwagie[6], Wim van Criekinge[1,6,7], Rene WM van der Hulst[8], Chris JJ Mulder[5], Beatriz Carvalho[1], Remond JA Fijneman[1], Connie R Jimenez[2#], Gerrit A Meijer[1#]

[1]Department of Pathology, Netherlands Cancer Institute, Amsterdam, the Netherlands. Department of [2]Medical Oncology, [3]Clinical Epidemiology and Biostatistics, [4]Pathology, [5]Gastroenterology and Hepatology, VU University Medical Center, Amsterdam, the Netherlands. [6]MDxHealth SA, Herstal, Belgium. [7]Department of Mathematical Modelling, Statistics and Bioinformatics, Ghent University, Ghent, Belgium. [8]Department of Gastroenterology and Hepatology, Kennemer Gasthuis, Haarlem, The Netherlands.

Background The fecal immunochemical test (FIT) is used in many countries for non-invasive screening for colorectal cancer (CRC), but its characteristics leave room for improvement. We aimed to identify novel stool-based protein markers in stool that outperform or complement hemoglobin in detecting CRC.

Methods A total of 313 stool specimens (91 CRC, 40 advanced adenoma (AA), 43 nonadvanced adenoma (A) and 139 controls) were analyzed by in-dept mass spectrometry GeLC-MS/MS workflow (Q-Exactive). CART analysis and logistic regression revealed combinations of 4 proteins as the most optimal combination to differentiate CRC from controls. Antibody assays (MSD) were performed on a validation set of 72 FIT fluid samples set (14 CRC, 16 AA, 18 A and 24 controls).

Results Of the 468 human proteins quantified in the discovery set, 93 were significantly enriched in CRC vs controls ($p<0.05$). Of these, 29 were significantly found enriched in CRC vs controls in a validation set ($q<0.05$). Receiver operation characteristics analysis of a combination of four proteins detected CRC with a sensitivity of 73% as compared to 43% for hemoglobin (HBA1) alone at 95% specificity ($p=0.00003$). Similarly a combination of four proteins was identified for differentiation of AA from controls, which showed a sensitivity of 48% vs 8% for hemoglobin ($p=0.0002$) at 95% specificity. Selected proteins could be measured in small sample volumes used in FIT-based screening programs, and discriminated CRCs from controls ($P<0.001$).

Conclusions Proteome profiling on stool samples revealed 29 validated proteins significantly enriched in CRC samples compared to controls. A panel of four complementary protein markers outperformed hemoglobin for detection of CRC as well as AA. Proof-of-concept for detecting the proteins in FIT fluids confirmed the high potential of these markers for screening purposes in a non-invasive and cost-effective manner.

Linda JW Bosch[#], Meike de Wit[#], Thang V Pham, Veerle HM Coupé, Annemieke C Hiemstra, Sander R Piersma, Gideon Oudgenoeg, George L Scheffer, Sandra Mongera, Jochim Terhaar Sive Droste, Frank A Oort, Sietze T van Turenhout, Ilhame Ben Larbi, Joost Louwagie, Wim van Criekinge, Rene WM van der Hulst, Chris JJ Mulder, Beatriz Carvalho, Remond JA Fijneman, Connie R Jimenez[*], Gerrit A Meijer[*] **Novel stool-based protein biomarkers for improved colorectal cancer screening: a case-control study** Annals of Internal Medicine 2017, accepted manuscript. #Shared first and *shared senior authors.

Acknowledgements: This project was supported by CTMM-Decode and CTMM-Bioscreen and Su2C Medocc

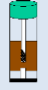
Improved colorectal cancer screening by new stool-based protein markers

Linda JW Bosch[1#], Meike de Wit[1,2#], Thang V Pham[2], Veerle HM Coupé[3], Annemieke C Hiemstra[1], Sander R Piersma[2], Gideon Oudgenoeg[2], George L Scheffer[4], Sandra Mongera[4], Jochim Terhaar Sive Droste[5], Frank A Oort[5], Sietze T van Turenhout[5], Ilhame Ben Larbi[5], Joost Louwagie[6], Wim van Criekinge[1,2,6,7], Rene WM van der Hulst[8], Chris JJ Mulder[5], Beatriz Carvalho[1], Remond JA Fijneman[1], Connie R Jimenez[2#], Gerrit A Meijer[1#].

[1]Department of Pathology, Netherlands Cancer Institute, Amsterdam, the Netherlands. Department of [2]Medical Oncology, [3]Department of Clinical Epidemiology and Biostatistics, [4]Pathology, [4]Gastroenterology and Hepatology, VU University Medical Center, Amsterdam, the Netherlands. [6]MDxHealth, Herstal, Belgium. [7]Department of Mathematical Modelling, Statistics and Bioinformatics, Ghent University, Ghent, Belgium. [8]Department of Gastroenterology and Hepatology, Kennemer Gasthuis, Haarlem, The Netherlands. [#] these authors contributed equally to this manuscript and should be considered co-first (LJWB and MdW) or co-senior (CRJ and GAM) authors.

Background

- **Colorectal cancer (CRC)** has a high cure rate when detected in an early stage
- **Adenoma** is a precursor lesion of CRC



- The **fecal immunochemical test (FIT)** is used for a population-wide screening for early detection of CRC.
- FIT detects small traces of **blood in stool**, which is not tumor-specific
- **Improvement** of FIT:
  - ➔ detect tumor-specific molecular changes
- **Measuring molecules** directly in stool can provide us with novel biomarkers that are stable in fecal environment

AIM: Identification and validation of **protein biomarkers for CRC screening** by proteome profiling of **stool**

Conclusions

- Stool proteome profiling revealed **29 candidate proteins biomarkers** significantly enriched in CRC stool samples.
- Multiple combinations of 4 proteins outperformed hemoglobin for discriminating CRC, as well as AA, from controls.
- Proof of concept for detecting the proteins in FIT samples confirms the high potential of these markers to **improve current CRC screening** in a non-invasive and cost-effective manner.
- Most promising protein biomarkers will be selected for validation and clinical assay development.

CRC: colorectal cancer, A: Adenoma, AA: Advanced adenoma

Results

- 834 human proteins were identified (**Figure 1a**), of which 29 were significantly enriched in CRC vs controls (Q<0.05) in two independent stool sample series (**Figure 1b**).
- Spectral counts for the α- and β-chain of hemoglobin (**Figure 1c**) and S100A8/S100A9 of calprotectin subunits (**Figure 1d**) were strongly correlated. Therefore, stool proteomics provides robust quantification of proteins.
- Multiple 4-protein combinations show significantly improved overall accuracy as compared to hemoglobin alone (**Figure 2**).
- The combinations of 4 proteins detects 30% more CRCs (**Figure 2a**), 23% more AA&CRCs (**Figure 2b**) and 40% more AAs (**Figure 2c**) than Hb does at 95% specificity.
- Candidate protein biomarkers can be quantified by antibodies in FIT samples and can significantly discriminate CRC from controls (**Figure 3**).

Number of identified proteins and subunit correlations

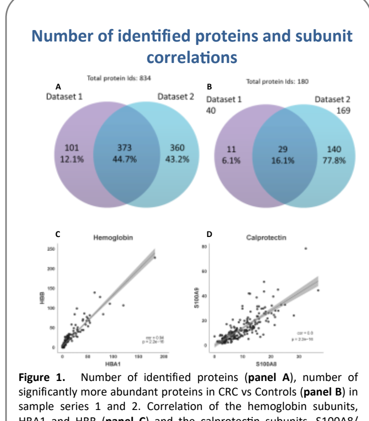


Figure 1. Number of identified proteins (**panel A**), number of significantly more abundant proteins in CRC vs Controls (**panel B**) in sample series 1 and 2. Correlation of the hemoglobin subunits, HBA1 and HBB (**panel C**) and the calprotectin subunits, S100A8/S100A9 (**panel D**).

ROCs of biomarker panels of 4 proteins

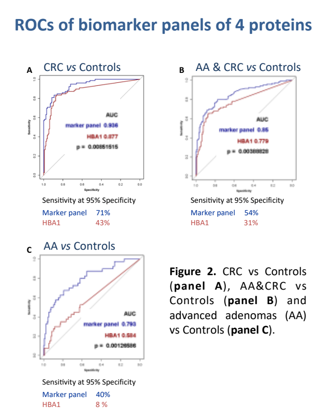


Figure 2. CRC vs Controls (**panel A**), AA&CRC vs Controls (**panel B**) and advanced adenomas (AA) vs Controls (**panel C**).

Antibody-based validation of 3 out of 4 markers in FIT samples

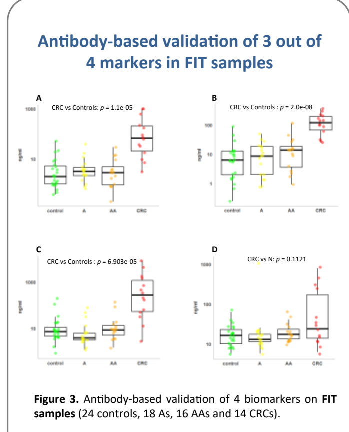


Figure 3. Antibody-based validation of 4 biomarkers on **FIT samples** (24 controls, 18 As, 16 AAs and 14 CRCs).

Material and methods

- Sample series 1: 22 stool specimens (12 CRC and 10 controls), isolated proteins were analyzed by in-dept mass spectrometry GeLC-MS/MS workflow (LTQ-FTMS).
- Sample series 2: 291 stool specimens (79 CRC, 40 AA, 43 A and 129 controls), were analyzed by single-shot mass spectrometry (Q-Exactive).
- Commercially available antibody assays (Meso scale discovery) for 4 candidate biomarkers were performed on an independent set of 72 FIT fluid samples set including 14 CRC, 16 AA, 18 A and 24 controls.
- CART and logistic regression analysis revealed protein combinations to differentiate CRCs and AAs from controls.

Workflow: MS-based **protein identification** in stool

Sample series 1 N=22 — Sample series 2 N=291

LTQ-FTMS — Protein biomarkers — QExactive

4-protein marker panels
By logistic regression and classification and regression tree (CART) analysis

This research was supported by the Center for Translational Molecular Medicine CTMM (DeCoDe and CRCBioscreen) and the Dutch digestive foundation.

This research is supported by an SU2C-DCS International Translational Cancer Research Dream Team Grant (SU2C-AACR-DT1415, MEDOCC).

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Exosomes Secreted by Apoptosis-Resistant Acute Myeloid Leukemia (AML) Blasts Harbor Regulatory Network Proteins Potentially Involved in Antagonism of Apoptosis

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Background Expression of apoptosis-regulating proteins (B-cell CLL/lymphoma 2 - BCL-2, Myeloid Cell Leukemia 1 - MCL-1, BCL-2 like 1 - BCL-X and BCL-2-associated X protein - BAX) in acute myeloid leukemia (AML) blasts at diagnosis is associated with disease-free survival. We previously found that the initially high apoptosis-resistance of AML cells decreased after therapy, while regaining high levels at relapse. Herein, we further explored this aspect of dynamic apoptosis regulation in AML.

Aim Identify the microenvironment protein factors involved in apoptosis regulation in AML

Results First, we showed that the intraindividual *ex-vivo* apoptosis-related profiles of normal lymphocytes and AML blasts within the bone marrow of AML patients were highly correlated. The expression values of apoptosis-regulating proteins were far beyond healthy control lymphocytes, which implicates the influence of microenvironmental factors.
Second, we demonstrated that apoptosis-resistant primary AML blasts, as opposed to apoptosis-sensitive cells, were able to up-regulate BCL-2 expression in sensitive AML blasts in contact cultures (p= 0.0067 andp= 1.0, respectively).
Using secretome proteomics, we identified novel proteins possibly engaged in apoptosis regulation. Intriguingly, this analysis revealed that major functional protein clusters engaged in global gene regulation, including mRNA splicing, protein translation, and chromatin remodeling, were more abundant (p= 4.01E-06) in secretomes of apoptosis-resistant AML. These findings were confirmed by subsequent extracellular vesicle proteomics.
Finally, confocal-microscopy-based colocalization studies show that splicing factors-containing vesicles secreted by high AAI cells are taken up by low AAI cells.

Conclusions The current results constitute the first comprehensive analysis of proteins released by apoptosis-resistant and sensitive primary AML cells. Together, the data point to vesicle-mediated release of global gene regulatory protein clusters as a plausible novel mechanism of induction of apoptosis resistance. Deciphering the modes of communication between apoptosis-resistant blasts may in perspective lead to the discovery of prognostic tools and development of novel therapeutic interventions, aimed at limiting or overcoming therapy resistance.

Wojtuszkiewicz A, Schuurhuis GJ, Kessler FL, Piersma SR, Knol JC, Pham TV, Jansen G, Musters RJ, van Meerloo J, Assaraf YG, Kaspers GJ, Zweegman S, Cloos J, Jimenez CR. Exosomes Secreted by Apoptosis-Resistant Acute Myeloid Leukemia (AML) Blasts Harbor Regulatory Network Proteins Potentially Involved in Antagonism of Apoptosis. *Mol Cell Proteomics.* 2016 Apr;15(4):1281-98.
Acknowledgements: This project is supported by VUmc-CCA

Exosomes secreted by apoptosis-resistant AML blasts harbor regulatory network proteins potentially involved in antagonism of apoptosis

Anna Wojtuszkiewicz[1,2], Gerrit J. Schuurhuis[2], Floortje L. Kessler[2], Sander R. Piersma[3], Jaco C. Knol[3], Thang V. Pham[3], Gerrit Jansen[4], René J. P. Musters[5], Johan van Meerloo[2], Yehuda G. Assaraf[6], Gertjan J.L. Kaspers[1], Sonja Zweegman[2], Jacqueline Cloos[1,2*], Connie R. Jimenez[3*]

[1]Dept. of Pediatric Oncology/Hematology, [2]Dept. of Hematology, [3]OncoProteomics Laboratory, Dept. of Medical Oncology, [4]Dept. of Rheumatology, VU Institute for Cancer and Immunology (V-ICI), [5]Dept. of Physiology, ICaR-VU, VU University Medical Center, Amsterdam, The Netherlands, [6]Dept. of Biology, Technion-Israel Institute of Technology, Haifa, Israel

*Shared senior authorship

Background

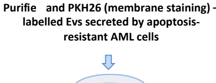
- Acute Myeloid Leukemia (AML) is a bone marrow malignancy.
- Anti-apoptosis index [AAI=(BCL-2*BCLX*MCL1)/BAX] as measured by flow cytometry is associated with disease-free survival (the occurance of relapse).
- The AAI of normal lymphocytes in the AML patients paralleles that of AML blasts of the same patient pointing to the role of microenvironment in disease outgrowth into relapse.

Aim: To characterize the microenvironment generated in the BM with apoptosis-resistant AML cells, in terms of its protein composition.

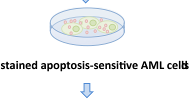
Methods

Mass-spectrometry based proteomics

Apoptosis-sensitive primary AML blasts — Apoptosis-resistance primary AML blasts



N=6 — whole secretome comparison — N=5



Purifie extracellular vesicles (Evs) comparison — ultra-centrifugation

N=2 — N=1

Cytoscape analysis (BiNGO and ClusterONE plugins)

Confocal imaging

Purifie and PKH26 (membrane staining) – labelled Evs secreted by apoptosis-resistant AML cells

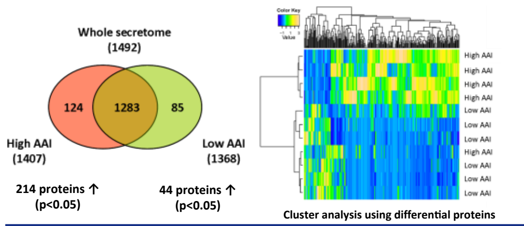
unstained apoptosis-sensitive AML cells

Confocal imaging of vesicle uptake + staining for splicing factors (SF3B3 and hnRNPH3)

- co-localization analysis

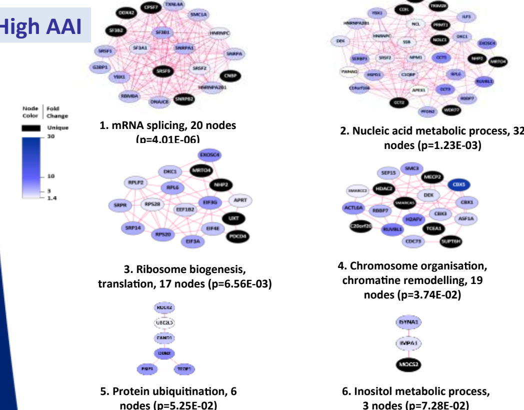
Results

1. Proteomics analysis points to distinct prefil s of secretome generated by high AAI and low AAI AML cells.

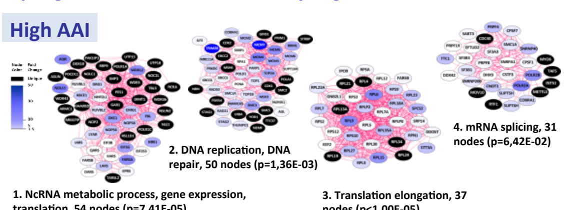


Cluster analysis using differential proteins

2. Unbiased functional cluster analysis reveals apoptosis-resistance associated networks of secreted proteins.

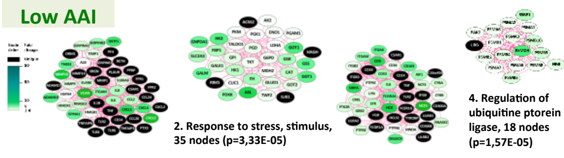


1. mRNA splicing, 20 nodes (p=4.01E-06)
2. Nucleic acid metabolic process, 32 nodes (p=1.23E-03)
3. Ribosome biogenesis, translation, 17 nodes (p=6.56E-03)
4. Chromosome organisation, chromatine remodelling, 19 nodes (p=3.74E-02)
5. Protein ubiquitination, 6 nodes (p=5.25E-02)
6. Inositol metabolic process, 3 nodes (p=7.28E-02)



1. ER associated catabolic process, 8 nodes (p=5.10E-04)
2. Small molecule metabolic process, 7 nodes (p=1.80E-03)
3. Chemotaxis, response to stimulus, 6 nodes (p=3.50E-03)
4. Proteolysis, 3 nodes (p=1.10E-02)
5. Transferrin transport, 3 nodes (p=7.60E-02)

3. Protein networks involved in gene expression are upregulated in EVs secreted by high AAI AML cells.

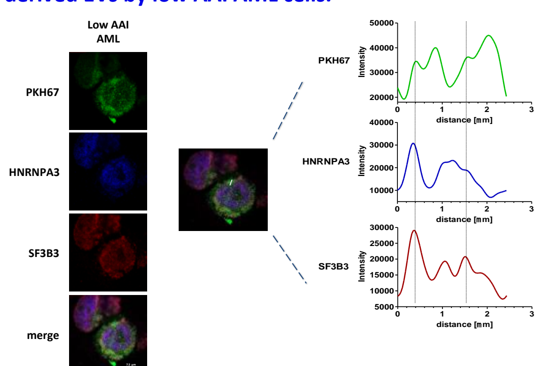


1. NcRNA metabolic process, gene expression, translation, 54 nodes (p=7,41E-05)
2. DNA replication, DNA repair, 50 nodes (p=1,36E-03)
3. Translation elongation, 37 nodes (p<1,00E-05)
4. mRNA splicing, 31 nodes (p=6,42E-02)

Low AAI

1. Defense, inflammatory response, 49 nodes (p=4,65E-04)
2. Response to stress, stimulus, 35 nodes (p=3,33E-05)
3. Small molecule metabolic process, 45 nodes (p=2,33E-02)
4. Regulation of ubiquitine ptorein ligase, 18 nodes (p=1,57E-05)

4. Confocal imaging points to uptake of high AAI AML-derived EVs by low AAI AML cells.



Conclusion

- EVs secreted by apoptosis-resistant AML cells are enriched in proteins involved in gene regulation as compared to EVs of apoptosis-sensitive AML cells.
- Low AAI cells are able to take up EVs secreted by high AAI cells.
- Functional impact of EVs derived from high AAI AML on apoptosis regulation in the recipient cells warrants further investigation.

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Identification of candidate prostate cancer biomarkers in urinary extracellular vesicle proteins by label-free LC-MS/MS-based proteomics.

Irene V. Bijnsdorp[1], L. Ayse Erozenci[1,2], Sander R. Piersma[2], Bart Westerman[3], Thang V. Pham[2], Andre N. Vis[1], R. Jeroen van Moorselaar[1], Connie R. Jimenez[2]

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Background Prostate cancer (PCa) is the most common cancer in males in Western countries. Extracellular vesicles (EVs) secreted by the prostate (cancer) cells can be isolated from urine, of which the collection is minimally-invasive. The cargo of urinary-EVs therefore represents an attractive source for biomarkers to detect PCa. Recent developments in proteomics analysis allow deep profiling of clinical samples, including urinary-EVs.

Methods Urinary-EVs were isolated by ultracentrifugation from 3 control, 3 intermediate and 3 advanced PCa patients. Protein profiles in 1D-gel-separated fractions (5 bands/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, FunRich, Cytoskape, R2-software and transmembrane proteins were predicted using TMHMM-database.

Results In total, 3950 proteins were identified in urinary-EVs. This reached depth outperformed previously reported datasets. Cluster analysis clearly separated advanced from intermediate/controls. Differential proteins of intermediate PCa were associated to migration and transport, and profiles of advanced PCa were associated with metabolism, cell growth and transport. For metastatic PCa, epithelial to mesenchymal transition (EMT) is frequently observed. 311 candidate EV-proteins for advanced PCa (>1,5 fold change, $P<0.05$) highly correlated to the expression of 11 EMT related-genes in PCa-tissues of the Jenkins-dataset (GSE46691), in contrast to the intermediate PCa corresponding 135 candidate proteins. This demonstrates that urinary-EVs are useful for an early and minimally-invasive read-out for PCa-stage. For ELISA-based EV-capture, transmembrane proteins are important. Predictive analysis of transmembrane proteins indicated that 17/135 proteins more abundant in urine-EVs of intermediate PCa and 16/ 311 more abundant in advanced PCa have a transmembrane protein. These proteins represent attractive new candidates for disease-stage specific EV-enrichment.

Conclusion The identified protein profiles provide novel insights that may ultimately translate to a minimally-invasive detection method for PCa.

Acknowledgements: This project is supported by the Dutch Cancer Society (KWF EMCR 2015-8022)

Identification of candidate prostate cancer biomarkers in urinary extracellular vesicle proteins by label-free proteomics

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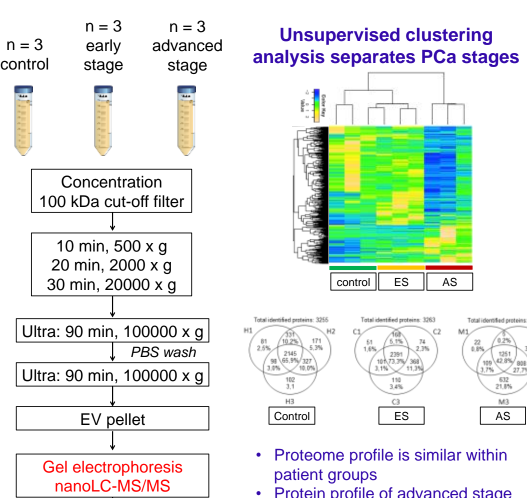
INTRODUCTION

- Prostate cancer (PCa) is the most common cause of cancer in males in Western countries.
- Clinical PCa markers currently in use hold little diagnostic/prognostic value on their own.
- A more specific test is needed that indicates which PCa stage the patient has.
- Extracellular vesicles (EVs) secreted by PCa cells are present in the urine and reflect in part PCa stage.
- Urinary Evs and their protein cargo therefore may provide a non-invasive source for PCa biomarkers.

AIM

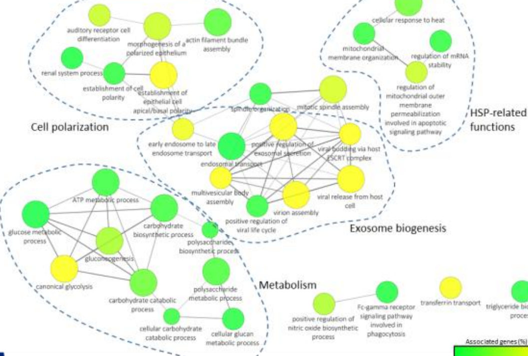
To determine PCa-stage related protein signatures by mass spectrometry-based profiling of urinary EVs

APPROACH & RESULTS



- Proteome profile is similar within patient groups
- Protein profile of advanced stage patients is more heterogenous

Core exosome proteins are related to exosome biogenesis, metabolism and cell polarization

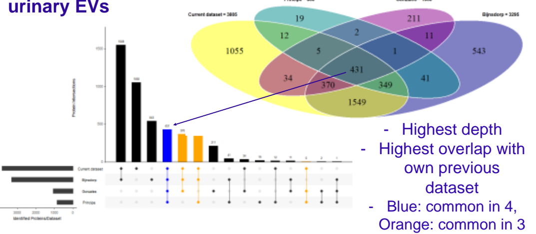


CONCLUSIONS

- We observed a distinct proteome signature in urinary EVs between different PCa stages.
- Candidate EV protein signatures will be validated in a larger series using a novel EV capture method* to ultimately improve PCa diagnosis and forecast disease progression and outcome.

RESULTS

Overlap of current dataset with independent datasets on urinary EVs

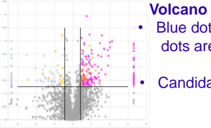


- Highest depth
- Highest overlap with own previous dataset
- Blue: common in 4, Orange: common in 3

Selection of candidate protein markers in early and advanced stage PCa urinary EVs

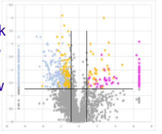
All proteins = 3950

Early stage (ES)	Advanced stage (AS)
Significant p<0.05 ES vs control → *372 proteins*	**Significant** p<0.05 ES vs AS → *642 proteins*
Fold change > 2 Control vs ES → *203 up, 103 down*	**Fold change** > 2 ES vs AS → *122 up, 450 down*
Abundance: Average count > 5 → *75 up, 33 downregulated*	**Abundance:** Average count > 5 → *42 up, 114 downregulated*
Filtering: early vs advanced +/- 1.2 fold → ***19 up, 13 down***	**Filtering:** early vs advanced +/- 1.2 fold → ***35 up, 65 down***



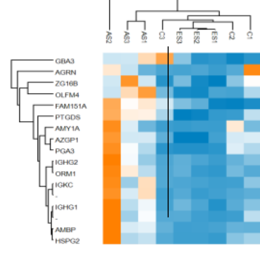
Volcano plots for ES and AS

- Blue dots are 2 fold down, pink dots are 2 fold up and p<0.05 proteins
- Candidates are marked yellow



Supervised clustering with ES all candidates

Supervised clustering with AS upregulated candidates



METHODS

- Urine samples were collected after informed consent from total of 9 patients and EVs were isolated by ultracentrifugation.
- Protein profiles in 1D-gel separated fractions (5 bands/sample) were measured by label free gunshot LC-MS/MS proteomics (QExactive).
- Protein identification and quantification was performed using MaxQuant
- Protein profiles were analyzed using the beta-binominal test, FunRich, Cytoscape and R2-software.
- *REF Bijnsdorp et al., JEV 2017, EV methods paper

Information:
www.oncoproteomics.nl
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Peptide-mediated ‘miniprep’ for high-throughput isolation of extracellular vesicles coupled to phosphoproteomics

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Background

Extracellular vesicles (EVs) are cell-secreted membrane vesicles enclosed by a lipid bilayer that may be derived from endosomes or from the plasma membrane. Since EVs are released into body fluids, and their cargo includes tissue-specific and disease-related molecules, they represent a rich source for disease biomarkers. Here we explore the use of a, high-throughput EV capture method that we previously bench-marked by proteomics, for the analysis of phosphoproteins.

Methods

Extracellular vesicles were isolated from concentrated cancer cell line secretome using a peptide-mediated ‘miniprep’ isolation kit, the ME™ kit (New England Peptide). The peptide in the kit binds to canonical Heat Shock Proteins on the exterior of exosomes and EVs that subsequently can be precipitated centrifugation. The extracellular vesicles pellets were lysed, digested and subjected to TiOx based stagetip phosphopeptide enrichment followed by subsequent label-free single shot LC-MS/MS (Q-Exactive) and data was searched using MaxQuant.

Results

Phosphoproteomics of EVs of the colorectal cancer cell lines HCT116 and HT29 yielded 11,185 phosphopeptides. These 11,185 phosphopeptides map back to 8052 phosphosites, derived from 2764 phosphorylated proteins including 163 phosphokinases. Current analyses focus on the glioblastoma cell lines U87 and EGFR mutant U87 .

Conclusions

Our data shows the feasibility of EV phosphoproteomics using a high-throughput, easy to use isolation method on cancer cell line secretome. We aim to further implement this method on different biological fluids, to enable development of non-invasive, liquid biopsy-based (predictive) diagnostics.

Peptide-mediated 'miniprep' for high-throughput isolation of extracellular vesicles coupled to phosphoproteomics

T.Schelfhorst[1], M. Luinenburg[1], R.R de Haas[1], A.A. Henneman[1], T. V. Pham[1], J. C. Knol[1], S.R. Piersma[1], H.M.W. Verheul[1] & C. R. Jimenez[1]

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Introduction

Here we explore the use of a high-throughput EV capture method that we previously benchmarked by proteomics[1], for the analysis of phosphoproteomics using a TiOX enrichment.

Aim

To explore the potential of EV phosphoproteomics. To this end, we performed a comparitive analysis of a glioblastoma model system of U-87 wild-type and ΔEGFRvIII mutant cells.

Approach

3 x 35ml conditioned cell medium

triplicate U-87 WT

3 x 35ml conditioned cell medium

triplicate U-87 ΔEGFRvIII

35-fold concentration

wt mut

peptide isolation using the ME-kit (New England Peptide)

EV wt mut

2x PBS wash lyse in Urea

lyse cells in Urea

Cell wt mut

trypsin digestion protein peptide

TiO_2: pS,T,Y enrichment

LC-MS/MS

Data analysis

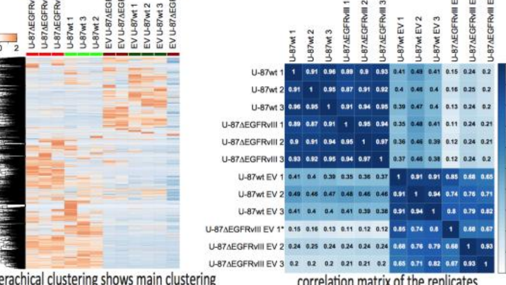
Results

[1] Data overview showing overall data quality

	phosphopeptides	peptides	% enrichment	phosphosites	%S	%T	%Y	kinases	cv	id reproducibility
GBM Cell Line U87 WT lysate	6535	17612	37.11	5396	89.49	9.99	0.52	118		
GBM Cell Line U87 WT lysate	6206	18178	34.14	5102	89.47	10	0.53	114	22.00%	58.30%
GBM Cell Line U87 WT lysate	6503	17867	36.4	5389	89.79	9.69	0.52	122		
GBM Cell Line U87ΔEGFRvIII lysate	6788	17846	38.04	5622	88.97	10.35	0.68	132		
GBM Cell Line U87ΔEGFRvIII lysate	6525	17594	37.09	5420	88.87	10.46	0.66	130	18.00%	63.70%
GBM Cell Line U87ΔEGFRvIII lysate	6634	18370	36.11	5505	89.03	10.41	0.56	129		
GBM Cell Line U87 WT EV	5525	9692	57.01	4551	88.73	10.92	0.35	99		
GBM Cell Line U87 WT EV	5384	9820	54.83	4410	88.82	10.79	0.39	107	20.30%	55.00%
GBM Cell Line U87 WT EV	4994	8727	57.22	4111	89.01	10.65	0.34	108		
GBM Cell Line U87ΔEGFRvIII EV*	*3548	*7368	*48.15	*2887	*88.19	*11.36	*0.45	*50		
GBM Cell Line U87ΔEGFRvIII EV	5570	9573	58.18	4581	88.93	10.54	0.52	118	50.00%	39.00%
GBM Cell Line U87ΔEGFRvIII EV	4982	8820	56.49	4089	88.8	10.69	0.51	102		

* sample was of much lower quality and thus removed in further analysis

[2] Clustering showing group separation and correlation matrix



hierachical clustering shows main clustering on cellular fractions and subclustering on EGFR status

correlation matrix of the replicates

[3] Bargraphs of several EV markers to check enrichment

CD9 CD63 CD81 TSG101

PDCD6IP CLTC SDCBP YWHAE

cell EV cell EV

wt mut

[4] EGFR related signaling upregulated in both the cellular lysate and the EVs

Cells

up in EGFR VIII vs. wildtype in the cells

Focal Adhesion
Signaling by ERBB2
Signaling by ERBB4
SHC1 events in ERBB2 signaling
PI3K events in ERBB4 signaling
SHC1 events in ERBB4 signaling
Nuclear signaling by ERBB4
GRB7 events in ERBB2 signaling
Downregulation of ERBB2:ERBB3...
GRB2 events in ERBB2 signaling

-log10 P-value

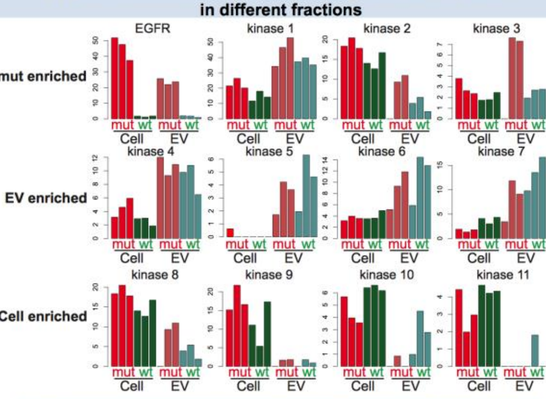
EV fraction

up in EGFR VIII vs. wildtype in the EVs

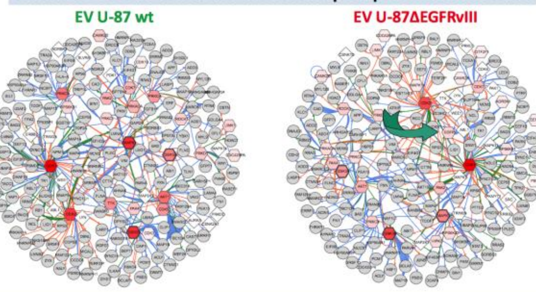
Signaling by ERBB2
Signaling by ERBB4
SHC1 events in ERBB2 signaling
PI3K events in ERBB4 signaling
SHC1 events in ERBB4 signaling
Nuclear signaling by ERBB4
GRB7 events in ERBB2 signaling
Downregulation of ERBB2:ERBB3...
GRB2 events in ERBB2 signaling
PI3K events in ERBB2 signaling

-log10 P-value

[5] phosphokinome bargraphs showing different regulation in different fractions



[6] Kinase-substrate networks of the wt EVs and the EGFRvIII EVs indicate EGFR as one of the differential phosphokinase activities



Conclusions

- Good depth of analysis in comparison to cellular fraction (~5000 phosphopeptides per EV prep versus ~6500 phosphopeptides per cell lysate)
- EV phosphoproteomics is feasible using the peptide-mediated EV isolation
- EV proteins are enriched for exosome markers
- EGFR-associated signaling is transmitted into exosomes
- Certain kinases are secreted more into vesicles.

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Proteomic analysis of extracellular vesicles from clinically available volumes of CSF: application to Alzheimer's disease

Davide Chiasserini[1], Tim Schelfhorst[1], Sander R. Piersma[1], Thang V. Pham[1], Pier Luigi Orvietani[2], Lucilla Parnetti[3], Connie R. Jiménez[1].

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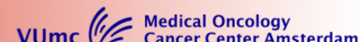
Background: Cerebrospinal fluid (CSF) contains extracellular vesicles (EVs) with undisclosed biomarker potential for neurodegenerative diseases. CSF EVs have been studied mainly using chromatography and ultracentrifugation methods. However, these techniques do not allow the establishment of a high-throughput proteomic method to analyze samples from clinically available volumes of CSF (0.5-1 mL). Here we explored the use of a peptide affinity method (Vn96 peptide) to isolate an EV-enriched fraction from CSF and we used this new approach to identify potential Alzheimer's disease (AD) biomarkers in CSF EVs.

Methods: CSF samples were obtained from the biobank of the University of Perugia. EV isolation was carried out on 1 mL of CSF using the peptide-affinity method that precipitates EVs based on binding to heat shock proteins on the vesicle surface. High-resolution mass spectrometry was used to characterize the proteome of CSF EVs, while functional annotations were investigated using enrichment analysis.

Results: In total, 936 proteins were consistently identified in all the CSF EV samples obtained *via* the peptide-affinity method. More than 60 frequently identified exosomal proteins were found in CSF EVs, including proteins involved in exosomes biogenesis, heat shock proteins, and tetraspanins. Substantial overlap (63%) was found between the CSF EV proteome isolated by the new method in comparison to gold-standard ultracentrifugation.
When applied to pooled CSF from AD and control subjects we found 39 protein up-regulated in AD EVs, while 25 were down regulated compared to CTRL EVs. Up-regulated proteins in AD EVs were related to extracellular matrix organization and cell adhesion, while down-regulated protein were associated with immune response and B-cell signaling.

Conclusions: The peptide-affinity method allows the isolation of EVs from small volumes of CSF and is suitable for the analysis of CSF EV proteomes from patients with neurodegenerative diseases.



PROTEOMIC ANALYSIS OF EXTRACELLULAR VESICLES FROM CLINICALLY AVAILABLE VOLUMES OF CSF: APPLICATION TO ALZHEIMER'S DISEASE

Davide Chiasserini[1], Tim Schelfhorst[1], Sander R. Piersma[1], Thang V. Pham[1], Pier Luigi Orvietani[2], Lucilla Parnetti[3], Connie R. Jiménez[1].

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3 Department of Medicine, section of Neurology, University of Perugia, Perugia, Italy

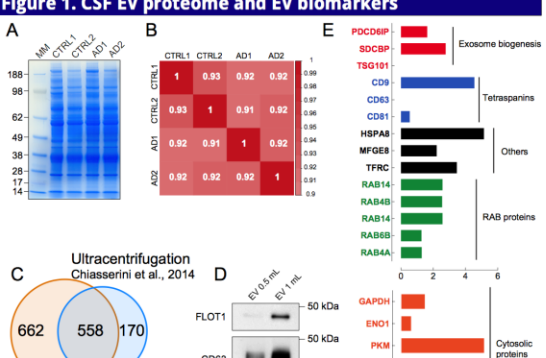
INTRODUCTION

Cerebrospinal fluid (CSF) contains extracellular vesicles (EVs) with undisclosed biomarker potential for neurodegenerative diseases. CSF EVs have been studied mainly using chromatography and ultracentrifugation methods. However, these techniques are not suitable as high-throughput proteomic methods to analyze samples from clinically available volumes of CSF (0.5-1 mL) and large series of patients.

AIM: To explore the use of a new peptide affinity method (ME™ kit, Vn96 peptide) to isolate CSF EVs and to apply this new approach in a pilot analysis of Alzheimer's disease (AD) CSF to identify potential AD biomarkers.

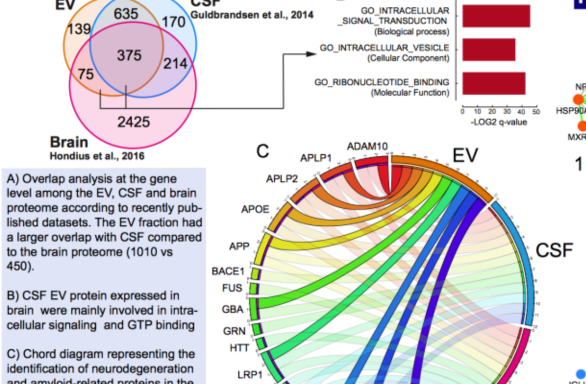
RESULTS

Figure 1. CSF EV proteome and EV biomarkers



A) SDS-PAGE of CSF EV fractions; B) Correlation plot of protein abundance across technical replicates; C) Overlap of protein identification between this study and previously reported CSF EV dataset using ultracentrifugation; D) Immunoblot of EV markers on 0.5 and 1 mL of CSF EV obtained with the ME™ kit; E) Protein abundance (spectral counts) of EV markers in CSF EV fractions.

Figure 2. Overlap analysis among EV, CSF and brain proteome



A) Overlap analysis at the gene level among the EV, CSF and brain proteome according to recently published datasets. The EV fraction had a larger overlap with CSF compared to the brain proteome (1010 vs 450).

B) CSF EV protein expressed in brain were mainly involved in intracellular signaling and GTP binding

C) Chord diagram representing the identification of neurodegeneration and amyloid-related proteins in the three datasets; a subset of them is present in CSF EVs, including APOE, APP, ADAM10 and TREM2.

APPROACH

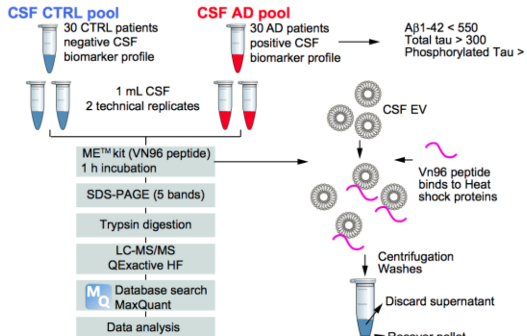
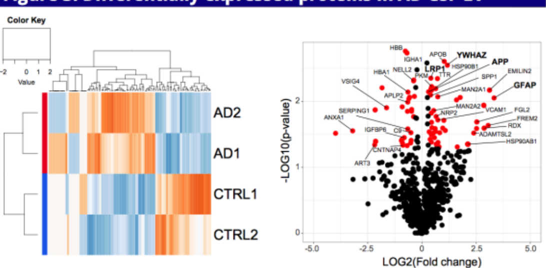


Figure 3. Differentially expressed proteins in AD CSF EV



Supervised clustering of differentially expressed proteins in CTRL and AD pools. 39 proteins were up-regulated while 25 were down regulated in AD CSF EV (FC <1.5, p-value <0.05).

Volcano plot of differentially regulated proteins in AD CSF EV. Among them, several proteins involved in amyloid metabolism were identified.

Figure 4. Network of differential expressed proteins in AD EV

Up-regulated in AD

NOMO2, OTUB1, FGL2, PCDH10, HSP90B1, APLP2, F5, FREM2, PTPRZ1, NPEPPS, ACAT2, APOB, CSPG4, GPC1, HSP90AB1, PKM, LRP1, PAM, HSP90AA1, GFAP, APP, SPP1, ECM2, RDX, UST, NCAN, MXRA5, MYH9, VCAM1, SCG5, COMP, YWHAZ, CSRP1, TTR, CHGA, SEZ6L2, EMILIN2, MAN2A2, LAMB2, LAMA2, MAN1A2, MAN2A1, MGAT5, LAMC1, LAMA4, ST8SIA4, RECK, LAMC3, LTBP2, NRP2

Biological process

1. Cellular localization, folding
2. Extracellular structure organization, neuron differentiation, vesicle trafficking
3. Proteoglycan metabolic process, dermatan-sulfate metabolic process
4. Extracellular matrix organization, biological adhesion
5. Protein N-glycosylation, N-glycan processing

Down-regulated in AD

CD14, SERPINA3, CFH, HBA1, ANXA1, VSIG4, ITIH4, C9, SERPING1, PLG, IGLL5, HP, AHSG, CFHR1, CNTNAP4, CPE, HBB, OMG, RTN4RL2, IGFBP6, ECM1, PTPRM, EFEMP1

Fold change | Experimental interaction

1. Complement activation, defense response
2. Response to ROS, oxidative stress response
3. Neuron projection development
4. NS

CONCLUSIONS

- The peptide-affinity method allows the isolation of EVs from small volumes of CSF (1mL).
- The EV isolated with the peptide-affinity kit show the expression of EV markers yet there is potential room for improvement through the use of detergents to further reduce background proteins
- The CSF EV proteome in AD patients show differentially expressed proteins involved in the pathogenesis of AD

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Early-stage cancer biomarkers uncovered in the platelet proteome

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*These authors contributed equally to this work

Introduction: 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 can be used as a source of biomarkers of early-stage cancer.

Methods: 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 was used to quantify and compare the platelet proteome of patients to controls. Also, samples before and after surgery were compared.

Results: Analysis revealed that the platelet proteome of patients with early-stage cancer is altered as compared to that of controls. In addition, the platelet proteome normalized after tumor resection. Using the above data, in conjunction with quantitative filtering, we were able to select seven potential platelet-derived biomarkers of early-stage cancer.

Conclusion: This pioneering study on the platelet proteome in cancer patients clearly identifies platelets as a new source of protein biomarkers of early-stage cancer.

Manuscript in revision for J. Proteomics

Lipopolysaccharide-regulated secretion of soluble and vesicle-based colorectal cancer cell proteins

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SIGNIFICANCE

- In order to mimic the environment during colorectal cancer (CRC) surgery, where epithelium barrier is disrupted and bacterial products translocate freely, six CRC cell lines were treated with bacterial lipopolysaccharide (LPS).
- The repertoire of secreted proteins (secretome), both soluble and extracellular vesicle-based, in response to bacterial endotoxin from these six CRC cell lines was analyzed by label-free proteomics. A subset of proteins has been previously associated with CRC hepatic metastasis, providing insight in endotoxin-induced factors potentially involved in distant metastatic niche preparation.

Background Surgery remains the mainstay curative treatment for primary colorectal cancer (CRC). However, some patients with stage II and III CRC may develop hepatic metastases. Surgery causes a disruption of the bowel barrier that allows the translocation of endotoxic bacterial products across the colon and promotes inflammation, which can stimulate metastatic ability of CRC cells.

Aim To investigate the response of six CRC cell lines after exposure to the bacterial cell-wall product lipopolysaccharide (LPS).

Results We identified the repertoire of secreted proteins, through extracellular vesicles (EVs) and both classically and non-classically secreted proteins, by mass spectrometry-based proteomics and evaluated NF-κB activation. Most of the regulated proteins after the treatment were found in the EV fraction. Among those LPS-regulated proteins, integrins, inflammatory cytokines and previously reported CRC-metastasis related proteins were found.

Conclusions Our findings suggest that proteins released upon LPS-treatment by CRC cell lines are preferentially secreted through EVs. Interestingly, tumor EVs in vivo have been shown to play a role in preparing the distant metastatic site. The bacterial endotoxin-induced release of these proteins may play a role in creating a permissive environment for CRC liver metastasis, especially through EV-mediated communication.

Tumor cells that phenocopy migratory behavior *in vivo* transport signaling networks through EVs

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# These authors contributed equally.

Background Tumors consist of heterogeneous populations of cells with various abilities to metastasize. Recent data suggest that tumor cells from different sub-types with distinct metastatic potential transplanted in mice 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 sub-type, and only subtle molecular differences drive metastatic heterogeneity.

Aim To characterize the content of EVs shed *in vivo* by two clones of melanoma (B16) tumors with distinct metastatic potential.

Results By mass-spectrometry and RNA sequencing, we show that EVs shed by these clones into the tumor microenvironment contain networks of interconnected RNAs and proteins. Moreover, using the Cre-LoxP system and intravital microscopy we show that cells from these distinct clones phenocopy their behavior through EV exchange.

Conclusions Our results show that cells from one tumor subtype can copy behavior through EVs, and suggest that small amounts of EV cargo may be effective if the transferred biomolecules are interconnected into biological networks.

Submitted manuscript to EMBO J.

Response and toxicity prediction by MALDI-TOF-MS serum peptide profiling in patients with non-small cell lung cancer.

Rovithi M[1], Lind JS[2], Pham TV[3], Voortman J[1], Knol JC[3], Verheul HM[1], Smit EF[2], Jimenez CR[3].
[1]Department of Medical Oncology; [2] Department of Pulmonary Diseases; [3] OncoProteomics Laboratory, Department of Medical Oncology, VU University Medical Center

PURPOSE: We validated a previously reported proteomic signature, associated with treatment outcome, in an independent cohort of patients with non-small cell lung cancer (NSCLC). A novel peptide signature was developed to predict toxicity.

EXPERIMENTAL DESIGN: Using automated magnetic C18 bead-assisted serum peptide capture coupled to MALDI-TOF MS, we conducted serum peptide profiling of 50 NCSLC patients participating in a phase II trial of erlotinib and sorafenib. On the obtained peptide mass profiles, we applied a previously described proteomic classification algorithm. Additionally, associations between observed side effects and peptide profiles were investigated.

RESULTS: Application of the previously acquired algorithm successfully classified the new cohort of patients in groups significantly associated with the outcome. The "poor" group exhibited shorter median progression-free survival (PFS) and overall survival (OS) of 1.35 and 1.98 months (with $p = 0.00677$ and $p = 0.00002$, respectively) while the "good" group had significantly longer PFS and OS (10.63 and 14.4 months with $p = 0.00142$ and $p = 0.00002$, respectively), compared to average OS and PFS. Two specific peptides were detected in the sera of all patients that developed severe toxicity.

CONCLUSIONS AND CLINICAL RELEVANCE: Our results provide an algorithm that, following prospective validation in larger cohorts, could assist treatment selection of patients with NSCLC in the first line setting.

Rovithi M, Lind JS, Pham TV, Voortman J, Knol JC, Verheul HM, Smit EF, Jimenez CR. Response and toxicity prediction by MALDI-TOF-MS serum peptide profiling in patients with non-small cell lung cancer. *Proteomics Clin Appl.* 2016 Jul;10(7):743-9.

Appendix 1. Abstracts & poster summaries of OPL core research

OncoProteomics: Method development (wet and dry)

Evaluation of different phospho-tyrosine antibodies for label-free phosphoproteomics.

van der Mijn JC, Labots M, Piersma SR, Pham TV, Knol JC, Broxterman HJ, Verheul HM, Jiménez CR
Department of Medical Oncology VU University Medical Center, Amsterdam

BACKGROUND Mass spectrometry based phosphoproteomics emerged as advantageous approach for the analysis of tyrosine phosphorylation on proteins and tyrosine kinase signaling. Immunoaffinity purification is required for comprehensive analysis. Here we compared the performance of two antibodies for label-free phosphotyrosine-based phosphoproteomics.

METHODS: Phosphopeptide immunoprecipitation of six technical replicates corresponding to 10mg protein from HCT116 cells was performed using agarose bead-coupled phosphotyrosine antibodies P-Tyr-1000 (N=3) and 4G10 (N=3). NanoLC-MS/MS was performed using a Q Exactive mass spectrometer. For relative quantitation of protein phosphorylation, spectral counts of phosphoproteins and ion intensities of phosphopeptides were determined using MaxQuant.

RESULTS: From the 3 samples incubated with P-Tyr-1000 a total of 689 phosphopeptides were identified with 60% ID reproducibility. The phosphopeptide capture using 4G10 resulted in a total of 421 at 46% ID reproducibility. The P-Tyr-1000 was applied to EGFR mutated U87 cells. Erlotinib reduced EGFR phosphorylation with 59% at y978, y1125, y1138, y1172, and y1197. EGFR inhibition was accompanied by enhanced phosphorylation of FYN, MET, PTK2, DYRK1A, MAPK1 and EPHA2.

CONCLUSION: The P-Tyr-1000 phosphotyrosine antibody performs superiorly when compared to 4G10 antibody for label-free phosphotyrosine-based phosphoproteomics. This workflow allows evaluation of drug target phosphorylation and may give insights in the pharmacodynamic effects of tyrosine kinase inhibitors.

CLINICAL SIGNIFICANCE: In the past decade multiple tyrosine kinase inhibitors (TKIs) have been implemented in standard treatment regimens for patients with cancer. Unfortunately the majority of patients develops resistance to these drugs. Reliable tools for analysis of pharmacodynamic effects and drug resistance mechanisms are therefore warranted. Phosphoproteomic analyses have meanwhile emerged as a sophisticated approach for the determination of protein phosphorylation. These analyses rely on antibodies for enrichment of tyrosine-phosphorylated peptides. Here we compared two commercially available phosphotyrosine antibodies and show that P-Tyr-1000 yields 64% more phosphopeptides than 4G10 antibody, while including almost all 4G10 captured phosphopeptides. The workflow can be reproducibly performed at intermediate protein input levels of 10mg. Furthermore, application of the P-Tyr-1000 antibody in a standardized phosphoproteomics workflow allows relative quantitation of drug target inhibition and provides insights in alternative signaling pathways in cancer cells. This article is part of a Special Issue entitled: HUPO 2014.

van der Mijn JC, Labots M, Piersma SR, Pham TV, Knol JC, Broxterman HJ, Verheul HM, Jiménez CR. Evaluation of different phospho-tyrosine antibodies for label-free phosphoproteomics ***J Proteomics.*** **2015** Sep 8;127(Pt B):259-63.

pTyr-phosphoproteomics of serial tumor biopsies from patients with advanced cancer treated with protein kinase inhibitors

M. Labots[1], J.C. Knol[1], R. de Goeij-de Haas[1], T.V. Pham[1], R.J. Honeywell[1], M. Neerincx[1], R. Beekhof[1], H. Dekker[1], S.R. Piersma[1], J.C. van der Mijn[1], M. Rovithi[1], D.L. van der Peet[2], M. R. Meijerink[3], N.C.T. van Grieken[4], G. J. Peters[1], C.R. Jiménez*[1], H.M. Verheul*[1]

[1]Department of Medical Oncology, VU University Medical Center, De Boelelaan 1117, 1081 HV, Amsterdam, The Netherlands [2]Department of Surgery, VU University Medical Center, VU University Medical Center, De Boelelaan 1117, 1081 HV, Amsterdam, The Netherlands [3]Department of Radiology, VU University Medical Center, VU University Medical Center, De Boelelaan 1117, 1081 HV, Amsterdam, The Netherlands [4]Department of Pathology, VU University Medical Center, VU University Medical Center, De Boelelaan 1117, 1081 HV, Amsterdam, The Netherlands * Shared senior authors

Background Mass spectrometry-based phosphoproteomics of tumor tissue lysates provides a potential personalized medicine approach based on its global information on aberrantly activated signaling pathways and potential drug targets. Here, we report results of a clinical trial (NCT01636908) to evaluate the effect of protein kinase inhibitor (PKI) treatment on tyrosine (pTyr) phosphoproteomic profiles of serial tumor needle biopsies in patients with advanced solid tumors.

Methods Tumor biopsies from 31 patients with advanced cancer were obtained before and after 2 weeks treatment with sorafenib (SOR), erlotinib (ERL), dasatinib (DAS), vemurafenib (VEM), sunitinib (SUN) or everolimus (EVE). Using matched protein input for paired biopsies per patient, down-scaled phosphopeptide immunoprecipitation was performed (P-Tyr-1000, PTMScan®) followed by LC-MS/MS (Q Exactive™) measurement. MaxQuant was used for phosphopeptide/site identification and quantification; fold-changes (Fc) were determined per biopsy pair. Tumor drug concentrations ([PKI]) were determined by LC-MS.

Results For pTyr-phosphoproteomics, biopsy pairs were profiled with median protein input of 2.0 mg per biopsy (range 0.8-2.9) for SOR/ERL/DAS cohorts and 1.1 mg (0.5-2.0) for VEM/SUN. Per biopsy, on average 440 ± 97 phoshopeptides were identified in SOR/ERL/DAS and 178 ± 64 in VEM/SUN, respectively. Unsupervised clustering based on phosphopeptide intensities showed clustering of biopsies from individual patients. Supervised clustering based on differential phosphopeptides (Fc > 5 in ≥ 3/5 patients per cohort) separated pre- and on-treatment groups. Differentially expressed phosphopeptides were drug-specific. Inhibition of (target) kinase activities related to clinical response was observed. Median [PKI] for SOR, ERL, DAS, SUN and EVE ranged between 2-10 μM, while median [VEM] was 1326 μM.

Conclusions In this study we were able to detect, for the first time, in tumors from patients treatment-induced specific changes in the phosphoproteome related to drug concentrations. This approach improves our understanding of individual tumor biology and will enable development of phosphoproteomics-based personalized medicine.

Acknowledgements: This work was supported by VHS Health Services

OPL ONCO Proteomics Laboratory

VUmc Medical Oncology Cancer Center Amsterdam

11609

Mass spectrometry-based phosphoproteomics of tumor needle biopsies from patients with advanced solid tumors during treatment with protein kinase inhibitors

M. Labots[1], R. Beekhof[1], J.C. Knol[1], M. Neerincx[1], R. de Goeij-de Haas[1], H. Dekker[1], T.V. Pham[1], S.R. Piersma[1], J.C. van der Mijn[1], M. Rovithi[1], D.L. van der Peet[2], M. R. Meijerink[3], N.C.T. van Grieken[4], C.R. Jiménez [1] *, H.M. Verheul [1] *
[1]Department of Medical Oncology, [2]Department of Surgery, [3]Department of Radiology, [4]Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands

Introduction

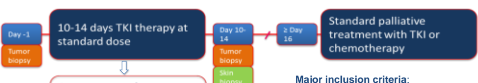
Mass spectrometry-based phosphoproteomics of tumor tissue lysates provides a potential personalized medicine approach based on its global information on aberrantly activated signaling pathways and potential drug targets. We have recently shown that tyrosine (pTyr) phosphoproteomics can be down-scaled to clinically achievable levels of protein input: 1 mg obtained by needle biopsy.[1] Here, we report results of a clinical trial (NCT01636908) to evaluate the effect of protein kinase inhibitor (PKI) treatment on pTyr phosphoproteomic profiles of serial tumor needle biopsies in patients with advanced solid tumors.

AIM:

- To determine the feasibility of repeated tumor profiling for pTyr phosphoproteomics during PKI treatment
- To determine whether drug-specific phosphopeptide regulation and inhibition of (target) kinase activity can be observed

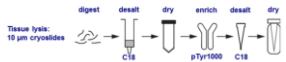
Methods

Study design and pTyr-phosphoproteomics workflow



Major inclusion criteria:
- Adult patients with advanced solid tumors
- Any histology
- Indication for standard palliative treatment (targeted therapy or chemotherapy)

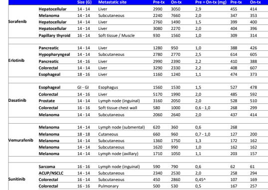
pTyr-phosphopeptide capture



Sample preparation and data analysis:
- Immunoprecipitation: P-Tyr-1000 (PTMScan®, Cell Signaling Technology)
- LC-MS/MS: QExactive™ , 2-hr gradient
- MaxQuant database searching
- Fold-changes (Fc) determined per biopsy pair

Results: unsupervised clustering analysis based on all phosphopeptides per cohort

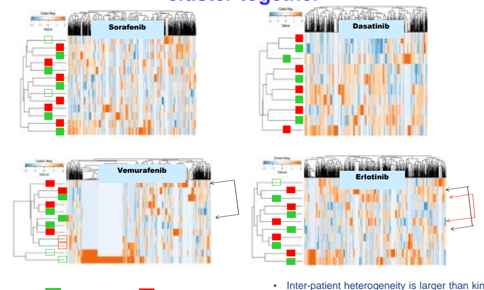
Patient and biopsy characteristics



Drug cohort	Tumor type	Tumor biopsy		Protein yield (ug)		Protein input	Phosphopeptides (#)	
		Size (G)	Metastatic site	Pre-tx	On-tx	Pre = On-tx (mg)	Pre-tx	On-tx
Sorafenib	Hepatocellular	14 - 14	Liver	2990	3050	2,9	455	414
	Melanoma	14 - 14	Subcutaneous	2240	7660	2,0	347	353
	Hepatocellular	14 - 14	Liver	2760	1490	1,5	399	400
	Hepatocellular	14 - 14	Liver	3080	2270	2,0	404	396
	Papillary thyroid	16 - 14	Soft tissue / Muscle	930	1560	1,0	309	314
Erlotinib	Pancreatic	14 - 14	Liver	1280	950	1,0	388	426
	Hypopharyngeal	14 - 14	Subcutaneous	2780	2770	2,5	614	605
	Pancreatic	14 - 16	Liver	2990	2390	2,2	410	388
	Colorectal	14 - 14	Liver	3290	2330	2,2	408	607
	Esophageal	18 - 16	Liver	1160	1240	1,1	474	373
Dasatinib	Esophageal	GI - GI	Esophagus	1560	1530	1,5	527	478
	Colorectal	14 - 16	Liver	5170	1990	2,0	485	592
	Prostate	14 - 14	Lymph node (inguinal)	3160	2050	2,0	528	510
	Colorectal	16 - 16	Soft tissue chest wall	580	1000	0,6 - 1,0	268	299
	Melanoma	14 - 14	Subcutaneous	2060	2640	2,0	437	414
Vemurafenib	Melanoma	14 - 14	Lymph node (submental)	620	360	0,6	268	
	Melanoma	18 - 18	Cutaneous	660	960	0,7 - 1,0	127	200
	Melanoma	14 - 14	Subcutaneous	1360	1750	1,3	172	162
	Melanoma	14 - 14	Subcutaneous	1620	990	1,0	162	162
	Melanoma	14 - 14	Lymph node (axillary)	1710	1050	1,1	203	157
Sunitinib	Sarcoma	16 - 16	Lymph node (inguinal)	590	790	0,6	62	61
	ACUP/NSCLC	14 - 14	Subcutaneous	2340	2530	2,0	258	294
	Colorectal	16 - 14	Subcutaneous	450	2860	0,45^	107	169
	Colorectal	16 - 16	Pulmonary	500	530	0,5	167	257
	Renal cell	14 - 14	Lymph node (subclavicular)	4990	340	0,35^	NE	NE

Pre- and on-treatment phosphoproteomics profiling is feasible using tumor needle biopsies
- Median protein input 2.0 mg per biopsy for SOR/ERL/DAS and 1.1 mg for VEM/SUN
- Number of identified phosphopeptides is related to protein input: low numbers result from (too) small biopsies

Tumor biopsies from individual patients tend to cluster together

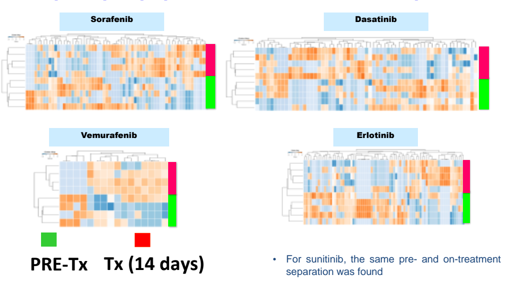


PRE-Tx Tx (14 days)

- Inter-patient heterogeneity is larger than kinase inhibitor-induced changes on the phosphoproteome.
- Data sunitinib not shown due to limited protein input

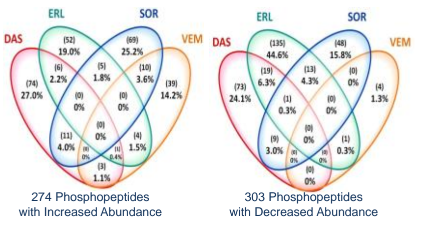
Results: supervised clustering analysis of differential phosphopeptides per cohort

Pre- and on-treatment groups are separated based on phosphopeptides with Fc [illegible] patients



- For sunitinib, the same pre- and on-treatment separation was found

Most differential phosphopeptides were drug-cohort specific

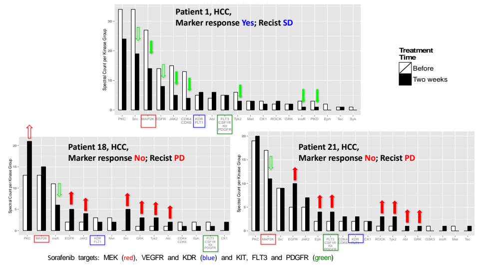


274 Phosphopeptides with Increased Abundance

303 Phosphopeptides with Decreased Abundance

Results: sorafenib effects on phopshophoproteome in 3 HCC patients

Multiple kinase activities down-regulated in responding patient



Common sorafenib effects on the phosphoproteome can be found at 14 days on-treatment (Fc> 2 in > 3/5 pts)
- Alterations in signaling, actin filament organisation, regulation of protein ex/import from/into nucleus
- Phosphorylation of cell death and T cell activation is UP on-Tx

Conclusions

In this study we show, for the first time, clinical feasibility of repeated pTyr-phosphoproteomics in tumor needle biopsies from patients with advanced cancer. Drug-specific changes in the phosphoproteome were found at 14 days on-treatment related to response. This approach will improve our understanding of individual tumor biology and enable development of phosphoproteomics-based personalized medicine.

Ref 1: Phosphoproteomics scaled-down to biopsy level for analysis of individual tumor biology and treatment selection. Labots et al, Submitted for publication

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Phosphotyrosine-based-phosphoproteomics scaled-down to biopsy level for analysis of individual tumor biology and treatment selection.

Labots M[1], van der Mijn JC[1], Beekhof R[1], Piersma SR[1], de Goeij-de Haas RR[1], Pham TV[1], Knol JC[1], Dekker H[1], van Grieken NCT[2], Verheul HMW[1], Jimenez CR[1].

[1] Department of Medical Oncology; [2] Department of Pathology VU University Medical Center

Background Mass spectrometry-based phosphoproteomics of cancer cell and tissue lysates provides insight in aberrantly activated signaling pathways and potential drug targets. For improved understanding of individual patient's tumor biology and to allow selection of tyrosine kinase inhibitors in individual patients, phosphoproteomics of small clinical samples should be feasible and reproducible.

Aim We aimed to scale down a pTyr-phosphopeptide enrichment protocol to biopsy-level protein input and assess reproducibility and applicability to tumor needle biopsies.

Approach To this end, phosphopeptide immunoprecipitation using anti-phosphotyrosine beads was performed using 10, 5 and 1mg protein input from lysates of colorectal cancer (CRC) cell line HCT116. Multiple needle biopsies from 7 human CRC resection specimens were analyzed at the 1mg-level.

Results The total number of phosphopeptides captured and detected by LC-MS/MS ranged from 681 at 10mg input to 471 at 1mg HCT116 protein. ID-reproducibility ranged from 60.5% at 10mg to 43.9% at 1mg. Per 1mg-level biopsy sample, >200 phosphopeptides were identified with 57% ID-reproducibility between paired tumor biopsies. Unsupervised analysis clustered biopsies from individual patients together and revealed known and potential therapeutic targets.

SIGNIFICANCE: This study demonstrates the feasibility of label-free pTyr-phosphoproteomics at the tumor biopsy level based on reproducible analyses using 1mg of protein input. The considerable number of identified phosphopeptides at this level is attributed to an effective down-scaled immuno-affinity protocol as well as to the application of ID propagation in the data processing and analysis steps. Unsupervised cluster analysis reveals patient-specific profiles. Together, these findings pave the way for clinical trials in which pTyr-phosphoproteomics will be performed on pre- and on-treatment biopsies. Such studies will improve our understanding of individual tumor biology and may enable future pTyr-phosphoproteomics-based personalized medicine.

Labots M, van der Mijn JC, Beekhof R, Piersma SR, de Goeij-de Haas RR, Pham TV, Knol JC, Dekker H, van Grieken NCT, Verheul HMW, Jimenez CR. Phosphotyrosine-based-phosphoproteomics scaled-down to biopsy level for analysis of individual tumor biology and treatment selection. *J Proteomics.* 2017 Apr 23. pii: S1874-3919(17)30140-9. doi: 10.1016/j.jprot.2017.04.014. [Epub ahead of print] PubMed PMID: 28442448.

Is less enough? Phosphoproteomics scaled down to tumor biopsies for future treatment selection

Mariette Labots[1], Koen van der Mijn[1], Sander Piersma[1], Richard de Haas[1], Jaco Knol[1], Thang Pham[1], Inge de Reus[1], Gerrit Meijer[2], Nicole van Grieken[2], Henk Verheul[1], Connie Jiménez[1]

[1]Dept. Medical Oncology, [2]Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands

Introduction

Mass spectrometry-based phosphoproteomics of cancer cell- and tissue lysates provides a unique approach to evaluate the cell signaling network, revealing information on aberrantly activated signaling pathways and potential drug targets.

To enable phosphoproteomics-based treatment selection for improved efficacy of targeted therapies in patients with advanced solid tumors, needle biopsies should provide reproducible profiles, representative of the individual tumor phosphoproteome.

AIM:

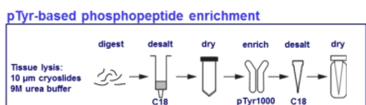
- To downscale the pTyr phosphoproteomics protocol to 1 mg protein input and assess reproducibility using HCT116 CRC cell line and CRC tissue as model
- To assess intra- and inter-tumor heterogeneity in CRC tumor needle biopsies

Methods

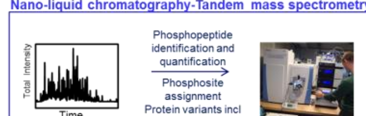
Phospho-proteomics strategy for identification of candidate driver kinases

Tumor resection specimen and needle biopsies

pTyr-based phosphopeptide enrichment



Nano-liquid chromatography-Tandem mass spectrometry



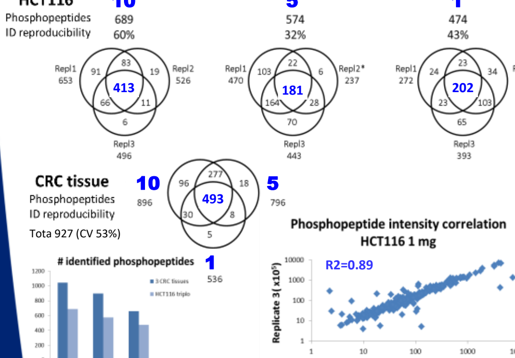
2 hr gradient on QExactive™
MaxQuant 1.4.1.2 database searching
Phosphopeptide intensities normalized on the median per sample

Data mining to select driver kinases

(Comparative) analysis, phospho-protein/kinase ranking
Kinase activity loop analysis and motif search
Integrative signaling network analysis

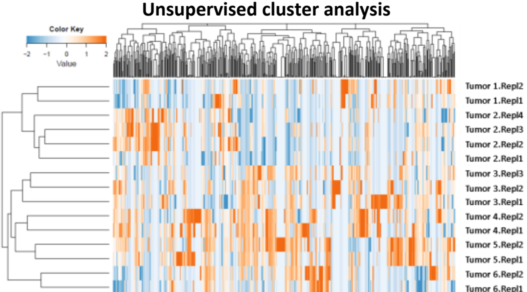
Results: pTyr-phosphoproteomics at 10-5-1 mg protein input

Good reproducibility of label-free phosphopeptide identification and quantification down to 1 mg level



Results: heterogeneity in CRC biopsies

Intra-tumor heterogeneity is smaller than inter-tumor heterogeneity in CRC needle biopsies profiled at 1 mg

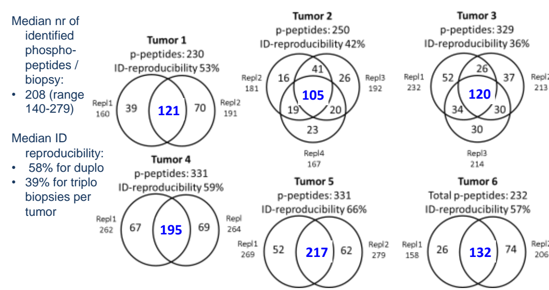


- Multiple 14G needle biopsies collected from 6 CRC resection specimen
- Biopsies with ≥ 50% tumor (H&E) processed using 1 mg protein per sample
- Unsupervised clustering based on phosphopeptide intensities:
- **Biopsies from one tumor cluster together, indicating intra-tumor heterogeneity is smaller than inter-tumor heterogeneity**

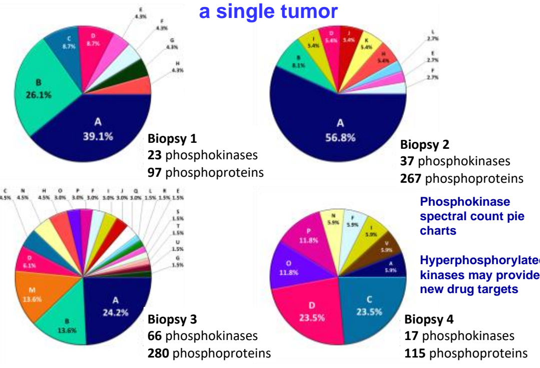
Overlap and differences of biopsies within tumors

Median nr of identified phospho-peptides / biopsy:
- 208 (range 140-279)

Median ID reproducibility:
- 58% for duplo
- 39% for triplo biopsies per tumor



Hyperphosphorylated kinases in biopsies of a single tumor



Phosphokinase spectral count pie charts

Hyperphosphorylated kinases may provide new drug targets

Conclusions

- This scale-down study demonstrates the feasibilty of label-free pTyr-phosphoproteomics at 'biopsy-level' of protein input
- Unsupervised analysis shows that needle biopsies of the same tumor cluster together with *intra*-tumor heterogeneity is smaller than *inter*-tumor heterogeneity
- Down-scaled pTyr-based phosphoproteomics of patient tumor biopsies may enable future phosphoproteomics-based treatment selection

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Comparison and optimization of global pSTY and pY-specific enrichment methods for mass spectrometry-based phosphoproteomics

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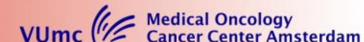
OncoProteomics Laboratory, Dept. Medical Oncology, VU University Medical Center

Background Protein (de)phosphorylation is a key step in cellular signaling . For comprehensive profiling by tandem mass spectrometry phosphopeptide enrichment is required. Over the past decade phosphopeptide enrichment methods including TiO_2, IMAC and anti-pTyr antibodies have been reported and improved. Here we present a comparison and optimization of global pSTY and pY-specific enrichment methods.

Methods Global pSTY enrichment methods were compared in single-shot (120 min) label-free LC-MS/MS: (1) TiO_2 beads using lactic acid or TFA to reduce acidic peptide binding. (2) Subsequently, we compared the optimized TiO_2 method with a new magnetic bead IMAC approach (vendor). (3) For pY-specific enrichment the effect of detergents on background peptide capture was assessed. (4) Additionally, the batch-wise pY enrichment method will be compared to a packed-tip format to reduce a-specific binding.

Results (1) Lactic acid reduces acidic, non-phosphopeptide binding to TiO_2 beads: 89% phosphopeptides vs 78% for TFA. However the overall phosphopeptide yield was higher for TFA: 7484 phosphopeptide IDs for TFA vs. 6053 for lactic acid per enrichment at 500 µg peptide input.
(2) IMAC showed superior selectivity for phosphopeptides compared to TiO_2: 94% vs 78% phosphopeptides per enrichment. Moreover the overall yield was also higher for IMAC: 7752 phosphopeptides compared to 7529 for TiO_2.
(3) 1% of the neutral detergents lauryl maltoside and octyl glucoside in the IP and wash buffers did not change the yield of pY phosphopeptides compared to control (no detergent). The typical phosphopeptide enrichment was 5% with an average yield of 541 phosphopeptides at 5 mg peptide input.

Conclusions Although TFA improved the phosphopeptide yield in TiO_2-based enrichment compared to lactic acid, IMAC proved to be the best performing method with respect to yield and selectivity. Background binding of non-phosphorylated peptides in pY IP remains an unsolved issue. Detergents do not improve selectivity for phosphopeptides.



Optimisation and comparison of global and pTyr-specific phosphopeptide enrichment methods

Sander R. Piersma, Richard R. de Haas, Jaco C. Knol, Thang V. Pham, H.M.W. Verheul, Connie R. Jiménez.

OncoProteomics Laboratory, Department of Medical Oncology, Cancer Center Amsterdam,
VU University Medical Center, Amsterdam, the Netherlands.

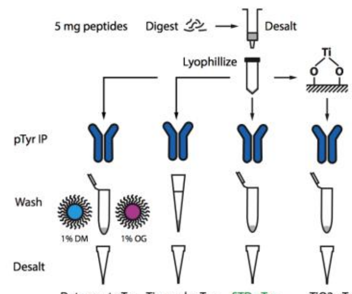
Introduction

Protein phosphorylation/dephosphorylation is the key step in cellular signaling. However, phosphopeptide enrichment is still required for in-depth phosphoproteome analysis. Over the past decades phosphopeptide enrichment methods including TiO2, IMAC and anti-pTyr antibodies have been developed and improved. Here we present a comparison of global pSTY and pTyr-specific enrichment methods.

Optimisation and comparison of enrichment methods

- -pTyr IP unphosphorylated background peptides reduction by non-ionic detergents
- -pTyr IP batch washing vs. in-tip washing method
- -TiO2 based phosphopeptide pre-enrichment prior to pTyr IP
- -Lactic acid vs. TFA in TiO2 global phosphopeptide enrichment
- -TiO2 vs. IMAC global phosphopeptide enrichment

Figure 1. pTyr peptide enrichment workflows



Standard pTyr IP method [1]

-pTyr-1000 antibody agarose beads (Cell Signaling) [2]
-5 mg HCT116 CRC cell line tryptic digest
-Desalt on 500 mg HLB OASIS cartridge (Waters)
-Bind and **wash** in 0.1% TFA
-Elute in 50% ACN/0.1%TFA
-Lyophillize for 48 hrs.
-Dissolve peptides in IP buffer (20 mM Tris HCl pH 7.2/10 mM Na-Phosphate/50 mM NaCl
-Incubate 2 hrs with 2µl IP beads/mg peptides @4 degr.
-Wash 3X with IP buffer and 2X with MQ
-Elute in 2X 0.15% TFA
-Desalt on a 200 µl SDB-XC (3M) stage tip
-Dry and **dissolve** in 20 µl 4% ACN/0.5% TFA

Figure 2. effect of 1% non-ionic detergents on pTyr IP

5.0 mg digest input

Total 646 p-peptides — 106 | 426 (65.9%) | 114 — STD — Pearson correlation 0.85

Total 641 p-peptides — 80 | 456 (71.1%) | 105 — DM (dodecyl maltoside) — Pearson correlation 0.90

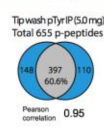
Total 646 p-peptides — 130 | 431 (66.7%) | 85 — OG (octyl glucoside) — Pearson correlation 0.89

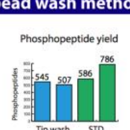
Phosphopeptide enrichment: 5.1% 5.1% 5.1% 5.5% 5.2% 4.4% (STD, DM, OG)

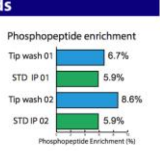
Phosphopeptide yield: 532 540 536 561 561 516 (STD, DM, OG)

- Label-free pTyr IP workflow yields reproducible phosphopeptide ID and Quant
- 1% dodecyl maltoside or 1% octyl glucoside in the binding and wash buffers does not reduce unphosphorylated background peptide binding

Figure 3. different pTyr IP bead wash methods







Phosphopeptide enrichment: Tip wash 01 6.7%; STD IP 01 5.9%; Tip wash 02 8.6%; STD IP 02 5.9% (Phosphopeptide Enrichment (%))

- Washing the agarose pTyr IP beads in a glass-fiber fritted tip results in higher phosphopeptide enrichment compared to the standard bulk wash method in a tube.
- Overall peptide yield in the standard method is higher than using a tip and shows similar phosphopeptide ID and Quant reproducibility

LC-MS/MS

All samples were analysed by label-free LC-MS/MS in a single shot analysis 90 min gradient (120 min inject-to-inject) on a Q Exactive and Q Exactive HF instrument in data-dependent acquisition mode. phosphopeptides were separated on a 40 cm × 75 µm ID fused silica column packed with 1.9 µm 120 Å ReproSil Pur C18 aqua particles maintained at 35°C. Eluting peptides were ionized at +2 kVa. Intact masses were measured at resolution 70K in the Orbitrap using an AGC target value of 3E6. The top 10/15 peptide signals (charge-states 2+ and higher) were submitted to MS/MS in the HCD cell (1.6 m/z isolation width, 25% normalized collision energy). MS/MS spectra were acquired at resolution 17.5K in the Orbitrap using an AGC target value of 1E6 an underfill ratio of 0.5% and a maxIT of 80/60 ms. Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 30 s. Raw files were searched using MaxQuant 1.5.3.2 against the human Swissprot FASTA file using default MaxQuant settings and the match between runs option

Acknowledgments:

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Figure 4. Pre-pTyr IP TiO2 phosphopeptide enrichment

STD pTyr IP (5 mg) Total 980 p-peptides — 103 | 770 (78.6%) | 107

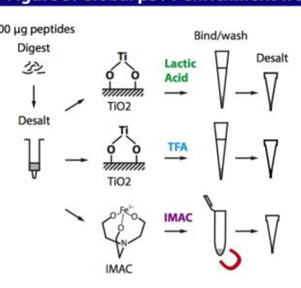
pTyr IP after TiOx (4.7 mg) Total 908 p-peptides — 118 | 636 (70.0%) | 154 — p-peptides pre-enriched by 25 mg TiO2 with 6% TFA

Phosphopeptide yield: 873 877 754 790 (STD, pre IP TiO2)

Phosphopeptide enrichment: TiOx-IP 01 49.7%; STD IP 01 11.3%; TiOx IP 02 44.2%; STD IP 02 11.6% (Phosphopeptide Enrichment (%))

- Pre-pTyr IP phosphopeptide enrichment results in lower unphosphorylated background peptides
- TiO2 phosphopeptide enrichment prior to pTyr IP does not improve the number of identified phosphopeptides compared to the standard pTyr IP method

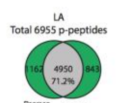
Figure 5. Global pSTY enrichment workflows

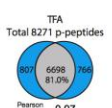


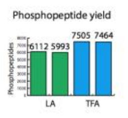
global pSTY enrichment methods

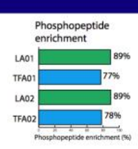
-TiO2 (10 µm GL Sciences)
-500 µg HCT116 CRC cell line tryptic digest
-Desalt on 60 mg HLB OASIS cartridge (Waters)
-Bind peptides to 2.5 mg TiO2 and wash in 0.5 ml 80% ACN/0.1% TFA/300 mg/ml Lactic Acid **(LA)** in a 200 µl C8 STAGE tip [3,4]
-Elute in 0.5 and 5% piperidine
-Desalt on a 200 µl SDB-XC (3M) STAGE tip
-Instead of LA, 6% TFA is used in the TFA method, keeping all other steps identical
-Incubate 500 µg desalted peptides (1 µg/µl in 80% ACN/0.1% TFA) with 100 µl Fe-NTA IMAC magnetic beads slurry (Cell Signaling)
-Wash 2X in binding buffer
-Elute 2x in 50% ACN/5% ammonia
-Desalt on a SDB-XC STAGE tip
-Dry and **dissolve** in 20 µl 4% ACN/0.5% TFA

Figure 6. TiO2: Lactic acid vs. TFA



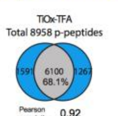


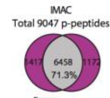


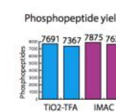


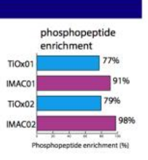
- 6% TFA in binding and washing buffers results in ±15% more phosphopeptide identifications and higher phosphopeptide ID reproducibility
- Lactic acid results in higher phosphopeptdide enrichment (89% vs. 77%) compared to TFA

Figure 7. TiO2-TFA vs. magnetic Fe-NTA IMAC









- TiO2-TFA and Fe-IMAC show highly similar phosphopeptide yield and perform similarly in phosphopeptide ID and Quant.
- IMAC is superior in phosphopeptide enrichment (95% vs 78% for TiO2-TFA, and 89% for TiO2-LA).
- The magnetic IMAC bead format is convenient an can potentially be scaled to 96-well format

Conclusions

- Phosphotyrosine enrichment using pTyr-1000 agarose beads reproducibly yields **550** (QE) to **870** (Q-HF) phosphopeptides with 60-75% ID reproducibility from 5 mg cell digest and good Quant with a Pearson r >0.85 for workflow replicates.
- Unphosphorylated background in pTyr IP is not lowered by detergents or alternative washing formats. Pre-enrichment by TiO2 does help but does not increase phosphopeptide yield.
- Global pSTY enrichment by TiO2 yields more phosphopeptides using 6% TFA, and is is similar to Fe-IMAC with **7700** (QE) phosphopeptides from 500 µg HCT116 digest at 70-80% ID reproducibility and good Quant with a Pearson r >0.88 for workflow replicates.

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[4] Piersma SR, Knol JC, de Reus I, Labots M, Sampadi BK, Pham TV, Ishihama Y et al. Feasibility of label-free phosphoproteomics and application to base-line signaling of colorectal cancer cell lines. J Proteomics. 2015 127 (Pt B):247-58.

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

Davide Chiasserini[1], Sander R. Piersma[1], Alex A. Henneman[1,2], Thang V. Pham[1], Tim Schelfhorst[1], Evelyn Fessler[3], Joan de Jong-Odding[3], H.M.W.Verheul[1], J.P. Medema[3], Connie R. Jiménez[1].

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Background: Colorectal cancer (CRC) is a heterogeneous disease, 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.

Methods: 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.

Acknowledgements: This project is supported by the CCA, KWF and NWO-Middelgroot

DATA INDEPENDENT VS DATA DEPENDENT ACQUISITION MASS SPECTROMETRY

APPLICATION TO THE CLASSIFICATION OF COLORECTAL CANCER SUBTYPES

Davide Chiasserini[1], Sander R. Piersma[1], Alex A. Henneman[1,2], Thang V. Pham[1], Tim Schelfhorst[1], Evelyn Fessler[3], Joan de Jong-Odding[3], H.M.W. Verheul[1], J.P. Medema[3], Connie R. Jiménez[1].

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3 Laboratory of Exp. Oncology and Radiobiology, Academic Medical Center, Amsterdam, The Netherlands

INTRODUCTION

Colorectal cancer (CRC) is a heterogeneous disease, molecular subtyping may help to predict drug response and outcomes. Recently, a transcriptome-based classification on consensus molecular subtypes (CMS) was proposed. The CMS classification includes 4 subtypes with different molecular features: immune activation (CMS1), WNT and MYC signaling (CMS2), metabolic activation (CMS3), stromal invasion, angionesis and TGF-β signaling (CMS4).

Aiming at developing a protein classifier for CMS subtyping, we evaluated here the performance of a data independent (DIA) acquisition mass spectrometry workflow vs. our standard data dipendent (DDA) method. DIA methods have been proposed as highly reproducible proteomic workflows to measure protein abundance in clinical samples, with low variability at the peptide and protein levels and less missing values compared to DDA.

APPROACH

Pooled CRC samples
10 patients x CMS group

DDA: CMS1 CMS2 CMS3 CMS4
DIA: CMS1 CMS2 CMS3 CMS4

SDS-PAGE (1 band)
Trypsin digestion

LC-MS/MS
QExactive HF

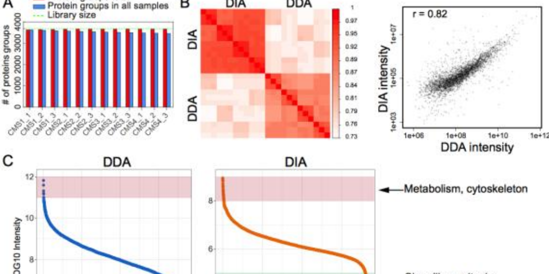
DDA: 1.5m/z isolation, Top15 precursors, 350-1500 m/z Top 10 MS2, ~1 sec/cycle → MaxQuant → DDA data

Spectral library → Spectronaut

DIA: 24 variable width MS2 windows: 20x20, 2x40, 2x60, 400-1000 m/z, ~2 sec/cycle → Spectronaut → DIA data

RESULTS

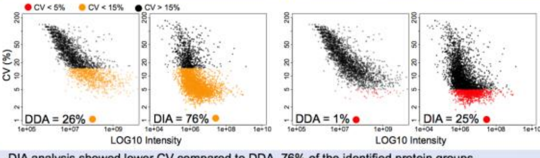
Figure 1. Protein identification and depth of analysis



C: DDA — 6 orders of magnitude; DIA — 5 orders of magnitude. LOG10 Intensity vs Ranked proteins. Metabolism, cytoskeleton; Signalling, mitosis, transcription.

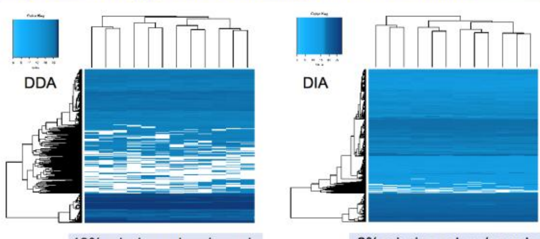
A) DIA protein groups recovery from DDA spectral library; B) Correlation between protein abundance using DDA and DIA methods across all the pools (left) and globally (right); C) Dynamic range of DDA and DIA. In both cases high abundance proteins were involved mainly in metabolic processes while proteins with low abundance were transcription factors and signaling proteins.

Figure 2. DIA analysis is more reproducible than DDA



DIA analysis showed lower CV compared to DDA. 76% of the identified protein groups showed a CV < 15%, while those with a CV < 5% were 25% (DDA = 26% and 1% respectively). Median CV for DDA was 16% while for DIA was 5%.

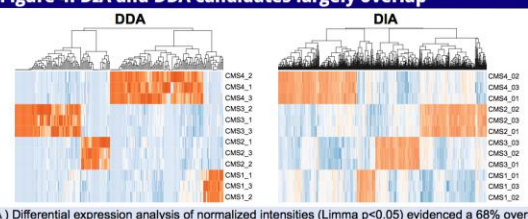
Figure 3. Strongly reduced missing values in DIA data



19% missing values/sample

2% missing values/sample

Figure 4. DIA and DDA candidates largely overlap



A) Differential expression analysis of normalized intensities (Limma p<0.05) evidenced a 68% overlap of DIA vs DDA up-regulated candidates across CMS groups; B) Gene set analysis of the up-regulated proteins in the CMS groups was remarkably similar between DIA and DDA analyses.

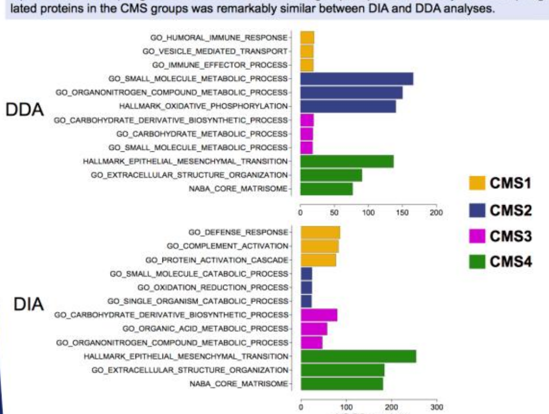
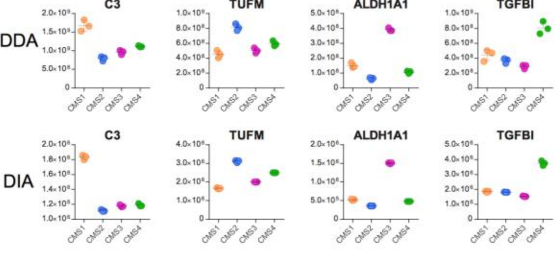


Figure 5. More precise quantitation of CMS markers using DIA



DIA workflow allowed a precise quantification of CMS specific biomarkers, with a lower variability compared to DDA

CONCLUSIONS

- DIA-MS allows better reproducibility compared to DDA and less missing values at the expense of depth of analysis
- Differential expression analysis using similar statistical workflow evidenced an overlap of ~70% of the candidates with similar functional enrichment and similar trend of specific CMS biomarkers
- DIA-MS is suitable for CRC biomarkers discovery and subtyping

ACKNOWLEDGEMENTS

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Feasibility of urinary extracellular vesicle proteome profiling using a robust and simple, clinically applicable isolation method.

Bijnsdorp IV[1], Maxouri O[2], Kardar A[2], Schelfhorst T[2], Piersma SR[2], Pham TV[2], Vis A[1], van Moorselaar RJ[1], Jimenez CR[2].

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Background Extracellular vesicles (EVs) secreted by prostate cancer (PCa) cells contain specific biomarkers and can be isolated from urine. Collection of urine is not invasive, and therefore urinary EVs represent a liquid biopsy for diagnostic and prognostic testing for PCa. However the gold-standard ultracentrifugation method for EV isolation is not compatible with the clinical practice.

Aim To benchmark a new promising highthroughput EV isolation method for urine EV isolation and downstream proteomics analysis. This urinary EV isolation protocol is based on an easy peptide-based isolation (using the Vn96-peptide), takes little time (≈1.5 h) and does not employ special equipment, as opposed to the lengthy ultracentrifugation protocol (>3.5 h), making the new protocol clinically feasible.

Approach In this study, we optimised urinary EV isolation using a method based on heat shock proteins and compared it to gold-standard ultracentrifugation.

Results We compared the isolated vesicles of both ultracentrifugation and Vn96-peptide by proteome profiling using mass spectrometry-based proteomics ($n = 4$ per method). We reached a depth of >3000 proteins, with 2400 proteins that were commonly detected in urinary EVs from different donors. We show a large overlap (>85%) between proteins identified in EVs isolated by ultracentrifugation and Vn96-peptide. Addition of the detergent NP40 to Vn96-peptide EV isolations reduced levels of background proteins and highly increased the levels of the EV-markers TSG101 and PDCD6IP, indicative of an increased EV yield.

Conclusion The Vn96-peptide-based EV isolation procedure is clinically feasibly and allows large-scale protein profiling of urinary EV biomarkers.

Bijnsdorp IV, Maxouri O, Kardar A, Schelfhorst T, Piersma SR, Pham TV, Vis A, van Moorselaar RJ, Jimenez CR. Feasibility of urinary extracellular vesicle proteome profiling using a robust and simple, clinically applicable isolation method. *J Extracell Vesicles.* 2017 Apr 28;6(1):1313091.
Acknowledgements: This project is supported by the Dutch Cancer Society (KWF/Alpe d'Huzes EMCR 2015-8022)

Identification of prostate cancer protein biomarkers by proteomics of urine extracellular vesicles

Irene V. Bijnsdorp[1], Aarzo Kardar[1,2], Tim Schelfhorst[2], Thang V. Pham[2], Jaco C. Knol[2], Sander R. Piersma[2], Albert A. Geldof[1], R. Jeroen van Moorselaar[1], Connie R. Jimenez[2] (iv.bijnsdorp@vumc.nl)
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INTRODUCTION

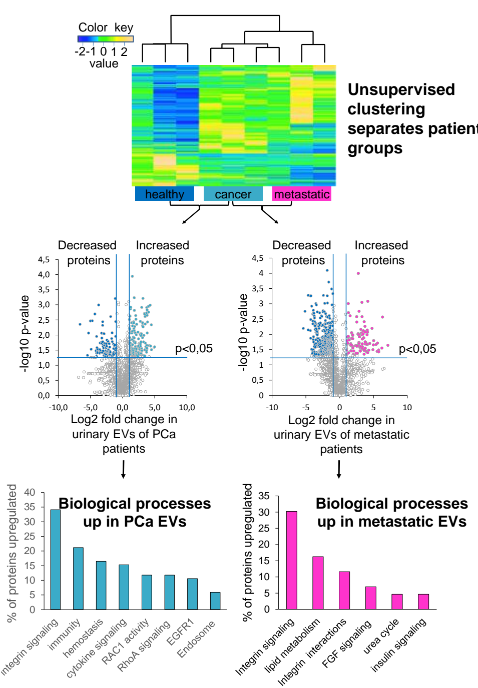
- Prostate cancer (PCa) is the most common cause of cancer in males in Western countries
- Marked developments in LC-MS/MS-proteomic technologies now enable very deep [illegible] of clinical samples, with high [illegible] and [illegible] accuracy.
- For clinical application of EVs as biomarker source, isolation should be fast and easy.
- VN96-peptide (New England Peptides) captures heat shock protein decorated EVs and offers isolation on mini-prep scale.

AIM

1. To identify differences in urinary EV-protein profiles between prostate cancer progression stages
2. To develop a mini-prep scale urinary EV isolation method

RESULTS

prostate cancer urinary EV-biomarker identification: aim 1



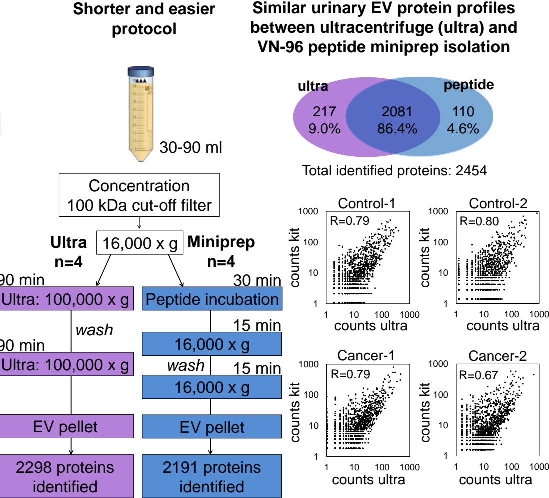
Conclusion: we identified several proteins related to (metastatic) prostate cancer. For clinical application we need an alternative isolation method than ultracentrifuge. See aim 2.

CONCLUSIONS

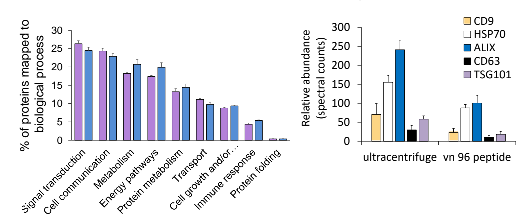
- We [illegible] functional differences between urinary EV profiles between prostate cancer progression stages.
- The revealed protein profiles provide novel insights that may ultimately translate to development of a PCa-[illegible] minimally invasive identification method.
- The VN-96 peptide offers a clinically applicable protocol for urinary-EV isolation with similar protein profile as that obtained by the ultracentrifuge.

RESULTS

Miniprep urinary EV-isolation method: aim 2



Similar protein patterns between ultracentrifuge and VN-96 peptide



METHODS

- Evs were isolated by either ultracentrifugation or by using the VN-96 peptide.
- Protein profiles were measured by label free gunshot LC-MS/MS proteomics.
- Protein profiles were analyzed by FunRich, Cytoskape and SPSS 20.0.
- Urine samples were collected after informed consent, and was approved by the local medical ethical committee.

Information:
www.oncoproteomics.nl
www.prostsatecancerresearch.eu

Feasibility of label-free phosphoproteomics and application to base-line signaling of colorectal cancer cell lines.

Piersma SR[1], Knol JC[1], de Reus I[1], Labots M[1], Sampadi BK[1], Pham TV[1], Ishihama Y[2], Verheul HM[1], Jimenez CR[1].

[1] OncoProteomics Laboratory, Dept. Medical Oncology, VU University Medical Center; [2]Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan.

Robust phosphopeptide enrichment methods with minimal fractionation are required to profile signaling network analysis in cancer cell lines and tissues. We assessed performance of single-shot LC-MS/MS label-free phosphoproteomics using TiOx-based phosphopeptide enrichment and report phosphopeptide identification reproducibility (75.8%), depth of identification (6014-6150 phosphopeptides) and reproducibility of label-free quantification (CV 17.8%). Subsequently, we have profiled the baseline global phosphorylation of 8 colorectal cancer (CRC) cell lines representing different CRC prognostic subtypes. Global single-shot phosphoproteomics can distinguish CRC subtypes previously identified by transcriptomics and identifies signaling proteins and processes associated with the CCS3 poor prognosis subtype. Data are available via ProteomeXchange with identifiers PXD001546 and PXD001550.

BIOLOGICAL SIGNIFICANCE: Label-free single-shot phosphoproteomics is a mature workflow that can be used for global quantitative profiling of biological cell lines and tissues to map signaling networks in comparative analyses. Here we show the feasibility of label-free profiling of CRC cell lines at sample input levels compatible with clinical samples such as tumor biopsies. This article is part of a Special Issue entitled: HUPO 2014.

Piersma SR, Knol JC, de Reus I, Labots M, Sampadi BK, Pham TV, Ishihama Y, Verheul HM, Jimenez CR. Feasibility of label-free phosphoproteomics and application to base-line signaling of colorectal cancer cell lines. ***J Proteomics.*** **2015** Sep 8;127(Pt B):247-58.

Simulated linear test applied to quantitative proteomics.

Pham TV, Jimenez CR.

OncoProteomics Laboraotry, Dept.Medical Oncology, VU University Medical Center

MOTIVATION: Omics studies aim to find significant changes due to biological or functional perturbation. However, gene and protein expression profiling experiments contain inherent technical variation. In discovery proteomics studies where the number of samples is typically small, technical variation plays an important role because it contributes considerably to the observed variation. Previous methods place both technical and biological variations in tightly integrated mathematical models that are difficult to adapt for different technological platforms. Our aim is to derive a statistical framework that allows the inclusion of a wide range of technical variability.

RESULTS: We introduce a new method called the simulated linear test, or the s-test, that is easy to implement and easy to adapt for different models of technical variation. It generates virtual data points from the observed values according to a pre-defined technical distribution and subsequently employs linear modeling for significance analysis. We demonstrate the flexibility of the proposed approach by deriving a new significance test for quantitative discovery proteomics for which missing values have been a major issue for traditional methods such as the t-test. We evaluate the result on two label-free (phospho) proteomics datasets based on ion-intensity quantitation.

AVAILABILITY AND IMPLEMENTATION: Available at
http://www.oncoproteomics.nl/software/stest.html

Pham TV, Jimenez CR. Simulated linear test applied to quantitative proteomics. ***Bioinformatics*. 2016** Sep 1;32(17):i702-i709.

Appendix 1. Abstracts of OPL collaborative research

Neuroproteomics (research hotel function)

Identification and validation of novel CSF biomarkers for early Alzheimer's disease by mass spectrometry-based proteomics

Davide Chiasserini[1*], Thorsten Müller[2*], Christina Loosse[2], Sander R. Piersma[1], Thang V. Pham[1], Silvina Fratantoni[1], Sanna-Kaisa Herukka[3], Hilkka Soininen[3], Jens Wiltfang[4], Hermann Esselmann[4], Wiesje M. van der Flier[5,6], Philip Scheltens[5], Lucilla Parnetti[6], Charlotte E. Teunissen[3], Katrin Marcus[2], Connie R. Jimenez[1].

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Background Cerebrospinal fluid (CSF) biomarkers are used to support the diagnosis of Alzheimer's disease (AD) and to discriminate patients presenting with mild cognitive impairment (MCI) who progress to AD dementia (MCI-AD). The core CSF biomarkers have a typical CSF profile in AD patients, with high levels of tau proteins and low levels of Aβ1-42 compared to controls and MCI not progressing to AD (MCI-S).

Aim Identify CSF protein biomarkers associated with early stages of AD CSF proteome. These proteins may have value for the molecular characterization of clinical AD.

Approach In-depth proteome profiling on CSF in two well-characterized cohorts of AD and MCI patients recruited from different European centres (Amsterdam n=20 and Bochum n=10) for biomarker discovery and used an independent cohort (Perugia, n=78) for biomarker verification. All data have been deposited in ProteomeExchange (accnr). We prioritized putative biomarkers using k-means clustering, selecting protein candidates showing either an increasing trend (UP-AD profile) or a decreasing trend (DOWN-AD profile) in AD patients.

Results About 1235 gene products were identified in the two discovery CSF datasets with an overlap of ~60%, and good correlation of protein quantities ($0.66<r<0.77$). After cluster and overlap analysis, 91 proteins associated with UP-AD CSF profiles, while 54 were included in the DOWN-AD profiles. UP-AD proteins were implicated in cell adhesion, glycolysis, axonogenesis and vesicle mediated transport, while the proteins in the DOWN-AD profiles showed enrichment in immune-related processes. Out of the 144 candidates, 11 were significantly regulated in the validation dataset and 5 of them showed also a similar CSF expression profile across the diagnostic groups, when compared to the discovery datasets. Specifically, 14-3-3 protein ζ (YWHAZ) and guanine deaminase (GDA) were significantly increased in AD vs CTRL, while rab GDP dissociation inhibitor alpha (GDI1) and aldolase A (ALDOA) were significantly increased in MCI-AD vs MCI-S patients. The best biomarker to distinguish AD vs CTRL was YWHAZ (AUC = 0.74) while for early AD diagnosis within MCI patients ALDOA showed the best performance (AUC = 0.82). The validated candidates showed a significant association with tau proteins and Aβ1-42, and were also associated with cognitive decline as measured by Mini Mental State Examination.

Conclusions This panel of new candidate AD biomarkers may contribute to the understanding of the pathological pathways involved in dementia onset and provide novel avenues for early detection of AD.

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Brain endothelial cell expression of SPARCL1 is specific to chronic multiple sclerosis lesions and is regulated by inflammatory mediators in vitro

Bridel C[1], Koel-Simmelink MJA[1], Peferoen L[2], Derada Troletti C[3], Durieux S[1], Gorter R[2], Nutma E[2], Gami P[2], Iacobeus E[4,5], Brundin L[4,5], Kuhle J[6], Vrenken H[7], Killestein J[8], Piersma SR[9], Pham TV[9], De Vries HE[3], Amor S[2,10], Jimenez CR[9], Teunissen CE[1].

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AIM 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-1expression 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.

Bridel C, Koel-Simmelink MJA, Peferoen L, Derada Troletti C, Durieux S, Gorter R, Nutma E, Gami P, Iacobeus 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.*** **2017** May 24.

MicroRNA-124 and -137 cooperativity controls caspase-3 activity through BCL2L13 in hippocampal neural stem cells.

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Background Adult neurogenesis continuously contributes new neurons to hippocampal circuits and the programmed death of a subset of immature cells provides a primary mechanism controlling this contribution. Epileptic seizures induce strong structural changes in the hippocampus, including the induction of adult neurogenesis, changes in gene expression and mitochondrial dysfunction, which may all contribute to epileptogenesis. However, a possible interplay between this factors remains largely unexplored.

Aim To investigate gene expression changes in the hippocampal dentate gyrus shortly after prolonged seizures induced by kainic acid, focusing on mitochondrial functions.

Results Using comparative proteomics, we identified networks of proteins differentially expressed shortly after seizure induction, including members of the BCL2 family and other mitochondrial proteins. Within these networks, we report for the first time that the atypical BCL2 protein BCL2L13 controls caspase-3 activity and cytochrome C release in neural stem/progenitor cells. Furthermore, we identify BCL2L13 as a novel target of the cooperative action of microRNA-124 and microRNA-137, both upregulated shortly after seizure induction.

Conclusion The cooperative microRNA-mediated fine-tuning of BCL2L13 expression controls casp3 activity, favoring non-apoptotic caspase-3 functions in NSPC exposed to KA and thereby may contribute to the early neurogenic response to epileptic seizures in the dentate gyrus.

Schouten M, Bielefeld P, Fratantoni SA, Hubens CJ, Piersma SR, Pham TV, Voskuyl RA, Lucassen PJ, Jimenez CR, Fitzsimons CP. Multi-omics profile of the mouse dentate gyrus after kainic acid-induced status epilepticus. ***Sci Data.*** **2016** Aug 16;3:160068.
Schouten M, Fratantoni SA, Hubens CJ, Piersma SR, Pham TV, Bielefeld P, Voskuyl RA, Lucassen PJ, Jimenez CR, Fitzsimons CP. MicroRNA-124 and -137 cooperativity controls caspase-3 activity through BCL2L13 in hippocampal neural stem cells.***Sci Rep.*** **2015** Jul 24;5:12448.

Novel diagnostic cerebrospinal fluid biomarkers for pathologic subtypes of frontotemporal dementia identified by proteomics.

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INTRODUCTION: Reliable cerebrospinal fluid (CSF) biomarkers enabling identification of frontotemporal dementia (FTD) and its pathologic subtypes are lacking.

METHODS: Unbiased high-resolution mass spectrometry-based proteomics was applied on CSF of FTD patients with TAR DNA-binding protein 43 (TDP-43, FTD-TDP, n = 12) or tau pathology (FTD-tau, n = 8), and individuals with subjective memory complaints (SMC, n = 10). Validation was performed by applying enzyme-linked immunosorbent assay (ELISA) or enzymatic assays, when available, in a larger cohort (FTLD-TDP, n = 21, FTLD-tau, n = 10, SMC, n = 23) and in Alzheimer's disease (n = 20), dementia with Lewy bodies (DLB, n = 20), and vascular dementia (VaD, n = 18).

RESULTS: Of 1914 identified CSF proteins, 56 proteins were differentially regulated (fold change >1.2, P < .05) between the different patient groups: either between the two pathologic subtypes (10 proteins), or between at least one of these FTD subtypes and SMC (47 proteins). We confirmed the differential expression of YKL-40 by ELISA in a partly independent cohort. Furthermore, enzyme activity of catalase was decreased in FTD subtypes compared with SMC. Further validation in a larger cohort showed that the level of YKL-40 was twofold increased in both FTD pathologic subtypes compared with SMC and that the levels in FTLD-tau were higher compared to Alzheimer's dementia (AD), DLB, and VaD patients. Clinical validation furthermore showed that the catalase enzyme activity was decreased in the FTD subtypes compared to SMC, AD and DLB.

DISCUSSION: We identified promising CSF biomarkers for both FTD differential diagnosis and pathologic subtyping. YKL-40 and catalase enzyme activity should be validated further in similar pathology defined patient cohorts for their use for FTD diagnosis or treatment development.

Teunissen CE, Elias N, Koel-Simmelink MJ, Durieux-Lu S, Malekzadeh A, Pham TV, Piersma SR, Beccari T, Meeter LH, Dopper EG, van Swieten JC, Jimenez CR, Pijnenburg YA. Novel diagnostic cerebrospinal fluid biomarkers for pathologic subtypes of frontotemporal dementia identified by proteomics. ***Alzheimers Dement (Amst).*** **2016** Jan 19;2:86-94.

Proteomic Identification of novel cerebrospinal fluid biomarker candidates of dementia with Lewy Bodies

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Background: Cerebrospinal fluid (CSF) is considered an optimal source for the discovery of in vivo biomarkers for Dementia with Lewy bodies (DLB). Thus far, no reliable biomarker with both high sensitivity and specificity is available for DLB. CSF biomarkers could improve the early diagnostic accuracy, monitor disease progression and treatment response. We set out a proteomics study to identify novel CSF biomarker candidates for DLB.

Methods: An in-depth proteomics workflow involving immune-depletion of high-abundant proteins, mono-dimensional SDS-PAGE in conjunction with nanoLC-MS/MS-based proteomics, database searching (MaxQuant) and label-free protein quantification was applied on CSF of 20 probable DLB patients and age and gender matched individuals with subjective cognitive decline (SCD, n=20). DLB patients had an abnormal (123)I-FP-CIT SPECT scan and normal CSF values of Aβ42, total tau and phosphorylated tau. Candidate biomarkers were selected based on the following criteria: (1) $p<0.05$, fold change>1.2, median normalized spectral count ≥2; (2) ≥50% separation between the two groups; and (3) identified peptide sequences covering ≥20% of the protein.

Results: Fifty-seven out of 2100 identified proteins were differentially expressed in DLB patients compared to SCD ($p<0.05$). 35 proteins were down-regulated and 22 proteins were up-regulated in DLB compared to SCD. We selected three proteins for validation by ELISA in a cohort including DLB, AD, Parkinson’s disease (PD) and SCD. This validation is currently in progress.

Conclusions: Our proteomics analysis of CSF identified several novel potential candidate biomarkers for DLB. Further validation of prioritized candidates in larger cohorts should delineate their potential as early diagnostic and/or progression biomarkers for DLB.

Identification of novel biomarker candidates in the cerebrospinal fluid proteome of drug-naïve Parkinson's disease patients

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Introduction: Cerebrospinal fluid (CSF) is in close contact with the extracellular fluid surrounding brain cells and therefore holds great promise as a source of biomarkers for Parkinson's disease (PD). PD biomarkers could improve the early diagnostic accuracy of PD, may be used to identify patients at risk of developing PD, stratify patients into subtypes and monitor disease progression.

Aim The current proteomics study was performed to identify novel CSF biomarker candidates for PD using two independent patient cohorts.

Approach and Methods: We first analysed the CSF proteome in a discovery cohort of 10 drug-naïve PD patients and 10 neurologically healthy controls. The proteomics workflow consisted of immuno-depletion of high-abundant proteins, mono-dimensional SDS-PAGE in conjunction with nanoLC-MS/MS-based proteomics and label-free protein quantification. Identified differentially expressed proteins were subsequently compared to a second proteomics dataset of an independent cohort of 12 medicated PD patients and 13 controls. Functional annotation as well as pathway and network analysis were performed to gain insight into the molecular processes associated with deregulated proteins.

Results: Ninety out of 1284 identified proteins in the discovery dataset were differentially expressed in PD patients compared to controls. Some proteins have previously been related to PD, such as chromogranin A (CHGA; down-regulated in PD). Ninety-seven percent of proteins of the discovery dataset overlapped with the 2115 proteins identified in the verification cohort. Three overlapping candidate biomarkers were found: myelin protein P0 (MPZ; up-regulated in PD), plastin-2 (LCP1; down-regulated in PD) and acid sphingomyelinase-like phosphodiesterase 3b (SMPDL3B; down-regulated in PD). Network analysis of proteins showing co-regulation between the two datasets showed subnetworks involved in complement activation, inflammation related processes and axon guidance.

Conclusion: Our proteomics analysis of CSF of PD patients and controls yielded various biomarker candidates for PD. Further clinical validation of prioritized candidates in larger cohorts should delineate their potential as early diagnostic, prognostic and/or progression biomarkers for PD.

Brain extracellular matrix is a source of extracellular vesicles: implications for Alzheimer's disease

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Introduction Perineuronal nets (PNNs) are reticular extracellular matrix (ECM) structures that surround the soma and proximal dendrites of many neurons in the central nervous system. In some brain areas, PNNs are predominantly found on interneurons, in particular fast-spiking parvalbumin-positive (PV+) interneurons. PNNs may serve different functions, including neuroprotection and the regulation of different types of neuronal plasticity. For instance, they have been suggested to control ion and neurotransmitter homeostasis, providing a stable and non-toxic microenvironment for fast-spiking PV+ cells. In addition, their development coincides with the ending of critical period plasticity in sensory cortical areas such as the visual and auditory cortices, and enzymatic breakdown or genetic disruption of PNNs can reinstate developmental plasticity in adulthood. Finally, adult plasticity underlying learning and memory limited by PNNs, and breakdown or disruption of PNNs can restore memory function, for instance in mouse models of Alzheimer's disease (AD).

Many studies have addressed the question how PNNs restrict plasticity in the brain. To this moment, three mechanisms have been proposed. Firstly, PNNs may form a physical barrier on the cell membrane to prevent formation of new synaptic contacts. Secondly, PNNs may restrict the lateral mobility of proteins, including neurotransmitter receptors, on the plasma membrane, thus limiting the insertion of new receptors at synaptic sites. Thirdly, PNNs may bind and contain inhibiting molecules that prevent the formation of new synaptic contacts or reduce their plasticity.

Aim We set out to identify PNN-contained factors that may limit neuronal plasticity and thus contribute to cognitive decline in AD.

Approach We previously showed that enzymatic breakdown of PNNs with chondroitinase ABC (chABC) restores both physiological plasticity in the hippocampus as well as hippocampal memory performance in APP/PS1 mice, a commonly used mouse model of AD. To test whether this rescue of plasticity and behaviour is mediated by factors that are contained within PNN structures, we exposed hippocampal slices of APP/PS1 mice and wildtype controls to chABC treatment ex vivo and analysed the releasates with mass spectrometry.

Results In a first experiment, we analysed total protein content of the releasates and observed a significant enrichment of proteins that are known to be part of extracellular vesicles (EVs). In a second experiment, we first purified EVs from the releasates using a peptide-affinity pull down approach. This allowed us to detect EVs and quantify differences in EV composition between APP/PS1 mice and controls with higher coverage and confidence than in the first experiment. In this second experiment we observed differential expression patterns in APP/PS1 mice and controls of several EV markers as well as ECM proteins.

Conclusion Based on a preliminary analysis of the data we conclude that (1) PNNs in the hippocampus are a source of EVs, (2) differences in PNN composition and/or density may contribute to altered EV binding or trapping in APP/PS1 mice compared with controls, and
(3) APP/PS1- and wildtype-derived EVs may differ in composition and content. In future research we will try to address the potential pathogenic nature of PNN-contained EVs by injecting wildtype mice with APP/PS1-derived EVs and vice versa, and studying their accumulation in PNNs and their effects on PV cell function, physiological plasticity and memory performance.

Appendix 1. Abstracts OPL collaborative research

Miscellaneous (phospho)proteomics (research hotel function)

Proteomics of urinary extracellular vesicles distinguishes patients with nephronophthisis-related ciliopathies from healthy controls

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Background Nephronophthisis is one of the leading genetic causes of end-stage renal disease in childhood. Early diagnostics and prognostics for nephronophthisis are limited because presenting symptoms are non-specific and phenotypic variability is substantial.

Aim We aimed to identify non-invasive protein biomarkers for nephronophthisis in urinary extracellular vesicles.

Approach Extracellular vesicles were isolated from urine of 12 patients with a nephronophthisis-related ciliopathy and 12 age- and gender-matched controls, followed by label-free LC-MS/MS proteomics analysis for protein identification and quantification by spectral counting.

Results We identified 156 differentially expressed proteins in patients compared to controls ($P<0.05$) with a fold change of at least four. Cluster analysis of the proteomic profiles almost completely separated patients from controls. 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 analyses of gene ontologies and pathways revealed enrichment of terms associated with 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.

Conclusions These findings represent the first step towards a non-invasive diagnostic test for nephronophthisis. Longitudinal serial testing of the same individuals over time may also provide prognostic and screening suggestions for asymptomatic patients and offer insights into the natural course of disease progression. Further research is needed to determine specificity of the biomarkers. In conclusion, proteomic profiles of urinary extracellular vesicles differentiate nephronophthisis-related ciliopathy patients from healthy controls.

Exploration of Dendritic Cell Lectin Receptors and their Immune Modulatory Signalling Pathways

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Dendritic cells (DCs) are key inducers of the adaptive immune response. They possess a multitude of different pattern recognition receptors (PPRs), including Toll-like receptors (TLRs), NOD-like receptors and C-type Lectin Receptors (CLRs) to elicit tailor-made immune response against invading pathogens. Over the last few decades, CLRs have gained much attention not only as endocytic PRRs, but also for their immune modulatory functions. Strikingly, several carbohydrate ligands are shared among different CLRs, yet each seems to propagate a unique signalling cascade. Additionally, CLR triggering can modulate the signalling pathways of other PRRs, such as the TLRS, to modify or prolong the TLR-induced response.
We recently showed that carbohydrates can have a huge impact on how DC polarize or suppress T cell responses. Carbohydrates such as High Mannose-, LewisX-, or LewisY-containing ligands displayed differential IL-10 and IL-12 expression profiles by DC after concomitant LPS stimulation. On the other hand α2-3- or α2-6-sialic acid-containing ligands skew DCs towards the induction of T regs. These carbohydrates target differential CLRs such as DC-SIGN and Siglec, respectively.

To gain further insight the immunogenic signalling pathways of the DC-expressed CLR DC-SIGN and its interference with TLR signalling, as well as the tolerogenic signalling pathways through Siglec, we coupled different carbohydrate ligands to a rigid dendrimeric structure, thereby offering multivalent presentation of these ligands. For insight in the underlying signalling pathways, we applied phosphoproteomics to investigate differences in DC protein phosphorylation upon specific CLR-ligand engagement, as well as next generation sequencing on a transcriptional level.
Analysis of the phosphoproteomics data demonstrated distinct signalling pathways towards a immunogenic or tolerogenic fingerprint upon stimulation of the DCs with specific carbohydrates.

Genome-wide siRNA Screen Identifies the Radiosensitizing Effect of Downregulation of MASTL and FOXM1 in NSCLC

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Lung cancer is the most common cancer worldwide and on top of that has a very poor prognosis, which is reflected by a 5-year survival rate of 5% to 15%. Radiotherapy is an integral part of most treatment regimens for this type of tumor, often combined with radiosensitizing cytotoxic drugs. In this study, we identified many genes that could potentially be exploited for targeted radiosensitization using a genome-wide siRNA screen in non-small cell lung cancer (NSCLC) cells. The screen identified 433 siRNAs that potentially sensitize lung cancer cells to radiation. Validation experiments showed that knockdown of expression of Forkhead box M1 (FOXM1) or microtubule-associated serine/threonine kinase-like (MASTL) indeed causes radiosensitization in a panel of NSCLC cells. Strikingly, this effect was not observed in primary human fibroblasts, suggesting that the observed radiosensitization is specific for cancer cells. Phosphoproteomics analyses with and without irradiation showed that a number of cell-cycle-related proteins were significantly less phosphorylated after MASTL knockdown in comparison to the control, while there were no changes in the levels of phosphorylation of DNA damage response proteins. Subsequent analyses showed that MASTL knockdown cells respond differently to radiation, with a significantly shortened G2-M phase arrest and defects in cytokinesis, which are followed by a cell-cycle arrest. In summary, we have identified many potential therapeutic targets that could be used for radiosensitization of NSCLC cells, with MASTL being a very promising and druggable target to combine with radiotherapy.

Nagel R, Stigter-van Walsum M, Buijze M, van den Berg J, van der Meulen IH, Hodzic J, Piersma SR, Pham TV, Jimenez CR, van Beusechem VW, Brakenhoff RH. Genome-wide siRNA screen identifies the radiosensitizing effect of downregulation of MASTL and FOXM1 in NSCLC. ***Mol Cancer Ther***. 2015 Mar 25. pii: molcanther.0846.2014. [Epub ahead of print] PubMed PMID: 25808837.

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

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Exosomes are endosome-derived small extracellular vesicles (EVs) implicated in cell-cell communication and secreted by Multivesicular Bodies (MVBs) fusing with the plasma membrane (PM). Current techniques to study exosome physiology are based on isolation procedures post-secretion, precluding direct dynamic insight into the mechanics of exosome biogenesis and the regulation of their release. Here we propose real-time visualization of MVB-PM fusion to overcome these limitations. Wedesigned tetraspanin-based optical reporters that spot MVB-PM fusions using live-TIRF and dynamic Correlative Light-Electron Microscopy (CLEM). Detailed single-cell analysis demonstrates that MVB-PM fusion activity is reduced by depletion of the tSNAREs SNAP23 and Syntaxin-4 and can be induced by stimulation of the Histamine H1 Receptor (H1HR). Interestingly, activation of this GPCR in HeLa cells increases Ser110 phosphorylation of SNAP23 promoting MVB-PM fusion. Using this single-cell resolution approach, we highlight the modulatory dynamics of MVB exocytosis that will increase our understanding of exosome physiology and help identify druggable targets in exosome-associated pathologies.

Frederik Johannes Verweij*, Maarten Paul Bebelman*, Connie Ramona Jimenez, Juan J Garcia-Vallejo, Hans Janssen , Jacques Neefjes , Jaco Knol , Richard de Goeij- de Haas , Sander Piersma , Rubina Baglio , Matthijs M Verhage , Jaap Middeldorp , Anoek Zomer , Jacco van Rheenen , Marc Coppolino , Ilse Hurbain , Graça Raposo , Martine Smit , Ruud Toonen , Guillaume van Niel, Michiel Pegtel. **Quantifying exosome release from MVBs in single cells reveals a modulatory role for GPCR signaling**. J. Cell Biology 2017, provisionally accepted manuscript.

Sensing of latent EBV infection through exosomal transfer of 5'pppRNA.

Baglio SR[1], van Eijndhoven MA[1], Koppers-Lalic D[2], Berenguer J[2], Lougheed SM[3], Gibbs S[4], Léveillé N[5], Rinkel R[6], Hopmans ES[1], Swaminathan S[5], Verkuijlen[1] SA, Scheffer GL[1], van Kuppeveld FJ[7], de Gruijl TD[3], Bultink IE, Jordanova ES, Hackenberg M, Piersma SR[3], Knol JC[3], Voskuyl AE, Wurdinger T[2], Jiménez CR[3], Middeldorp JM[1], Pegtel DM[1]

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Complex interactions between DNA herpesviruses and host factors determine the establishment of a life-long asymptomatic latent infection. The lymphotropic Epstein-Barr virus (EBV) seems to avoid recognition by innate sensors despite massive transcription of immunostimulatory small RNAs (EBV-EBERs).
Here we demonstrate that in latently infected B cells, EBER1 transcripts interact with the lupus antigen (La) ribonucleoprotein, avoiding cytoplasmic RNA sensors. However, in coculture experiments we observed that latent-infected cells trigger antiviral immunity in dendritic cells (DCs) through selective release and transfer of RNA via exosomes. In ex vivo tonsillar cultures, we observed that EBER1-loaded exosomes are preferentially captured and internalized by human plasmacytoid DCs (pDCs) that express the TIM1 phosphatidylserine receptor, a known viral- and exosomal target. Using an EBER-deficient EBV strain, enzymatic removal of 5'ppp, in vitro transcripts, and coculture experiments, we established that 5'pppEBER1 transfer via exosomes drives antiviral immunity in nonpermissive DCs. Lupus erythematosus patients suffer from elevated EBV load and activated antiviral immunity, in particular in skin lesions that are infiltrated with pDCs. We detected high levels of EBER1 RNA in such skin lesions, as well as EBV-microRNAs, but no intact EBV-DNA, linking non-cell-autonomous EBER1 presence with skin inflammation in predisposed individuals.
Collectively, our studies indicate that virus-modified exosomes have a physiological role in the host-pathogen stand-off and may promote inflammatory disease.

Baglio SR, van Eijndhoven MA, Koppers-Lalic D, Berenguer J, Lougheed SM, Gibbs S, Léveillé N, Rinkel RN, Hopmans ES, Swaminathan S, Verkuijlen SA, Scheffer GL, van Kuppeveld FJ, de Gruijl TD, Bultink IE, Jordanova ES, Hackenberg M, Piersma SR, Knol JC, Voskuyl AE, Wurdinger T, Jiménez CR, Middeldorp JM, Pegtel DM. Sensing of latent EBV infection through exosomal transfer of 5'pppRNA. ***Proc Natl Acad Sci USA***. 2016 Feb 2;113(5):E587-96.

Exosomal sorting of the viral oncoprotein LMP1 is restrained by TRAF2 association at signalling endosomes.

Verweij FJ, de Heus C, Kroeze S, Cai H, Kieff E, Piersma SR, Jimenez CR, Middeldorp JM, Pegtel DM.

Background The Epstein-Barr virus (EBV)-encoded oncoprotein latent membrane protein 1 (LMP1) constitutively activates nuclear factor κB (NFκB) from intracellular membranes to promote cell growth and survival. LMP1 associates with CD63 in intracellular membranes and is released via exosomes. Whether tumour necrosis factor (TNF) receptor-associated factors (TRAFs) mediate LMP1 NFκB signalling from endosomes and modulate exosomal sorting is unknown.

Aim The explore factors involved in LMP1 exosomal sorting

Results In this article, we show that LMP1-TRAF2 signalling complexes accumulate at endosomes in a palmitoylation-dependent manner, thereby driving LMP1-dependent oncogenicity. Palmitoylation is a reversible post-translational modification and is considered to function as a membrane anchor for proteins. Mutagenesis studies showed that LMP1-TRAF2 trafficking to endosomes is dependent on one single cysteine residue (C78), a known palmitoylation site of LMP1. Notably, growth assays in soft agar revealed that oncogenic properties of the palmitoylation-deficient LMP1 mutant C78A were diminished compared to wild-type LMP1. Since LMP1 recruitment of TRAF2 and downstream NFκB signalling were not affected by a disturbance in palmitoylation, the specific localization of LMP1 at endosomal membranes appears crucial for its transforming potential. The importance of palmitoylation for trafficking to and signalling from endosomal membranes was not restricted to LMP1, as similar observations were made for the cellular oncoproteins Src and Fyn.
Despite abundant LMP1-TRAF2 association at endosomal membranes TRAF2 could not be detected in exosomes by Western blotting or proteomics. Interestingly, point mutations that prevented TRAF binding strongly promoted the sorting and release of LMP1 via exosomes. These observations reveal that LMP1-TRAF2 complexes at endosomes support oncogenic NFκB activation and suggest that LMP1 dissociates from the activated signalling complexes upon sorting into intraluminal vesicles.

Conclusion We propose that "signalling endosomes" in EBV-infected tumour cells can fuse with the plasma membrane, explaining LMP1 release via exosomes.

Verweij FJ, de Heus C, Kroeze S, Cai H, Kieff E, Piersma SR, Jimenez CR, Middeldorp JM, Pegtel DM. Exosomal sorting of the viral oncoprotein LMP1 is restrained by TRAF2 association at signalling endosomes. ***J Extracell Vesicles.*** 2015 Apr 10;4:26334

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.

Tutakhel OA, Jeleń S, Valdez-Flores M, Dimke H, Piersma SR, Jimenez CR, Deinum J, Lenders JW, Hoenderop JG, Bindels RJ. Alternative splice variant of the thiazide-sensitive NaCl cotransporter: a novel player in renal salt handling. ***Am J Physiol Renal Physiol***. 2015 Nov 11:ajprenal.00429.2015.

Evaluation of potential circulating biomarkers for prediction of response to chemoradiation in patients with glioblastoma.

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Background Surgery followed by chemoradiation and adjuvant chemotherapy is standard of care for patients with a glioblastoma (GBM). Due to its limited benefit, an upfront method to predict dismal outcome would prevent unnecessary toxic treatment.

Results We searched for a predictive blood derived biomarker in a cohort of 55 patients with GBM. Increasing age (HR 1.03, 95 % CI 1.01-1.06), and postoperative tumor residue (HR 1.07, 95 % CI 1.02-1.15) were independently associated with unfavourable progression free survival (PFS) in these patients. Corticosteroid use before start of chemoradiaton was strongly predictive for outcome (HR 3.26, 95 % CI 1.67-6.39) with a mean PFS and OS in patients using corticosteroids of 7.3 and 14.6 months, versus 16.1 and 21.6 months in patients not using corticosteroids ($p = 0.0005$, $p < 0.0067$ respectively). Despite earlier reports, blood concentrations of YKL-40, Fetuin-a and haptoglobin were not predictive for response. In addition, serum peptide profiles, determined by MALDI-TOF mass spectroscopy, were not predictive as well.

In **conclusion**, further biomarker discovery studies are needed to predict treatment outcome for patients with GBM in the near future.

van Linde ME, van der Mijn JC, Pham TV, Knol JC, Wedekind LE, Hovinga KE, Aliaga ES, Buter J, Jimenez CR, Reijneveld JC, Verheul HM. Evaluation of potential circulating biomarkers for prediction of response to chemoradiation in patients with glioblastoma. *J Neurooncol*. 2016 Sep;129(2):221-30.

Mycosins Are Required for the Stabilization of the ESX-1 and ESX-5 Type VII Secretion Membrane Complexes.

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Pathogenic mycobacteria contain up to five type VII secretion (T7S) systems, ESX-1 to ESX-5. One of the conserved T7S components is the serine protease mycosin (MycP). Strikingly, whereas MycP is essential for secretion, the protease activity of MycP1 in Mycobacterium tuberculosis has been shown to be dispensable for secretion. The essential role of MycP therefore remains unclear. Here we show that MycP1 and MycP5 of M. marinum have similar phenotypes, confirming that MycP has a second unknown function that is essential for its T7S system. To investigate whether this role is related to proper functioning of the T7S membrane complex, we first analyzed the composition of the ESX-1 membrane complex and showed that this complex consists of EccBCDE1, similarly to what was previously shown for ESX-5. Surprisingly, while mycosins are not an integral part of these purified core complexes, we noticed that the stability of both the ESX-1 complex and the ESX-5 complex is compromised in the absence of their MycP subunit. Additional interaction studies showed that, although mycosins are not part of the central ESX membrane complex, they loosely associate with this complex. We hypothesize that this MycP association with the core membrane complex is crucial for the integrity and functioning of the T7S machinery.

IMPORTANCE:
Among the major virulence factors of pathogenic mycobacteria are the type VII secretion (T7S) systems. Three of these systems, ESX-1, ESX-3, and ESX-5, have been shown to be crucial for virulence or viability. Here we describe the function of mycosin proteases, which are conserved components within these systems. We show that MycP1 and MycP5 have a second, proteolytic-independent function which is essential for the T7S system. We additionally found that this second essential role is related to the stabilization and proper functioning of their respective ESX membrane core complexes. Finally, we found that this is mediated by a loose association of MycP with the complex. Understanding the essential role of mycosins in type VII secretion systems, which play central roles in the virulence and viability of pathogenic mycobacteria, may provide new intervention strategies to treat tuberculosis.

Van Winden VJ, Ummels R, Piersma SR, Jiménez CR, Korotkov KV, Bitter W, Houben EN. Mycosins Are Required for the Stabilization of the ESX-1 and ESX-5 Type VII Secretion Membrane Complexes. ***MBio.*** 2016 Oct 18;7(5). pii: e01471-16.

Essential Role of the ESX-5 Secretion System in Outer Membrane Permeability of Pathogenic Mycobacteria.

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Mycobacteria possess different type VII secretion (T7S) systems to secrete proteins across their unusual cell envelope. One of these systems, ESX-5, is only present in slow-growing mycobacteria and responsible for the secretion of multiple substrates. However, the role of ESX-5 substrates in growth and/or virulence is largely unknown. In this study, we show that esx-5 is essential for growth of both Mycobacterium marinum and Mycobacterium bovis. Remarkably, this essentiality can be rescued by increasing the permeability of the outer membrane, either by altering its lipid composition or by the introduction of the heterologous porin MspA. Mutagenesis of the first nucleotide-binding domain of the membrane ATPase EccC5 prevented both ESX-5-dependent secretion and bacterial growth, but did not affect ESX-5 complex assembly. This suggests that the rescuing effect is not due to pores formed by the ESX-5 membrane complex, but caused by ESX-5 activity. Subsequent proteomic analysis to identify crucial ESX-5 substrates confirmed that all detectable PE and PPE proteins in the cell surface and cell envelope fractions were routed through ESX-5. Additionally, saturated transposon-directed insertion-site sequencing (TraDIS) was applied to both wild-type M. marinum cells and cells expressing mspA to identify genes that are not essential anymore in the presence of MspA. This analysis confirmed the importance of esx-5, but we could not identify essential ESX-5 substrates, indicating that multiple of these substrates are together responsible for the essentiality. Finally, examination of phenotypes on defined carbon sources revealed that an esx-5 mutant is strongly impaired in the uptake and utilization of hydrophobic carbon sources. Based on these data, we propose a model in which the ESX-5 system is responsible for the transport of cell envelope proteins that are required for nutrient uptake. These proteins might in this way compensate for the lack of MspA-like porins in slow-growing mycobacteria.

Ates LS, Ummels R, Commandeur S, van de Weerd R, Sparrius M, Weerdenburg E, Alber M, Kalscheuer R, Piersma SR, Abdallah AM, Abd El Ghany M, Abdel-Haleem AM, Pain A, Jiménez CR, Bitter W, Houben EN. Essential Role of the ESX-5 Secretion System in Outer Membrane Permeability of Pathogenic Mycobacteria. ***PLoS Genet***. 2015 May 4;11(5):e1005190.

Accessory ESX-1 components EccA1, EspG1 and EspH have distinctive roles in secretion and virulence of *Mycobacterium marinum*

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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 substrates and accessory components is far from complete.
Here, we describe the functional characterization of the accessory ESX-1 components EccA1, EspG1 and EspH. These proteins are neither structural components of the membrane complex nor ESX-1 substrates.

Proteomic analysis revealed that EspG1 is crucial for ESX-1 secretion, since all detectable ESX-1 substrates were absent from the culture supernatant in an espG1 mutant. We also identified 3 new ESX-1 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, with the secretion of only EspE, EspF and EsxAB severely affected. While hemolytic activity was lost in all mutant strains, implying that hemolytic activity is not strictly correlated with EsxA secretion, in vitro infection experiments showed significant differences; EspG1, but not EccA1, plays a major role in early stages of infection. Surprisingly, while EspH is essential for successful infection of phagocytic host cells, deletion of espH resulted in a significantly increased virulence phenotype in zebrafish, linked to poor granuloma formation and extracellular outgrowth.
Together, these data show that by studying these accessory proteins we could show that different sets of ESX-1 substrates play different roles at various steps of the mycobacterial infection cycle.

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

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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 via the different type VII secretion (T7S) systems. The most recently evolved T7S 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 N-terminus. However, the role of these proteins remains largely elusive.
Here, we use proteomics to show that mutations of *ppe38* completely block secretion of two large subsets of ESX-5 substrates, i.e. PPE-MPTR and PE_PGRS, together more than 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 play an important role in virulence attenuation. Phylogenetic analysis revealed that deletion of ppe38 occurred at the branching point of 'modern' Beijing sublineage and is shared by Beijing outbreak strains worldwide, suggesting that this deletion may have contributed to their success and global distribution.

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

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Background Epicardial adipose tissue (EAT) is a metabolically active visceral adipose depot directly attached to heart. EAT is known to secrete fibro-adipokines which is a subset of proteins with fibrotic, inflammatory and anti-inflammatory capacities. 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, EAT secretes adipo-fibrokines and promotes fibrosis in the atria, which is an important substrate for atrial fibrillation (AF). Moreover, in patients who develop post-operative AF, various proteins are differentially expressed compared to patients who do not develop AF. Clinically, the patients with AF have a larger volume of EAT compared to the patients without AF. These findings suggest that EAT activity and/or its volume around the heart play an important role in AF pathology. However, the biological activity of EAT and its contribution to AF pathology remain largely unknown.

Research aim and our approach We aim to comprehensively understand the components of EAT secretome and its interaction with myocardium 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 (n=3) to those with no history of AF undergoing coronary artery bypass surgery (n=3).

Results We found in total 312 proteins significant differentially expressed in AF secretome ($p<0.05$, fold-change>1.2). Amongst them, expression of 119 and 193 proteins were increased and decreased respectively in EAT secretome from patients with AF. Gene ontology analyses showed that the highly expressed proteins mainly comprised groups involved in redox homeostasis and inflammation, such as aldehyde dehydrogenase [NAD(P)+] activity, oxygen transporter activity, and cellular response to pro-inflammatory cytokine Interleukin-6. On the other hand, proteins with decreased expression comprised various groups involved in regulation of protein processing and maturation in a response to stimuli.

Conclusion and outlook This small-scale study shows a substantial difference in the protein composition of EAT secretome between patients with and those without AF. These findings support the possibility that the biological activity of EAT in AF changes the redox and inflammatory status of heart tissue, affecting myocardial function, which consequently contributes to AF pathology. Our next research goals are to assess our findings in a larger scale study, and to perform functional analyses by targeting those differentially expressed proteins in EAT secretome from AF patients.

Proteomic landscape of Hypertrophic Cardiomyopathy

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Introduction: Hypertrophic cardiomyopathy (HCM) is the most common inherited cardiac disease. It is caused by one of >1400 mutations in contractile genes and characterized by increased wall thickness, impaired relaxation, fibrosis and increased risk of sudden cardiac death.

Aim: Since the molecular mechanisms that lead to disease outcome are still largely unknown, we want to compare the cardiac proteome of HCM patients of different genetic background with healthy controls.

Approach: Interventricular septum biopsies from patients were obtained during myectomy surgery (n=51). Control samples were obtained from two regions of non-diseased hearts (free left ventricular wall (n=6) and interventricular septum (n=8)). Tissue was homogenized in reducing SDS sample buffer and the proteins fractionated using SDS-PAGE. After in-gel digestion with trypsin, extracted peptides were separated using nano-LC followed by MS/MS analysis. Pathway analysis was performed with ClueGO.

Results: A total of 3811 proteins were identified. The comparison of all HCM patients (n=51) to septum control tissue (n=8) showed a differential regulation of 537 proteins ($p<0.05$) of which we found 186 to be up- and 351 to be downregulated. The proteins are predominantly involved in metabolic processes like oxidative phosphorylation, glycolysis and respiratory electron transport as well as extracellular matrix organization. Changes in metabolism are known to occur in hypertrophied hearts and can contribute to impaired relaxation and increased risk of sudden cardiac death.

Conclusion: This is the first large scale clinical tissue profiling of genetically characterized patient samples in HCM. The protein profile of HCM patients shows differential expression of metabolic pathways independent from their genetic background, which could help explain part of the disease pathology.

Human testis phosphoproteome reveals kinases as potential targets in spermatogenesis and testicular cancer

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Introduction Spermatogenesis is a complex cell differentiation process that takes place in the testis, within the seminiferous tubules. The process of spermatogenesis is very dynamic and disturbances in any of the steps would lead to fertility deficiencies. However, while hormonal regulation of spermatogenesis is well understood, other layers of regulation, such as signal transduction through phosphorylation, remain less well explored.

Aim To increase the knowledge of signal transduction through phosphorylation during sperm development by using high-throughput techniques, in order to unravel the molecular regulation of spermatogenesis and identify kinases that might be essential for the process.

Approach Metal oxide affinity chromatography using titanium dioxide (TiOx) method coupled to LC-MS/MS was conducted to profile the phosphoproteome of human testes with full spermatogenesis. To further investigate phosphoregulation, MS data was used to predict the most active and abundant kinases. For kinase ranking, data was extracted from web resources, including currently recognized protein kinases from KinBase and official gene symbols from HGNC. An algorithm provided on the Phomics website was used to identify kinase activation loop peptides.

Results A total of 8187 phosphopeptides derived from 2661 proteins were identified (6687-7210 phosphopeptides per replicate). The ratio of identified phosphopeptides to non-phosphopeptides ranged from 90.45 to 92.11%. Phosphosite localization analysis in MaxQuant detected around 89.7% of the phosphosites in serines, 9.8% in threonines and 0.5% in tyrosines. From the total of known testicular proteins, 32.9% were found to be phosphorylated in this study. 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, the data set contained 174 phosphorylated kinases, which covers the 32% of the human kinome. Information available at the Uniprot Knowledgebase (UniProtKB/Swiss-Prot) website allowed the identification of most of these active kinases as related to the functional annotations over-represented in the human testis phosphoproteome

Conclusions Our data confirm that phosphoregulation by protein kinases is highly active in sperm differentiation, and open a window to the detailed characterization and validation of potential targets for the development of drugs modulating male fertility, and tumor behavior.

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Investigation of huntingtin ubiquitination by LFQ-based mass spectrometry

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Huntington's disease (HD) is a neurodegenerative disorder hallmarked by the aggregation of polyglutamine (polyQ)-expanded N-terminal Huntingtin protein fragments (mHtt) in both the cytoplasm and nuclei of affected neurons. Aggregation of mHtt causes cellular toxicity hence improved clearance of mHtt prior to aggregation would be an interesting strategy to prevent or delay the onset of HD.
The ubiquitin-proteasome system (UPS) plays a key role in the degradation of proteins in the cell. Proteins are generally targeted for proteasome-dependent degradation by polyubiquitin chains.

We investigate the ubiquitination of wildtype (wtHtt) and polyQ-expanded mutant (mHtt) huntingtin in neuronal cells and brain tissues by diGly-peptide enrichment and Label Free Quantitative (LFQ) mass spectrometry. Our initial data shows that Htt K6 and Htt K9 are the main sites targeted for ubiquitination. Relative abundance of ubiquitination of these sites appear to differ between cell lines. Next, we will use immunoprecipitation and LFQ-based mass spectrometry to identify interaction partners of wtHtt and mHtt, thereby focusing on ubiquitinating- and deubiquitinating enzymes as manipulating their activities may enhance the targeting of mHtt for proteasomal degradation.

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Usp9X/Y is deubiquitating enzymes involved in regulation of RhoB expression and activation in endothelial cells

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Introduction RhoGTPases are small proteins involved in regulation of cell migration, apoptosis and cytoskeleton morphology. RhoA, B and C share high degree of sequence homology, but are remarkably different in terms of protein stability. Proteins stability is regulated by sequential addition of ubiquitin via concerted action of E1, E2 and E3 enzymes in ubiquitination cascade. We have shown that RhoB is rapidly degraded in endothelial cells due to Cullin3-KCTD10-Rbx1-dependent polyubiquitination and subsequent lysosomal and proteasomal degradation. There are several hundred E3 ligases which mediate protein ubiquitination and approximately 100 ubiquitin hydrolase responsible for deubiquitnation of proteins. Despite the important and well-studied role of RhoGTPases in cell biology, so far there are no deubiqitinating enzymes described to regulate their function. We applied mass spectrometry analysis of the interactome of C-terminus of RhoB to identify deubiquitnating enzymes for RhoB in endothelial cells.

Aim Identification of deubiquitinating enzymes involved in regulation of RhoB expression and activation in endothelial cells.

Approach We performed pulldown assay using biotinylated C-terminal peptide of RhoB containing TAT sequence as a bait and lysates of primary human endothelial cells. Biotinlyated TAT-sequence only was used as a negative control. Prior to lysis cells were either left untreated or they were stimulated with 10 ng/ml TNFalpha or 300 nM Cullin-RING ligase inhibitor MLN4924 for 4 hr. Proteins bound to C-terminus of RhoB were separated using SDS-PAGE and following trypsin digestion analysed using label free mass spectrometry. Peptides were identified using MaxQuant. The experiment was repeated three times independently.

Results We have identified several deubiquitinating enzymes as potential interactors of C-terminus of RhoB by MS approach. Usp5, Usp7 and Usp9X/Y were enriched in samples and were not detected in negative controls. The most prominent potential interactor or RhoB in our MS screen is Usp9X/Y with ($p = 9.85E-08$ all samples vs negative control). Additionally, interaction of Usp9X/Y with the C-terminus of RhoB was decreased upon MLN4924 treatment ($p=0.04$ untreated vs MLN4924 and $p=0.008$ TNF vs MLN4924).

Conclusion There are currently no deubiquitinating enzymes described for RhoGTPases in the literature. In our mass spectrometry analysis we identified Usp9X/Y as a potential interactor of RhoB. We are currently validating these results by immunoprecipitation and *in vivo* ubiquitination assays.

Insulin-induced myocardial microvascular recruitment is increased by exercise, regulated by endothelial AMPK and a determinant of left ventricular function

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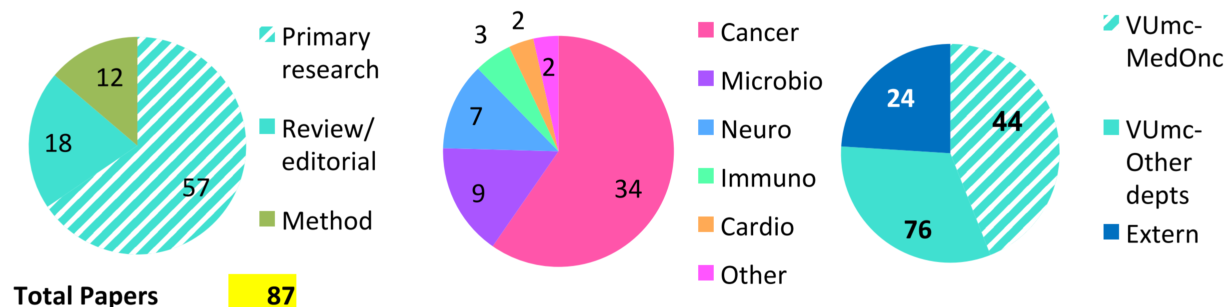
Rationale Exercise activates 5'AMP-activated kinase (AMPK) in endothelium and enhances myocardial perfusion, insulin sensitivity and ventricular function. Myocardial perfusion determines ventricular function and insulin increases myocardial perfusion by recruiting microvascular blood volume (IMVR), but the relationship between exercise, AMPK and IMVR in the heart is unknown.

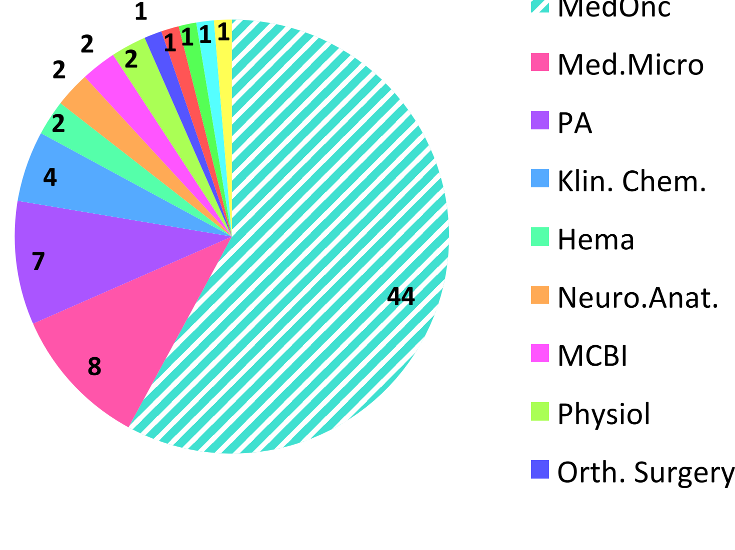
Objective To investigate whether exercise increases insulin-induced microvascular recruitment (IMVR) in the myocardium and whether endothelial 5'AMP-activated kinase determines myocardial IMVR and function.

Methods and results In human subjects, we analyzed whether physical activity relates to IMVR using myocardial contrast echocardiography (MCE). In mice, we tested whether treadmill running increases IMVR. IMVR correlated with physical activity ($r=0.50, p<0.05$) and increased upon treadmill running. IMVR correlated with left ventricle contractility in men (dp/dt, $r=0.49, p<0.05$).
In both inducible and constitutive endothelium specific AMPK knockout ($AMPK^{EC\text{-}KO}$) mice we performed MCE, pressure-volume loops and radiolabeled hyperinsulinemic euglycemic clamps. $AMPK^{EC\text{-}KO}$ decreased IMVR (0.41 ± 0.10-0.31 ± 0.08, $p<0.05$), left ventricle stroke volume (25.3 ± 5.8µl-18.3 ± 5.2µl, $p<0.05$), stroke work (1008 ± 283mmHg*µl-698 ± 449mmHg*µl, $p=0.05$) and induced ventriculo-arterial uncoupling. Quantitative mass spectrometry revealed that deletion of endothelial AMPK activity altered expression of clusters of myocardial proteins related to muscle contraction (eg.Myh1,Myh4), cytoskeletal organization (Nrap,Synpo2,Myom2) and response to stress (Lyz1,Fibrinogen).

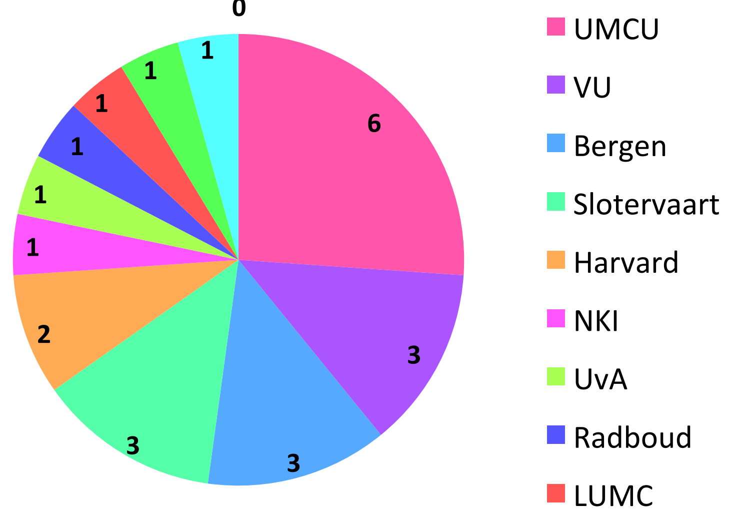
Conclusion Physical activity determines insulin-stimulated myocardial blood volume in man and mice, and the exercise mediator AMPK in endothelium controls insulin-induced microvascular recruitment and left ventricular function by upregulation of contractile proteins.

Appendix 2 OPL production





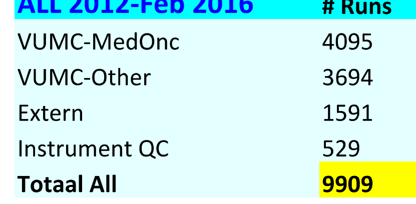
VUmc-Depts. 2006- 2016	# Papers
MedOnc	44
Med.Micro	8
PA	7
Klin. Chem.	4
Hema	2
Neuro.Anat.	2
MCBI	2
Physiol	2
Orth. Surgery	1
Klin.Gen.	1
Surgery	1
Urologie	1
KNO	1
Total Papers	**76**



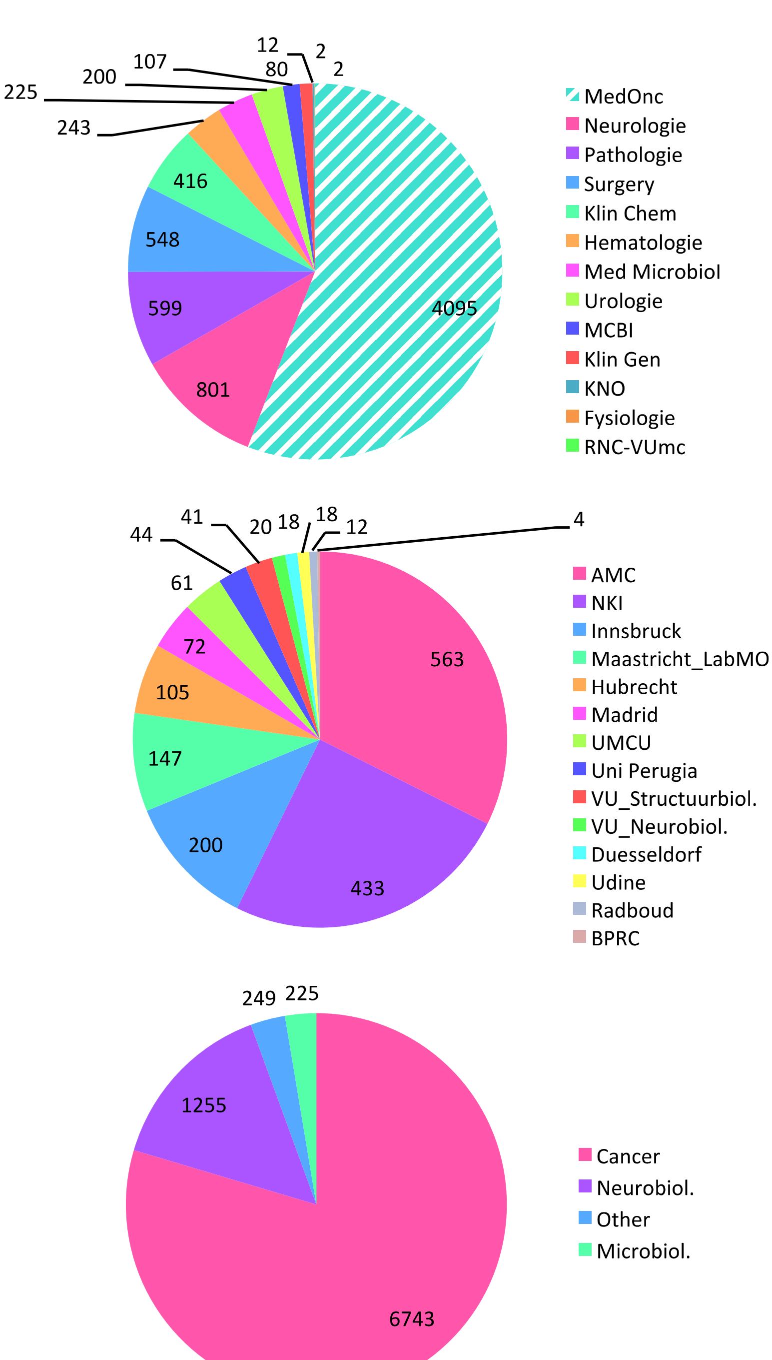
EXTERN. 2006- 2016	# Papers
UMCU	6
VU	3
Bergen	3
Slotervaart	3
Harvard	2
NKI	1
UvA	1
Radboud	1
LUMC	1
Kopenhagen	1
Innsbruck	1
Bochum	1
Totaal Papers	**24**

Mass spec runs 2012- Feb 2016

(2x QExactive; 1000K; 20% CCA)



ALL 2012-Feb 2016	# Runs
VUMC-MedOnc	4095
VUMC-Other	3694
Extern	1591
Instrument QC	529
Totaal All	**9909**



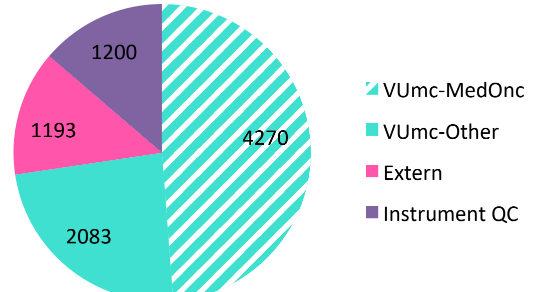
VUmc 2012-Feb 2016	# Runs
MedOnc	4095
Neurologie	801
Pathologie	599
Surgery	548
Klin Chem	416
Hematologie	243
Med Microbiol	225
Urologie	200
MCBI	107
Klin Gen	80
KNO	12
Fysiologie	2
RNC-VUmc	2
Total VUmc	**7330**

Extern 2012-Feb 2016	# Runs
AMC	563
NKI	433
Innsbruck	200
Maastricht_LabMO	147
Hubrecht	105
Madrid	72
UMCU	61
Uni Perugia	44
VU_Structuurbiol.	41
VU_Neurobiol.	20
Duesseldorf	18
Udine	18
Radboud	12
BPRC	4
Total Extern	**1738**

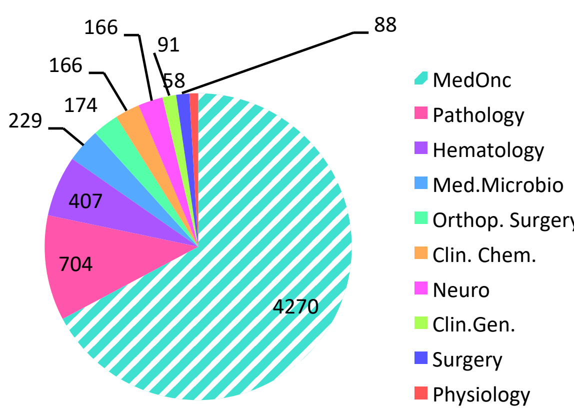
TOPIC 2012-Feb 2016	# Runs
Cancer	6743
Neurobiol.	1255
Other	249
Microbiol.	225
Total	**8472**

Mass spec runs 2007- 2010

(1x LTQ-FTMS; 1000K; CCA start-up grant)



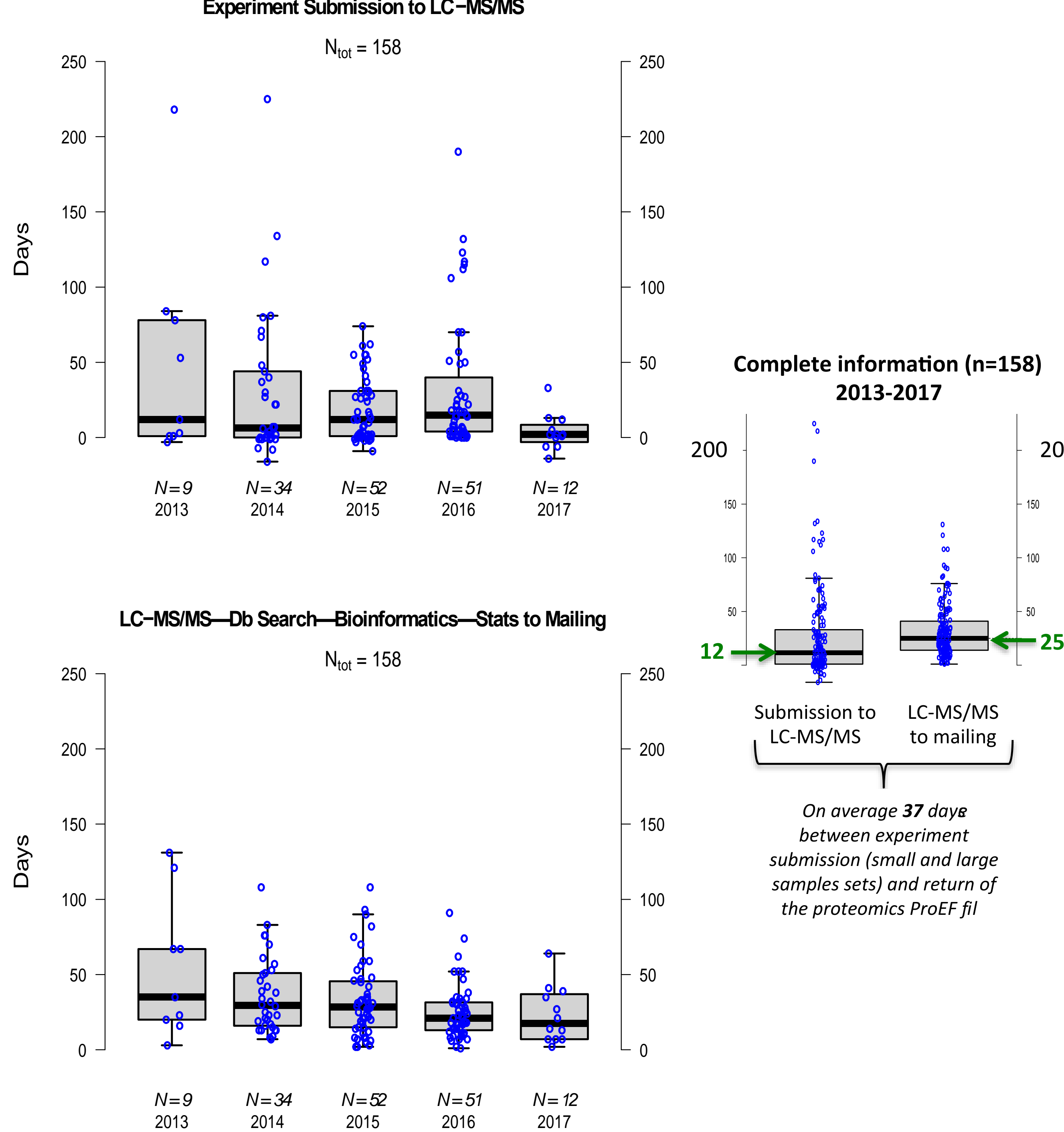
ALL 2007-2010	# Runs
VUmc-MedOnc	4270
VUmc-Other	2083
Extern	1193
Instrument QC	1200
Total All	**8746**



VUmc 2007-2010	# Runs
MedOnc	4270
Pathology	704
Hematology	407
Med.Microbio	229
Orthop. Surgery	174
Clin. Chem.	166
Neuro	166
Clin.Gen.	91
Surgery	88
Physiology	58
Total VUmc	**6768**

Extern 2007-2010	# Runs
UMCU	310
Harvard	189
VU	155
UvA	141
ID Lelystad	124
ProQInase	90
Bergen	62
Kopenhagen	60
Slotervaart	28
AMC	21
Magdeburg	13
Total Extern	**1193**

TOPIC 2007-2010	% Runs
Cancer	77%
Neuro	14%
Microbio	7%
Other	2%
Cardio	0,50%
Total	**100%**



All experiments with complete information in the OPL ProPEL database were included in the calculation. NB large experiments may take weeks of instrument time and data processing, thus have much longer analysis times. These were included as well in the calculation. Therefore we are happy with the result of an average turn-around time to user of 37 days for our proteome and phosphoproteome profiling.

From the left top and bottom panels it is clear that our waiting times reduced over the past 5 years due to more efficient organization of OPL core processes and pipelining some of the analysis steps. From the summary graph (right), it is clear that currently data analysis capacity is more limiting than MS capacity.

Appendix 3
What do our collaborators say about the OPL?

Quotes from CCA collaborators:

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, Tumor Biology section of the Dept. Otolaryngology-Head and 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, Head of the Oncogenetics Labs, 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.'

Drs. Mariette Labots M.D., Dept. Medical Oncology (clinical staff), collaborator 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, new collaborator:
"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**."

Quotes from non-CCA VUmc collaborators

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 in 2018. The identified disease modifiers will be tested in functional assays to establish their exact role in cardiac disease.

Quotes from external collaborators

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. Jan Paul Medema, AMC, 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."

Prof. Jacco van Rheenen, Dept. Cancer Biophysics, Hubrecht Institute, 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."

Quotes from the NL proteomics veld:

Support quotes regarding our funded NWO-MG proposal 'Next-generation proteomics to create digital cancer proteome maps for translational research and personalized medicine'

Prof. Rainer Bischoff (head of the Analytical Biochemistry Group at RUG and main participant in the Biomarker Development Center): "Digitalizing cancer proteomes will help to assess the individuality and heterogeneity of cancers in view of personalized cancer treatment''.

Dr. Arzu Umar (assistant professor and breast cancer proteome expert at EMC): "Dr. Jimenez is the **founding mother of the proteomics community in the Netherlands** with an enormous network in the proteomics field, both national and international, she is an expert in cancer proteomics, and has always been at the **forefront** of new developments and initiatives with the aim of translating state-of-the-art technologies and findings towards the clinic".