# OncoProteomics Laboratory

## Progress Report

## 2012-2014

CCA VUmc O P L ONCO Proteomics Laboratory

VUmc Cancer Center Amsterdam

# Progress Report 2012-2014

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*Cover: Spiegelretourschip de Soeverein, Travelydays*

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# Foreword

The progress report 2012-2014 covers the activities of the past three years of the OncoProteomics Laboratory (OPL) that was established at the VUmc department of Medical Oncology in April 2006 with support from the VUmc Cancer Center Amsterdam. In this report, you can read about our biomarker research, mass spectrometry infrastructure and workflows, including summaries of running core and collaborative projects.

In the past 3 years, using two state-of-the-art nanoLC-MS/MS discovery platforms that were acquired in 2012, we could expand our cancer proteomics research effort and significantly increase the sample size per experiment, important to address the heterogeneity of cancer and clinical samples. Here follows a brief overview of the highlights:

- Meike de Wit, Marc Warmoes and Benjamin Emmink defended their PhD theses that contained mutiple chapters with cancer proteomics data that advanced molecular insights into colorectal and breast cancer and pave the way for new biomarker applications.
- In the past 3 years, 37 peer-reviewed research papers were published, including papers in Cancer Research, British Journal of Cancer, Gut, PNAS and Molecular and Cellular Proteomics. This list also includes 8 invited reviews on (label-free) proteomics biomarker strategies, colon and breast cancer proteomics as well as a book chapter for the American Society Clinical Oncology Educational Book. This book chapter was associated to the ASCO2014 meeting to which I was invited as a speaker and as chair of the educational proteomics session. It was also an honor to be invited speaker and chair of the cancer proteomics session at the HUPO2014 meeting in Madrid, a meeting that we attented with almost the whole team!
- In the context of the CTMM valorisation project CRC-Bioscreen, we recently validated in a large series of faecal samples (300!) many of our candidate protein biomarkers for CRC screening that were identified in the CTMM-DeCoDe project. The collaboration with Dr. Remond Fijneman, Dr. Beatriz Carvalho and Prof. Gerrit Meijer will be expanded in the coming years as 2 new KWF projects were funded with focus on cancer-specific variants as CRC protein biomarkers (PhD student Gosia Komor) and phosphoproteomics in colorectal adenoma-to-carcinoma progression (vacancy post-doc).
- The successful research line on protein biomarkers for BRCA1 deficient breast cancer will be continued by post-doc Frank Rolfs with support of KWF. In collaboration with Dr. Jos Jonkers (NKI) and Prof. Paul van Diest (UMCU), protein biomarkers for homologous recombination (HR) deficient breast cancer will be identified and validated using patient-derived xenograft triple negative breast cancer tumors with known HR and BRCA status and patient material.
- I am also happy that the research line on protein biomarkers for cisplatin response prediction in NSCLC (our previous CTMM Airforce project) will be continued in a new KWF project with Prof. Egbert Smit, Dr. Sjaak Burgers and Dr. Katrien Grunberg.
- A new collaboration was initiated with Prof. Jan Paul Medema to identify protein biomarkers for CRC subtypes and prognosis in stage II-III disease. This research will be boosted by the in 2014 funded Alpes d'HuZes project Connection.
- Progress was made in the area of phosphoproteomics: the first three phosphoproteomics papers were recently submitted and funding was obtained for four projects that will employ phosphoproteomics to identify aberrantly activated signaling pathways and Achilles heel(s) in AML (CCA project, PhD student Carolien van Alphen), pancreatic cancer (VUmc-AMC alliance project, PhD student Tessa Le Large) and colorectal cancer (private, PhD student Robin Beekhof) and to develop a targeted MS-based kinome activity assay (CCA, vacancy post-doc). Our participation in the Center for Personalized Cancer Treatment will enable to explore the value of phosphoproteomics for drug response prediction in a genomic context.
- Phosphoproteomics was successfully scaled down to the level of tumor biopsy samples and applied in a proof-of-concept study to a set colorectal tumor biopsies. In close collaboration with Prof. Henk Verheul we hope to develop phosphoproteomics into a clinically applicable tool to enable patient selection for targeted anti-kinase therapy and personalized medicine.

Finally, I hope you will enjoy reading this report on OPL proteomics activities and that this report triggers new ideas and collaborative projects.

Dr. Connie Jimenez,
*Head of the OncoProteomics Laboratory, Associate Professor, Dept. Medical Oncology, VUmc*

## Members of the Research Group:

Group photo OncoProteomics Laboratory, June 2014

**Head, Associate Professor:**

Dr. Connie R Jimenez

**Research associates:**

Dr. Sander Piersma (OPL-core: nanoLC-MS/MS)
Dr. Thang V. Pham (OPL core: (bio)informatics)
Dr. Henk Broxterman

**Research technicians**

Dr. Jaco C. Knol (OPL core: down-stream data mining, back-up nanoLC-MS/MS)
Ing. Inge de Reus (lung cancer biomarkers, exosome proteomics)
Ing. Richard Goeij-de Haas (phosphoproteomics)

**Post-doctoral fellows:**

Dr. Meike de Wit (colon cancer biomarkers)
Dr. Tieneke Schaaij-Visser (NSCLC predictive biomarkers)
Dr. Davide Chasserini (CSF biomarkers)

**PhD students:**

Marc Warmoes (breast cancer biomarkers; thesis completed)
Joey Lam (breast cancer biomarkers)
Carolien van Alphen (AML phosphoproteomics)
Tessa Le Large (Pancreatic cancer phosphoproteomics
Robin Beekhof (CRC phospoproteomics)
Gosia Komor (CRC proteogenomics)
Koen van der Mijn (tumor profiling for response prediction)

**Translational scientists:** Mariette Labots (PhD student, oncologist), Prof. Henk Verheul

**Visiting scientists**

Cira Garcia de Durango Caveda (CRC exosome proteomics, 2014)

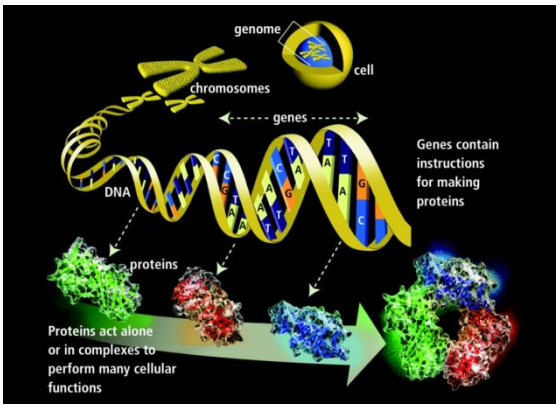
**Internship students**

Tim Schelforst (Testing a new method for exosome isolation, 2014)
Erik Meijer (CRC proteomics in relation to 5FU in vivo, 2014)
Niek de Klein (Statistical modeling of MS/MS peaks, 2013-2014)
Sushen Adhin (Spectral library searching for protein identification, 2013)
Valerie Dusseldorp (Proteomics of colorectal cancer exosomes, 2012-2013)
Barath Kumar (Phosphoproteomics of a panel of colon cancer cell lines, 2011-2012)
Sofia Koustoulidou (FFPE proteomics colon cancer, 2011-2012)
Hulya Ozturk (colon cancer secretome and exosome proteomics, 2011-2012)

# Introduction

The OncoProteomics Laboratory (OPL) of the VUmc department of Medical Oncology has been founded in April 2006 together with the establishment of the cancer research building of the VUmc-Cancer Center Amsterdam (CCA). The OPL was created to provide a state-of-the-art proteomics infrastructure and knowledge center for cancer researchers of the CCA/V-ICI research institute and beyond.

Proteomics is optimally suited to bridge the gap between genomic information on the one hand and biological functions and disease phenotypes at the other, since it studies global expression and/or post-translational modification (especially phosphorylation) of proteins - the major cellular players effectuating cellular functions - in biological specimens.




Mass spectrometry technology and (bio)informatic tools have matured to the extent that they can provide high-throughput, comprehensive, and quantitative protein inventories of cells, tissues and biofluids in clinical samples at low level.

## Mission of the OPL

The mission of the OPL is to improve (early) diagnostics and treatment of cancer through innovative proteomics and data analysis approaches.

To this end, we focus on next generation proteomics employing nano-liquid chromatography coupled to high-resolution tandem mass spectrometry (nanoLC-MS/MS) for in-depth label-free (phospho)protein profiling of tumor tissues, (proximal) biofluids, and cancer models. In addition, we use emerging proteogenomic approaches for the identification of tumor-specific protein variants, and targeted multiplex mass spectrometry strategies for large-scale biomarker validation.

Below we provide a discussion of proteomics strategies, research highlights, and summaries of running projects.

## OPL research activities

The projects of the OPL broadly can be subdivided into 3 categories: 1. OPL core research, ie., proteomics research to test and set up methods important for cancer biomarker discovery as well as oncoproteomics research projects initiated by the OPL, 2 Collaborative research projects, and 3. Service projects (protein identification work on a fee-per-sample basis). The far majority of the projects fall in category 1 and 2. See OPL research and collaboration diagram (figure 1) for an overview.

## Information exchange

- In weekly OPL group meetings (every Friday): discussion of all running proteomics projects
- In bi-monthly lunch meetings (every 2$^{nd}$ and 4$^{th}$ Tuesday): journal club
- All OPL people attend and present their projects in the weekly Medical Oncology Dept. seminars.
- Through our website (www.oncoproteomics.nl )

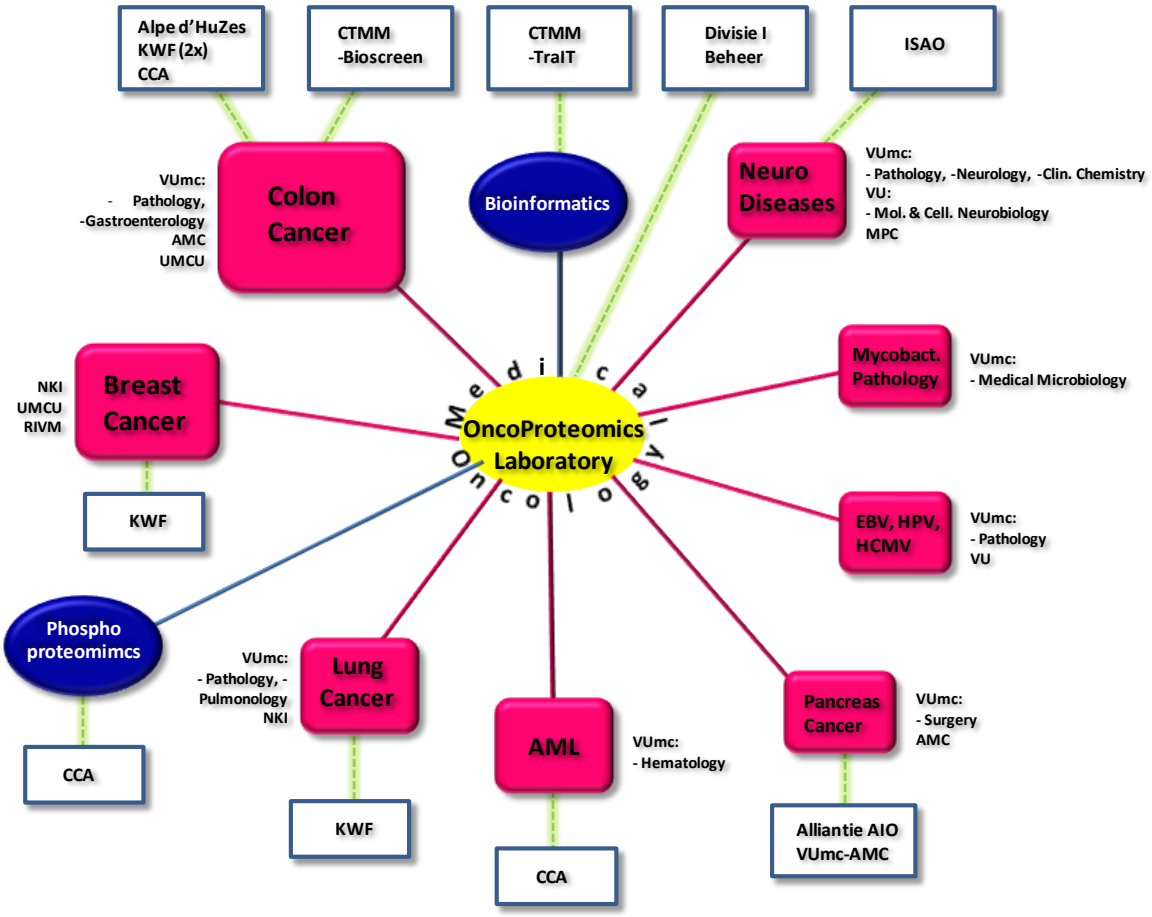



Figure 1. Areas of OPL proteomics activities and collaborating departments and institutes. The size of the boxes is an approximate indication of the magnitude of the effort. Sources of *current* research funding (anno dec 2014) are indicated by the blue boxes.

## Organisation of the OPL

The OncoProteomics Laboratory is a cancer research laboratory of the Department of Medical Oncology as well as a facilitating proteomics center where most projects are shaped in close interaction with the collaborators.

Head of the laboratory is a scientist (1 fte, Dr. Connie Jimenez, Associate Professor) who is leading a team of scientists (3 fte, a mass spectrometrist, Dr. Sander Piersma; a computer scientist, Dr. Thang Pham; and a biochemical research technician, Dr. Jaco Knol) as well as PhD and master students and post-docs, co-workers on temporary projects and visiting scientists on collaborative projects.

The OPL performs biomarker research in many collaborative projects within CCA, VUmc as well as at the national level (CTMM projects and Alpes d'HuZes consortium) (see figure 1) and in miscellaneous smaller projects without dedicated funding. The main focus is on proteomics strategies to enhance molecular insights into tumor biology, for early (non-invasive) detection of cancer and to enable patient selection for targeted therapy and prediction of therapy sensitivity/resistance.

## Infrastructure

**HOUSING** The OPL is housed on the first floor of the CCA building with two laboratory spaces: a small protein chemistry lab (CCA 1-52) and a mass spectrometry lab (CCA 1-47). The lab spaces were experienced overall as too small in 2012-2014 as the wet lab is at times very crowded. The MS lab is very noisy. Ear plugs and noise absorbing shields have provided some solution (though not ideal). The OPL office space was sufficient to accommodate everyone together on the first floor but like the lab space may need expansion in 2015.

**PLATFORMS** The mass spectrometry lab houses three tandem mass spectrumeters on-line coupled to nanoLC systems: Three discovery systems for global profiling: two next generation Orbitrap platforms (QExactive) (figure 2, left picture) and one system for targeted mass spectrometry: the QTrap 5500 (right picture).

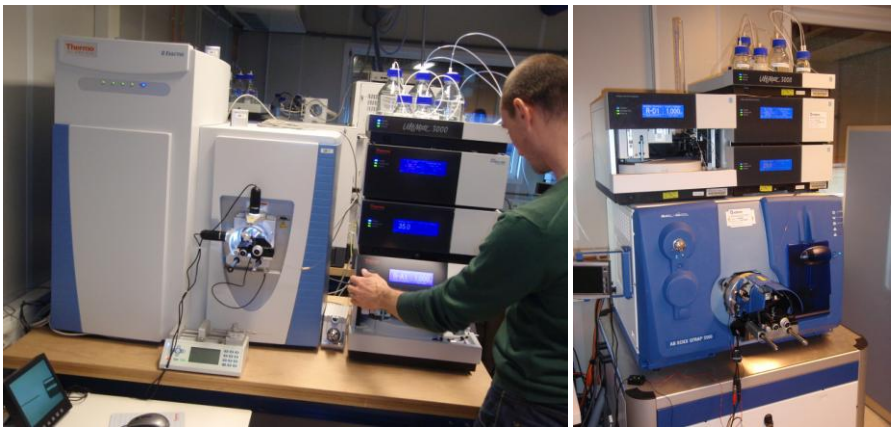


Figure 2. Tandem mass spectrometers at the OPL (CCA 1-47). Left: next generation Orbitrap, the QExactive (ThermoFisher) (one of the systems is shown). Right: nanoLC system (Ultimate3000) coupled to a QTrap 5500 platform (Applied Biosystems).

**BOX 1. What is Mass Spectrometry?**

- A mass spectrometer is an instrument that very accurately measures the masses of individual molecules that have been converted to ions; i.e., molecules that have been electrically charged.
- Since the invention of soft peptide ionisation methods (ie., electrospray and matrix-assisted laser desorption/ionisation (MALDI)) in 1988, mass spectrometry (MS) has become a central analytical technique for protein research
- In proteomics research, MS is used for large scale analysis, ie., detection, identification and quantification, of peptides and proteins in complex mixtures derived from biological or clinical samples
- For protein identification, powerful bioinformatics tools have been developed that link mass spectral molecular weight information of peptides and their fragments to amino acid sequence databases
- A wide range of new MS–based analytical platforms has been developed, including hybrid instruments such as the Orbitrap and Qtrap platforms that allow for high-resolution, super-fast and super-sensitive protein analysis.

**IT Infrastructure**
After data acquisition by nanoLC-MS/MS, raw data are processed by the software tool MaxQuant for peptide/protein identification and quantification. For large-scale projects, we employ the IDPicker tool to integrate database search results from multiple search engines, including MS-GF+ and Myrimatch, for identification and quantification. Data exports to Excel are used for further dedicated statistical analyses, which is also facilitated by the OPL. The CTMM TraIT project in which the OPL participates will facilitate the development of standardized processed data organization, enabling integrative analysis with other -omics domains.

The computer infrastructure to cope with the large data flows is constantly being upgraded. Currently, three servers are in a local network with the tandem MS data acquisition PCs and are connected to the VUmc network via a network switch (see figure 3). These servers are connected to the VUmc store4ever system for big data storage, as well as VUmc GreenQloud and the national life science grid for additional computing power.

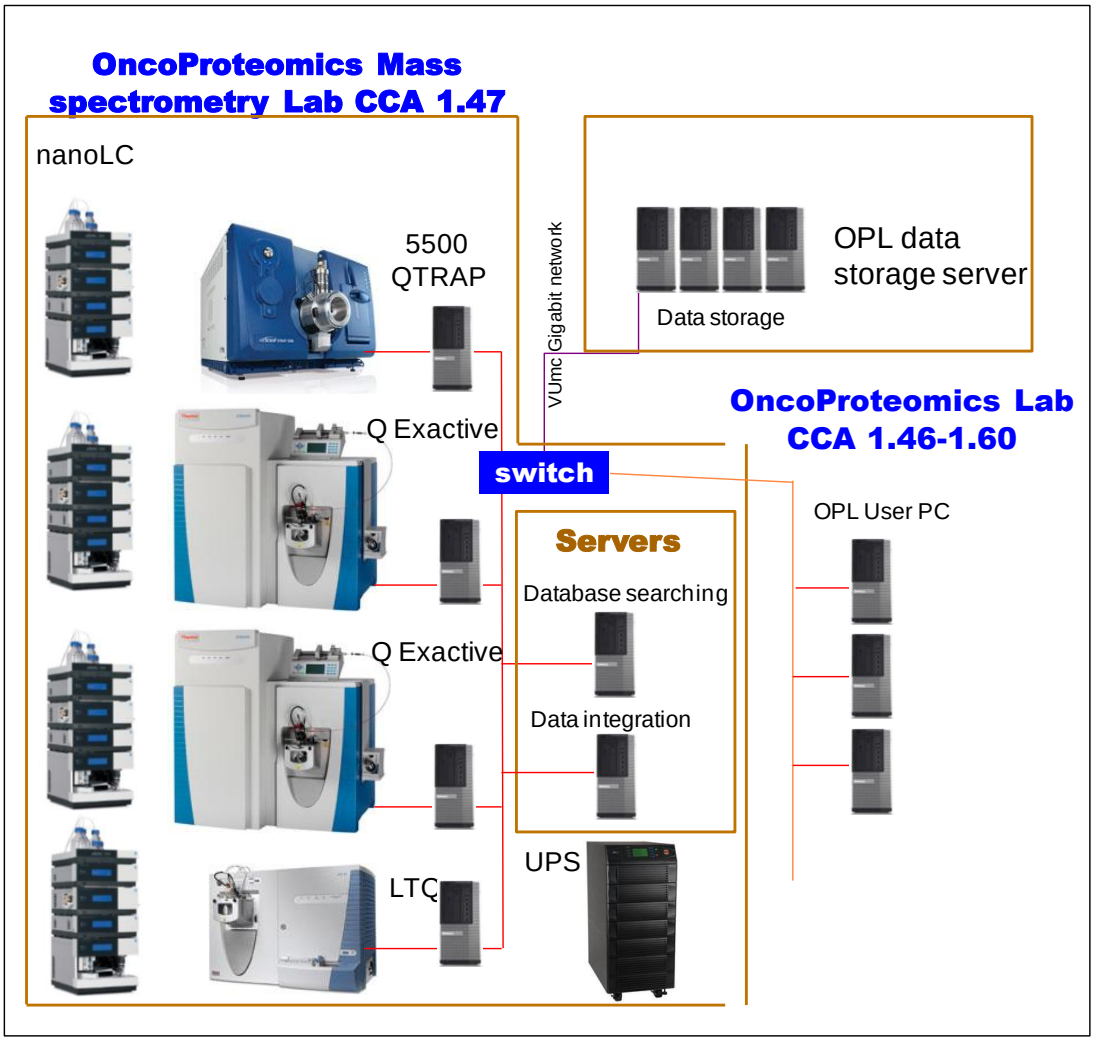



Figure 3. Proteomics infrastructure OncoProteomics Laboratory VUmc.

## Research strategy and projects

### Introduction

Proteomic analysis focuses on the large-scale identification and quantification of the proteome complement of the genome in cells, tissues, and biofluids. It benefits from the successful sequencing of the genomes of a wide variety of organisms, including humans. In contrast to the genome, the proteome is highly dynamic, depending on spatio-temporal conditions (e.g., subcellular location, developmental stage, environmental cues). The field of proteomics is particularly important because diseases, including cancer, are regulated at the protein level.

**Box 2. Key Points Proteomics**

- Current mass spectrometry platforms provide comprehensive and quantitative inventories of a large number of proteins (up to ~10.000) in complex biological and clinical samples
- For protein identification, proteins are tryptically digested to peptides to improve detectability in the mass spectrometer and to provide multiple measurements per protein. Bioinformatic tools link mass spectrometric information on the molecular weight of tryptic peptides and their *in situ* generated fragments to general amino acid sequence databases, or more recently to cancer genome-based protein sequences to enable the identification of protein variants (proteogenomics)
- Protein biomarkers are advantageous molecules for diagnostic applications as they are directly connected to (patho-)physiology and may be coupled to routine, antibody-based assays.
- Phosphoproteomics can uncover the activity of endogenous signaling networks in (cancer) cells and (tumor) tissues, and enables the identification of candidate driver kinases for therapeutic targeting in cancer
- Targeted mass spectrometry approaches have been developed into valuable high-throughput tools for specific and sensitive multiplexed validation of protein biomarkers derived from global discovery experiments as a first step towards their potential clinical application

Proteins are ideal biomarkers as they can be immuno-stained in routine paraffin-embedded specimen and immuno-detected in blood using conventional ELISA. Discovery proteomics allows for large scale protein identification quantification and may identify novel candidate biomarkers for cancer diagnosis, (early) detection and (prediction of) drug response (Fig. 4). Candidate-based, targeted proteomics can speed up the process of validation in larger sample sets as up to 100 candidates can be multiplexed in a single analysis. From these targeted analyses validated candidates may be prioritized for development of antibody-based tests. Cancer-related proteins validated in large cohorts may provide novel drug targets and candidate biomarkers for development into non-invasive (multiplex) antibody-based assays.

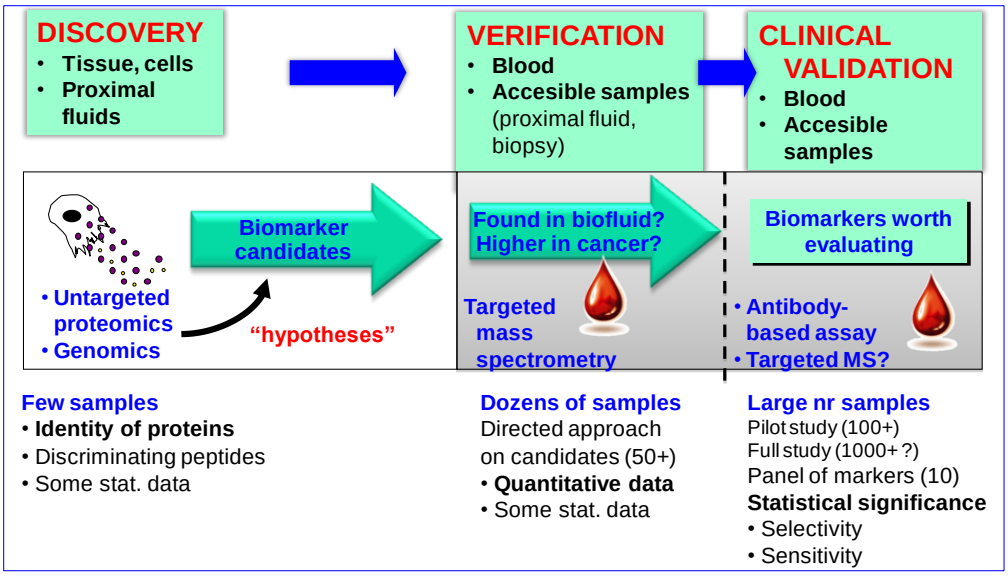



Figure 4. Complementary mass spectrometry-based proteomics approaches for discovery and validation of cancer signatures and biomarkers at the OncoProteomics Laboratory.

Figure 4 depicts the general pipeline for discovery and validation of biomarker candidates. Besides analytical considerations described below, important issues need to be taken into account when embarking on a biomarker discovery project to avoid bias. These include pre-analytical variables such as the quality of the clinical samples (collected using a standardized protocol?), the tissue composition (inspected by a pathologist?, how representative is the sample for the tissue?), and the study design that should avoid a potential bias among the compared groups. Finally, beyond discovery and verification studies, clinical validation requires a large(r)-scale case-control or cohort study to carefully examine the impact of other covariates on the proposed marker test, to determine the positive predictive values and false referral probabilities in real practice, and to compare or combine the new test with existing clinical tests. Clearly, biomarker discovery and validation by proteomics requires a multi-disciplinary effort.

Below, I discuss in more detail the requirements for label-free biomarker discovery and verification by MS-based proteomics and I will highlight our expertise and recent applications in model systems and clinical samples.

## Development and application of robust mass spectrometry-based methods for cancer biomarker discovery and validation

### EXPERTISE

- Label-free biomarker discovery workflow
- Candidate-based targeted mass spectrometry
- In-depth proteomics of cells and tissues
- Proteomics of biomarker-rich subproteomes (secretome, exosomes, cell surface, nucleus)
- Proteomics of in vivo biofluids and its components (CSF, stool, sputum, platelets, vesicles)
- Phosphoproteomics of cells and tissues
- Mining high-dimensional proteomics data

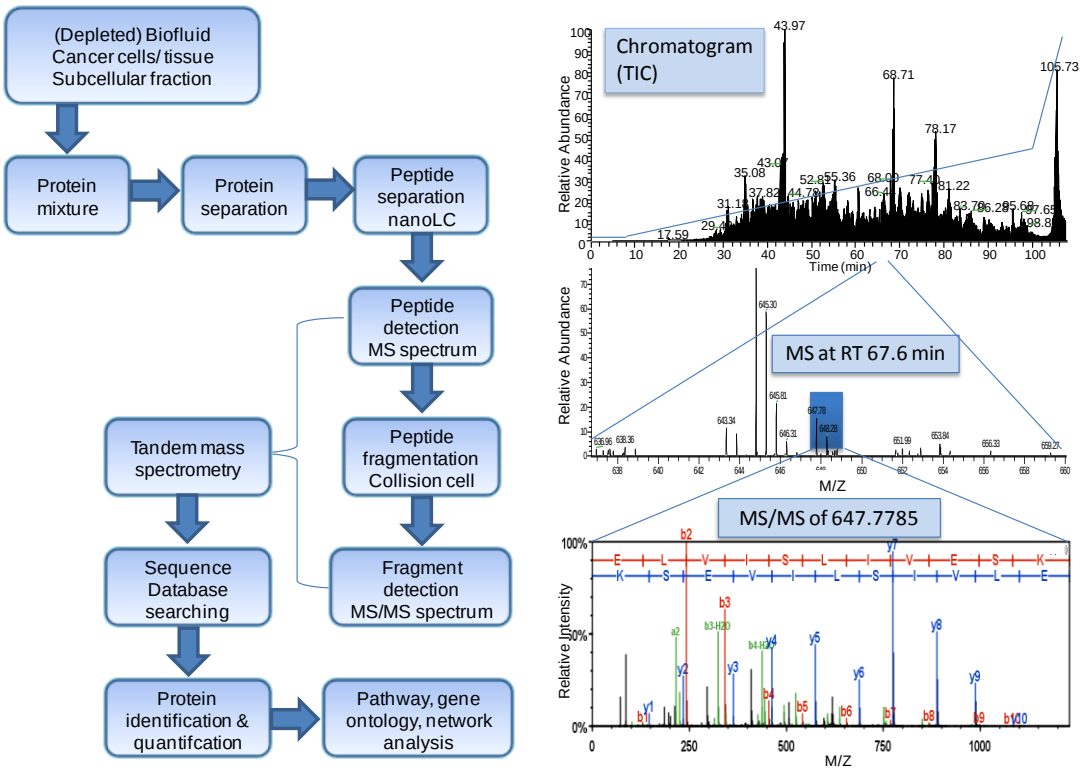



Figure 5. NanoLC-MS/MS-based proteomics workflow of implemented at the OPL (figure from Pham et al., Expert Rev. Mol. Diagnostics 2012). Label-free-based protein quantiation is based on counting the number of MS/MS spectra per peptide that maps to a protein.

**Label-free proteomics discovery workflow**
For in-depth proteome analysis, we perform two dimensions of fractionation: 1. Proteins in the biological/ clinical samples are fractionated by 1D gel electrophoresis 2. In-gel digested proteins are separated using nano-liquid chromatography (LC) on-line coupled to MS/MS sequencing of the peptides (Fig. 5). Together, this fractionation approach ensures unbiased proteome analysis at a large dynamic range of detection (~$10^6$) at intermediate throughput (2 hrs- 7,5 hrs per sample).

Label-free quantitative proteomics is an emerging field that we have pioneered (see our review in Expert Rev. Mol. Diagnostics: Pham et al., 2012). Label-free experiments have the advantage over experiments using labeling strategies in that they allow for profiling large series of (clinical) samples with the flexibility of multiple different comparisons, are cost-effective, and do not involve complex labeling steps/reagents. Except for one iTRAQ-based study (Rajcevic et al., 2009), all our quantitation has been performed label-free because of good results in our studies with regards to reproducibility and rediscovering known markers as well as new ones (eg.,De Wit et al., Gut. 2012; Warmoes et al., Mol. Cell. Proteomics 2012; Posthuma De Boer et al., British J Cancer 2013) (appendix pages 66, 73 and 40). We further stream-lined the label-free workflow by testing and implementing the 'whole gel' protocol for parallel sample processing prior to mass spectrometry (Piersma et al., Proteome Sci 2013). Dr. Thang Pham optimized and implemented dedicated beta-binomial-based statistics for the analysis of spectral count data (the quantitative measure of protein abundance) (Figure 6). Spectral counting turned out to be a robust, reliable approach (Pham et al., 2010; 2012; 2013; page 85). In addition, to exploit MS1 data in LC-MS datasets for quantitation, we have implemented the MaxQuant tool that allows for quantitation of peptide ion abundance (Figure 6), which yields an independent alternative label-free measure.

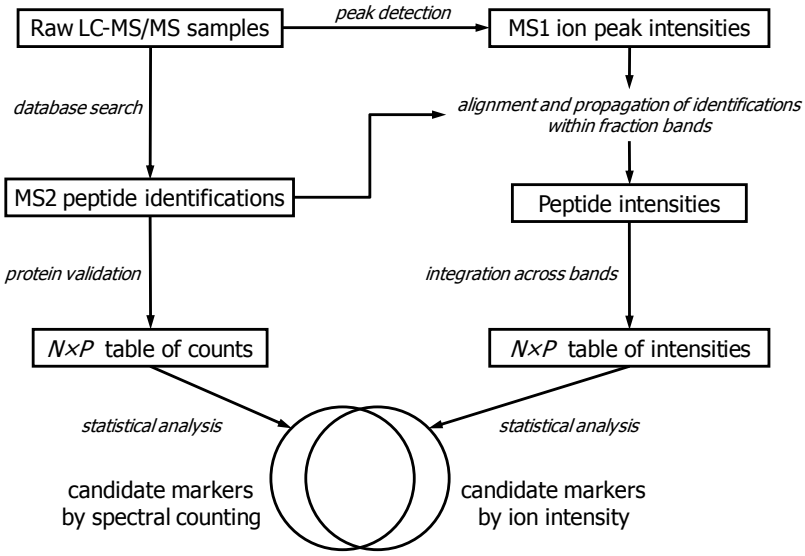



Figure 6 Label-free protein quantitation strategies implemented at the OPL (Figure from Pham et al., Expert Rev. Mol. Diagnostics, 2012).

**Candidate-based targeted mass spectrometry**
Discovery proteomics typically yields a host of candidate biomarkers of interest for validation studies in large series of independent samples. However, conventional antibody-based assays such as immunohistochemistry or ELISA typically measure only one protein per assay while simultaneous (multiplexed) antibody-mediated detection of several proteins requires more demanding assays. Moreover, specific and reliable antibodies are often not available, and immunoassays are not suitable for multiplexing a large number of proteins. With the development of targeted mass spectrometry methods like Multiple Reaction Monitoring (MRM) and parallel reaction monitoring (PRM), large numbers of proteins of interest can be quantified in one multiplexed analysis, without the need for antibodies. MRM has emerged as a sensitive method that can profile tenths of proteins at low-attomole sensitivity and high reproducibility (%CV generally <10%). Peptide detection and quantification is based on the combination of two consecutive stages of mass filtering, selecting a peptide precursor mass in the 1$^{st}$ quadrupole (Q1) and, after CID, a fragment ion mass in the 3$^{ed}$ quadrupole (Q3) in MRM (figure 7) or read-out of the full MS/MS spectrum as in PRM. The OPL has a dedicated platform for targeted MRM-MS (the QTrap 5500, see picture in figure 2) and PRM can be performed on the QExactive instrument. Because of the sensitivity of targeted mass spectrometry, instrument time can be reduced, allowing for the profiling of relatively large numbers of samples, thereby providing a suitable candidate biomarker validation platform.

In the context of various projects, we have developed multiplex assays for up to ~40 biomarkers candidates to enable hightroughput validation in large clinical sample sets. Based on these analyses small discriminatory panels will be selected for further antibody-based assay development and clinical testing.

**In-depth proteomics of cancer cells and tumor tissues**

If well-characterized tumor tissue is available for proteomics, we prefer to use tissue as the starting point for biomarker discovery. For in-depth analysis of total tissue lysates, 1-10 mg is enough (ie., biopsy level). If a large quantity is available (>50-100 mg), fractionation into tumor sub-proteomes may enhance the sensitivity of detection of selected proteins of interest (see below). Proteins isolated from total tissue lysates or subfractions are subjected to 1D gel electrophoresis and nanoLC-MS/MS analysis. This analysis yields typically 4000-5000 of identified and quantified proteins per sample.
One example of a project that employed proteomics of total tissue lysates is the comparative analysis of breast cancer tissue of genetic mouse models for BRCA1 deficient and proficient breast cancer (collaboration with Dr. Jos Jonkers, NKI). This work has yielded a novel diagnostic 45 protein signature that was validated in silico in public transcriptome datasets for identification of BRCA1 and BRCA2 deficient breast cancer in humans (Warmoes et al., Mol. Cell. Proteomics 2012) and is described in the research appendix on page 73. Another example of total lysate proteomics is our project using patient tumor tissue-derived colon cancer spheroids as a model system enriched for colon cancer stem cells (Emmink et al., Gasteroenterology 2011). The project with Dr. Onno Kranenburg at the UMCU has yielded a novel drug resistance proteins enriched in the stem cells (Van Houdt et al., Mol. Cellular Prot. 2011) (see abstracts, pages 67). Proteomics of resected liver metastases has identified proteins that correlate with early disease recurrence (Snoeren et al., British J Cancer, 2013; abstract page 94).
Finally, an exciting new development that will enable many biomarker projects in the coming years is protein profiling of FFPE material. We recently implemented a protocol for protein lysis from FFPE tissues (Piersma et al, Proteome Sci 2013) and we have used this protocol to investigate protein expression changes in colon adenoma to carcinoma progression (see abstract page 59).

**Proteomics of biomarker-rich organelles/subcellular compartments**

For certain biomarker applications, a focus on a sub-proteome or proximal fluid (fraction) is advantageous. Which sub-proteome depends on the sample type and research question (Figure 8). Sub-cellular fractions of special interest for cancer proteomics with operational OPL workflows are: 1. In vitro generated tumor secretomes and exosomes to identify candidate biomarkers that have an increased chance to be detected in biofluids. 2. cell surface/ plasma membrane to provide candidate biomarkers for molecular imaging and drug targeting and 3. sub-nuclear fractions (chromatin-binding fraction and the nuclear matrix) to learn more about mechanisms of chromosomal instability, chromatin regulation and identify cancer-related biomarkers.

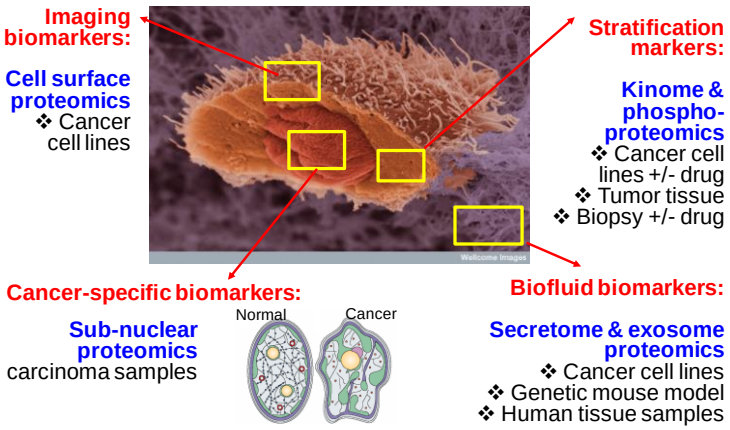



Figure 8. Biomarker-rich subproteomes with workflows at the OPL

From the analytical point of view, plasma is the most difficult biofluid to study, due to the diversity of proteins of different cellular origins and the vast dynamic range in protein concentrations. Therefore, alternative approaches have emerged to identify non-invasive cancer biomarkers using in vivo (eg., urine, saliva or cerebrospinal fluid) or in vitro generated proximal fluids.
In vitro proximal fluids or “secretomes” (proteins secreted in bathing fluid by tissue pieces or cancer cells) are a rich source of biomarkers because they contain tumor-derived proteins at high

concentrations. Furthermore, in vivo, the cancer secretome may contribute to the tumor microenvironment that plays a key role in tumor-promoting processes such as angiogenesis and invasion. The feasibility of secretome proteomics to identify serum-based markers was first explored by us in a mouse model system (Piersma et al., J. Proteome Res. 2010). In subsequent projects secretome proteomics was used to identify non-invasive biomarkers for colorectal cancer (Fijneman et al., Clin. Cancer Res. 2012; De Wit et al., J Prot 2014; abstract page 61), breast cancer (abstract pages 76-78), lung cancer (abstract page 82) and AML (abstract page 35). All these studies revealed unexpected proteins in the secretome, most notably nuclear protein complexes and triggered our interest in extracellular vesicles (exosomes) as source for these non-conventionally secreted proteins. Exosomes are small endosome-derived vesicles secreted by cancer cells and have been implicated in immune suppression and tumor progression. Exosome proteomics of a panel of cancer cell lines (abstract page 31) showed that nuclear protein complexes may constitute exosome cargo. Exosome proteomics of prostate cancer cells identified proteins involved in involved in migration/invasion that were more abundant in urine exosomes of metastatic patients compared to benign prostate hyperplasia or prostate cancer (Bijnsdorp et al., J Extracell Vesicles. 2013; abstract page 32-34). Exosome proteomics of pooled cerebrospinal fluid samples of various neurodegenerative diseases has generated a comprehensive dataset that shows its biomarker potential that warrants further study (abstract page 37).
Cell surface proteomics has been employed to identify novel biomarkers for molecular imaging of colorectal cancer (De Wit el al., Gut 2012) and to identify proteins as receptor for targeted drug delivery (Posthuma de Boer et al., British J Cancer 2013, abstract page 40; 66).
Subnuclear proteomics has been used to study colorectal adenoma to carcinoma progression and identified chromatin remodeling and transcription factors associated with progression (Albrethsen et al., Mol. Cell. Prot 2010; Knol et al., Biochim Biophys Acta. 2014).

**In-depth proteomics of in vivo biofluids and biofluid subproteomes**
Proximal fluids are in contact with the diseased organ and thereby provide an enriched in vivo source for biomarker discovery. Other in vivo biomarker-rich biofluid fractions that we investigate are platelets (pioneered by Piersma et al., J. Proteomics 2009; and see below) and plasma microparticles/ exosomes (Van der Mijn et al., 2014).

CSF provides an attractive source for biomarker discovery in brain and neurological diseases while sputum is the biofluid of choice for lung disease and faeces for colorectal cancer as these biofluids are in direct contact with the diseased tissues and contain disease-related proteins (Figure 9).

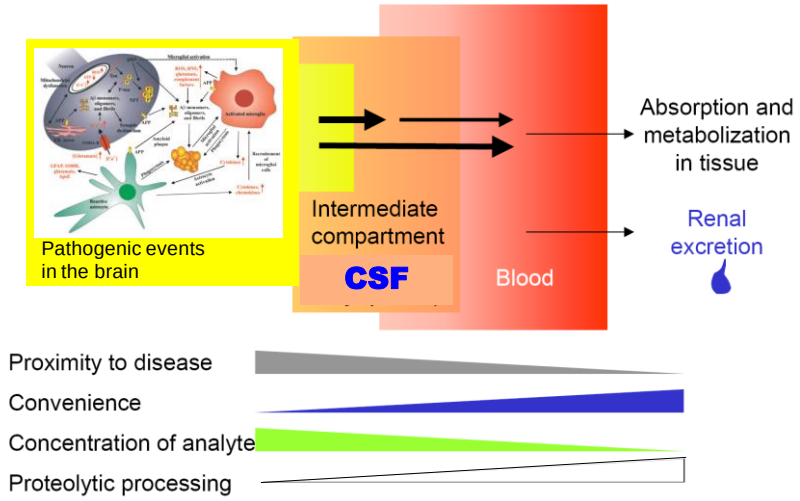



Figure 9. Proximal fluids provide an enriched source of in vivo pathogenic events and candidate biomarkers for biofluid-based diagnostics. (Modified from Schulz-Knappe and Schrader, 2003)

However, CSF still contains a wide dynamic range of protein expression levels spanning up to ~9-10 orders of magnitude. Therefore, additional steps comprising abundant protein depletion need to be performed prior to GeLC-MS/MS-based proteomics analysis. We have implemented affinity-based abundant protein depletion (Top14) to enable detection of low abundant target proteins in CSF, typically brain-derived plasmamembrane and secreted proteins (Fratantoni et al., Prot. Clinical Applic. 2010). In the past years this strategy has been applied to CSF samples of subjects with mild cognitive impairment, Alzheimer's Disease and controls and identified a set of promising candidate biomarkers for early detection and risk prediction of AD (abstract on page 111-113). In addition, this approach was used to CSF biomarkers for genetic and sporadic Parkinson's Disease (context EU Mefopa, page 116), for frontotemporal dementia (appendix page 115).

Less complex workflows simply comprising of protein extraction have been developed for faeces while for sputum some additional filtration is required. Analyses of these biofluids has revealed disease-specific protein profiles and identified candidate biomarkers (abstract pages 57 and 83).
Finally, platelets has been put forward as a circulating biomarker resource/ liquid biopsy. Platelet proteomics was pioneered by us in 2007 (Piersma et al.) and in recent years successfully applied to identify changes between normal subjects and patient with cancer and differences between platelet content before and during treatment of patients advanced solid tumors (abstract page 42-43).

**Proteogenomics: proteomics of genetically annotated tumors**
A new development is so-called “proteogenomics”. In this approach, the MS/MS data are searched against a sample-specific sequence database derived from transcriptome sequencing ("RNA-seq"), to enable detection of protein sequence variants among the proteomic data. General goals of proteogenomics analyses are to identify variant protein sequences corresponding to somatic mutations, to evaluate the relationship between mutation frequency and variant protein expression and to determine how copy number variation relates to protein expression differences. Determination of which DNA or RNA sequence variants are expressed as proteins provides a basis for prioritizing mutations for further study of their contribution to cancer phenotypes.
The proteogenomic approach is currently being applied in the context of colorectal cancer to pinpoint splice variants in CRC cell lines after down-modulation of splice factors and to identify tumor specific splice variants in colorectal tumors (abstract page 69). The proteogenomics workflow will also be applied in several other on-going projects with genomically annotated tumors (patient-derived xenograft models of colorectal, pancreatic and breast cancer).

**Phosphoproteomics**

Progress in mass spectrometry technologies, protocols for phosphopeptide enrichment, and bioinformatic tools for quantitative, high-confidence phosphopeptide identification, now allow for the large-scale phosphoproteomic analysis of complex biological systems. Phosphoproteomics provides a powerful approach to analyze global protein phosphorylation and signalling pathways, that may yield therapeutic targets and predictive markers (Figure 12). Unraveling endogenous phosphorylation events in vivo, in cancer cells and tissues, is the main aim of most phosphoproteomic studies, as they may provide clues to signaling defects when occurring at an aberrant level.

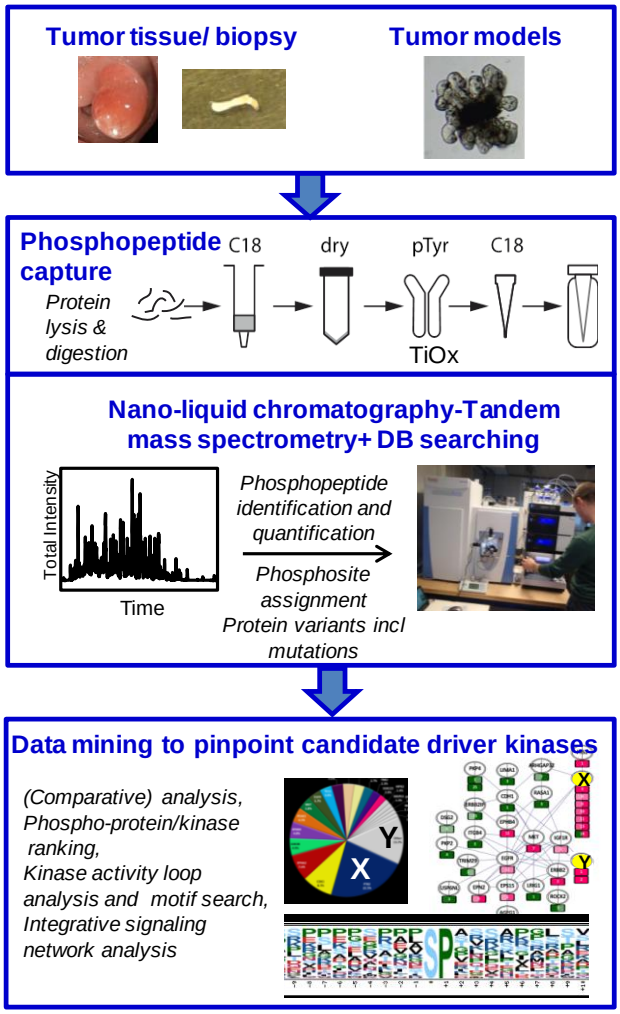



Figure 12. Phosphoproteomics workflow and analysis strategy to identify activated cancer signaling pathways and predictive biomarkers to enable personalized medicine.

Two affinity-based phosphopeptide enrichment methods have been implemented at the OPL: 1. A titanium-oxide-based capture method that can enrich for for all phosphopeptides containing pTyr, pThr, pSer and that works at low level and 2. An antibody-based method that can specifically capture phosphopeptides containing a phosphotyrosine residues. Figure 12 shows the steps in the phosphoproteomics workflow including some of the data mining strategies that may be employed. In our hands, the TiOx workflow allows for reproducible profiling up to 8000-10000 phosphopeptides on ~3000-4000 phosphoproteins in a single nanoLC-MS/MS run on the sensitive, fast scanning QE tandem mass spectrometer, with only 100-500 µg protein as starting material, while the pTyr-specific antibody workflow enables profiling of 200-600 phosphopeptides with 1-10 mg of protein as starting material (see abstracts on pages 44- 51).

We successfully applied phosphoproteomics in several studies using cancer cell lines (abstracts pages 46-47; 50-52) and recently also to the analysis of tumor biopsies (abstract page 54). For example, we explored sunitinib-induced alterations in the phosphotyrosine proteome of renal cancer cells (see page 50) and identified promising new kinase targets. Phosphoproteomics in a glioblastoma

model system with mutant EGFR identified the largest dataset to date (see page 44). In this analysis known oncogenic kinases such as EGFR and c-Met were identified as drivers in addition to many other activated tyrosine kinases and their modulation after erlotinib treatment. Phosphoproteomics of a panel of colorectal cell lines representing CRC subtypes pinpointed kinases and processes associated with the poor prognosis subtype (pag 51). Finally recently we down-scaled the phosphotyrosine workflow and demonstrated the feasibility of label-free pTyr-phosphoproteomics at the biopsy level. Unsupervised analysis showed that tumor needle biopsies from individual patients cluster together indicating that this analysis can identify patient specific phosphoproteomic (see abstract page 54).

Together the preliminary studies show that label-free phosphoproteomics provides a promising avenue for the identification of (hyper)activated protein kinases as candidate therapeutic targets and diagnostic markers which may enable future phosphoproteomics-based treatment selection.

**Mining of multi-dimensional proteomics datasets**

In-depth proteomics creates datasets with quantitative information on hundreds to thousands of proteins. We are applying web-based data mining tools for data organization, gene ontology mining, protein network and pathway analysis to go from large-scale data to new molecular knowledge about cancer pathways. Tools that we use include FatiGO, David, STRING, Cytoscape and sequence motif tools like SecretomeP, SignalP and MotifX. In our experience, proper data visualization is key to create a comprehensive overview of the large datasets and to enable new functional insights. Figure 13 shows examples of different modes of data visualization that are being employed by the OPL.

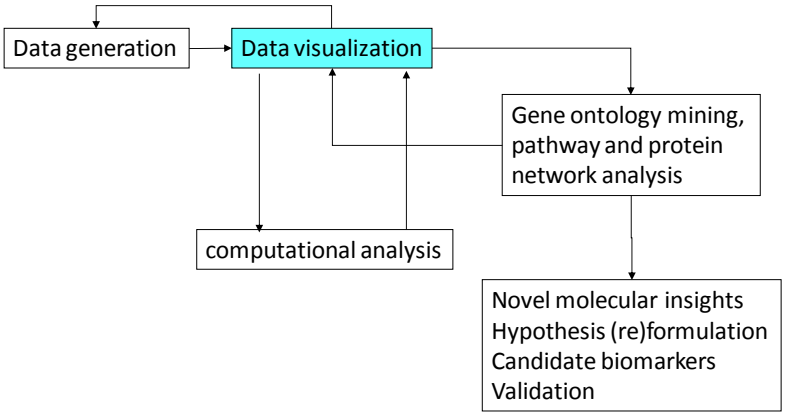
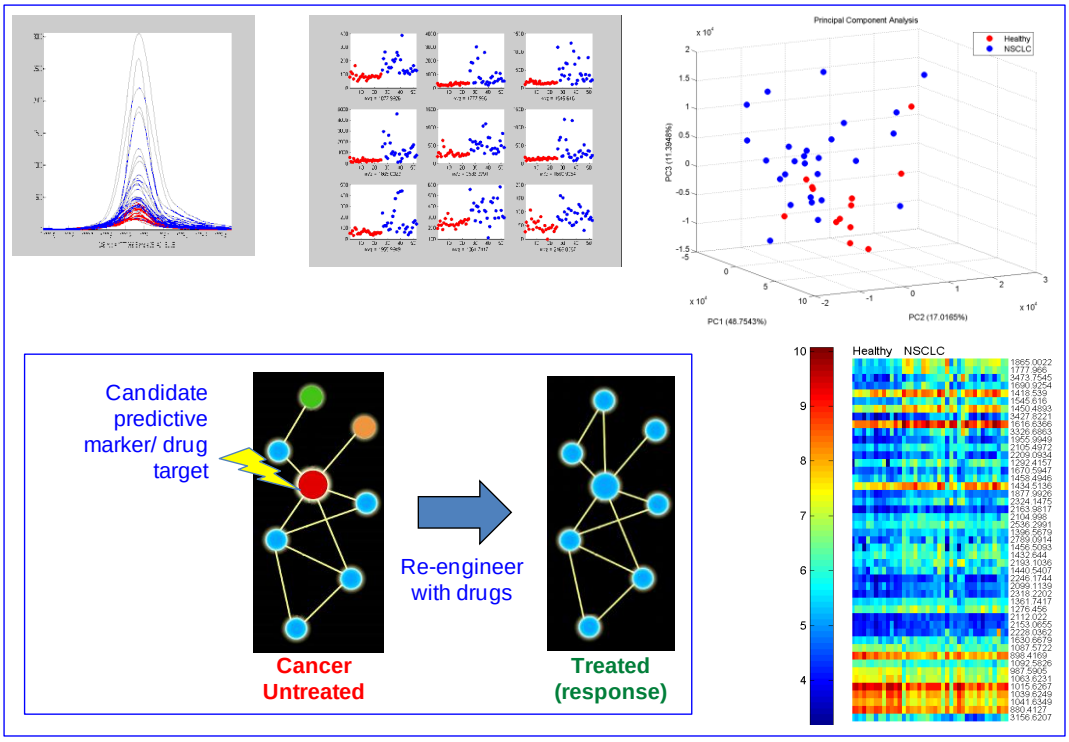



Figure 13. Computational analysis and data visualization is key in the analysis of highthroughput data. Upper panel: General scheme of OMICs data mining. Lower panel: Data may be visualized at different levels: raw data, peptide ion, spectral overlay, scatter plot, boxplot, clustering, heatmap, PCA plot and at an even higher analysis level: protein-protein interaction network with overlayed protein regulations



## Listing of projects

**OPL core research lines** See below for an overview of research lines and running projects. For more details, please read the abstracts in the appendix.

**1. Poteomics of biomarker-rich subproteomes: secretome, exosome, cell surface proteome, platelets** (pag. 29-43)

- Sub-project 1: Evaluation of a novel method for extracellular vesicle isolation: Assessment of exosome enrichment, sensitivity and reproducibility in comparison to the standard ultracentrifugation method (pag. 29-30)
- Sub-project 2: Proteomics of exosomes secreted by cancer cell lines and primary cells reveals oncogenic signaling and biomarker potential (pag.31)
- Sub-project 3: Exosomal ITGA3 interferes with non-cancerous prostate cell functions and is increased in urine exosomes of metastatic prostate cancer patients (pag. 32-33)
- Sub-Project 4: Proteomics of exosomes in urine of prostate cancer patients reveals potential biomarkers (pag 34)
- Sub-Project 5: Exosomes secreted by apoptosis-resistant AML blasts harbor regulatory proteins potentially involved in antagonism of apoptosis (pag 35-36)
- Sub-project 6: Proteomic analysis of cerebrospinal fluid extracellular vesicles: a comprehensive dataset (pag. 37-38)
- Sub-project 7: Analysis of AKT and ERK1/2 protein kinases in extracellular vesicles isolated from blood of patients with cancer. (page 39)
- Sub-Project 8: Surface proteomic analysis of osteosarcoma identifies EPHA2 as receptor for targeted drug delivery (pag 40-41)
- Sub-Project 9: Platelet proteomics for cancer biomarker discovery, study 1 (pag 42)
- Sub-Project 10: Platelet proteomics for cancer biomarker discovery, study 2 (pag 43)

**2. Phosphoproteomics for insight into cancer signaling, identification of drug targets and biomarkers for patient stratification** (pag. 44-56)

- Sub-project 1: Evaluation of different phospho-tyrosine antibodies for label-free phosphoproteomics (pag. 44-45)
- Sub-project 2: Phosphoproteomics of a panel of AML cell lines (pag. 46-47)
- Sub-project 3: Phosphoproteomics for therapy response prediction in pancreatic cancer (pag 48)
- Sub-project 4: Phosphoproteomics for therapy response prediction in colorectal cancer (pag 49)
- Sub-project 5: Phosphoproteomics analysis of renal cell cancer cells exposed to sunitinib
- reveals targets for new combination treatment (pag 50)
- Sub-project 6: Robust TiO2-based enrichment for single-shot phosphoproteomics; application to colorectal cancer cell lines representing different subtypes (pag. 51-52)
- Sub-project 7: Is less enough? Scaling down protein input for phosphoproteomics based treatment selection in patients with advanced solid tumors (pag. 54)
- Sub-project 8: Tumor concentrations of kinase inhibitors in correlation with pre- vs on-treatment profiling of patient derived tumor samples (pag. 55-56)

**3. Colorectal cancer (CRC) proteomics** (pag. 57-72)

- Sub-project 1: Stool proteomics reveals novel candidate biomarkers for colorectal cancer screening (pag 57-58)
- Sub-project 2: Proteomic Profiling of Colorectal Adenoma-to-Carcinoma Progression
- on FFPE Material (pag 59-60)
- Sub-project 3: Colorectal cancer candidate biomarkers identified by tissue secretome proteome profiling (pag. 61-62)
- Sub-project 4: Sub-nuclear proteomics: profiling of chromatin-associated proteins in colorectal adenoma and carcinoma tissues (pag. 63)
- Sub-project 5: Proteomic analysis of LPS-modified secretion in six colorectal cancer cell lines (pag 64-65)
- Sub-project 6: Cell surface proteomics identifies glucose transporter type 1 and prion protein as candidate biomarkers for colorectal adenoma-to-carcinoma progression (pag 66)

- Sub-project 7: Colorectal cancer tissue spheroids: an in-depth proteomics analysis (pag 67)
- Sub-project 8: The secretome of colon cancer stem cells contains drug-metabolizing enzymes (pag. 68)
- Sub-project 9: Tumor-specific protein biomarkers for early detection of colorectal cancer (pag 69)
- Sub-project 10: Proteomics analysis of the effect of fluorouracil (5-FU) and 5-FU/leucovorin (LV) on colorectal cancer in patients (pag 70-71)
- Sub-project 11: Connection proteomics: Protein-based stratification of colon cancers (pag 72)

**4. Breast cancer proteomics** (pag 73-80)

- Sub-project 1: Proteomics of mouse BRCA1-deficient mammary tumors identifies DNA repair proteins with diagnostic and prognostic value in human breast cancer (pag. 73-74)
- Sub-project 2: Proteomics of mouse breast cancer models identifies fatty acid metabolism proteins as predictive markers for cisplatin resistance (pag. 75)
- Sub-project 3: Proteomics of mouse BRCA1-deficient and proficient mammary cancer cell secretomes reveals candidate biomarkers for non-invasive testing (pag 76-77)
- Sub-project 4: Proteomic profiling of the murine mammary tumor secretome identifies candidate biomarkers for non-invasive breast cancer testing (pag 78)
- Sub-project 5: Proteomics of Brca1-deficient mouse tumors resistant for PARP inhibitors

**5. Lung cancer proteomics** (pag 81-86)

- Subproject 1: Novel candidate biomarkers for cisplatin response prediction in NSCLC (pag. 81-83)
- Sub-project 2: Proteomics of FFPE tumors of patients with lung cancer to identify prognostic/ predictive biomarkers for cisplatin response (pag. 84-85)
- Sub-project 3: Exploration of sputum to develop protein-based assays for early detection,
- prognosis and drug response of lung cancer (pag 86)

**10. Mining high-dimensional proteomics data** (pag. 87-94)

- Sub-project 1: An accurate paired sample test for count data (pag. 87-88)
- Sub-project 2: Computational analysis of phosphoproteomics data (pag. 89-91)
- Sub-project 3: Constructing spectral library from multiple proteomics experiments (pag 92)
- Sub-project 4: Accelerating the analysis of large-scale targeted proteomics data (pag 93)
- Sub-project 5: Simulated linear tests applied to quantitative proteomics (pag 94)

## **Other core/ collaborative projects** (pag 95- 133)

**1. Miscellaneous cancer proteomics** (pag. 95-110)

- Maspin is a marker for early recurrence in primary stage III and IV colorectal cancer (pag. 96)
- miR-200a-mediated suppression of non-muscle heavy chain IIb inhibits meningioma cell migration and tumor growth in vivo (pag 97)
- HP1-gamma's expression correlates with glioma grade and survival and is a putative marker of glioma stem like cells (pag 98)
- A factor that rebuilds immunity in mice and humans (pag 99)
- Target identification for microRNAs that play a role in the persistence of acute myeloid leukemia stem cells (pag 100-101)
- Identification and characterization of potential ligand(s) for the C-type Lectin-like Receptor CLEC12A (pag 102)
- Predictive biomarker(s) and therapeutic target(s) in radiation-resistant head and neck squamous cell carcinomas (HNSCC) (pag 103)
- Identifying interaction partners for the Hedgehog pathway regulator Smoothened (pag 104)
- Proteomics analysis of HUVEC (pag 105)
- Genome wide siRNA screen identifies the radiosensitizing effect of inhibition of MASTL and FOXM1 in NSCLC (pag 106)
- Secretome proteomics of breast and colon cancer cell lines: Carcinoma origin dictates differential functional macrophage phenotype (pag 107)
- Proteomics Profiling of cell lines from transgenic high-grade glioma mouse models (pag 108)

- Palmitoylation-dependent targeting of LMP1-TRAF2 complexes to endosomal membranes supports oncogenic NFκB activation and sorting into exosomes (pag 109)
- Exosome sorting of virus-encoded 5'ppp-small RNA supports a host sensing mechanism of latent EBV infected B cells (pag 110)

**Neuroproteomics** (pag 111-122)

- CSF biomarkers for early Alzheimer's disease (pag 111-112)
- CSF biomarkers for early Alzheimer's disease, study 2 (pag 113)
- BRI2-BRICHOS is increased in human amyloid plaques in early stages of Alzheimer's disease (pag 114)
- Identification of novel diagnostic CSF protein biomarkers for FTD with high discriminatory power (pag 115)
- Identification of novel biomarker candidates in the cerebrospinal fluid proteome of drug-naïve Parkinson's disease patients (pag 116)
- Proteome of Cerebral Capillary Amyloid Angiopathy: relevance for amyloid clearance in Alzheimer's disease (pag 117-118)
- MicroRNA-124&137 regulate caspase-3 activity in neural stem cells by cooperatively fine-tuning BCL2L13 (pag 119)
- Mass spectrometric detection of amyloid beta-peptide fragments in CSF of Alzheimer's disease patients and in mild cognitive impairment (pag 120-121)
- Search for CSF biomarkers for DISC1opathies (pag 122)

**Proteomics of signaling protein complexes and perturbation of cellular state** (pag 123-133)

- Protein network disturbance in response to oxidative stress (pag 123)
- Secretome analysis of TLR2-stimulated FLT3L bone marrow cultures (pag 124)
- Identification of novel signalling partners of the HCMV encoded viral GPCR US28 (pag 125)
- Threonine-594 of the Estrogen Receptor Alpha F domain is a phosphorylated residue involved in down-regulation of receptor activity (pag 126-127)
- Accurate quantitation of MHC-bound peptides by application of isotopically labeled peptide MHC complexes (pag 128)
- A proteomic analysis of the cardiac sodium channel macromolecular complex (pag 129)
- A quantitative phosphoproteomics study reveals a role for kinase X in cardiomyocyte proliferation (pag 130)
- PKCα-specific phosphorylation of the troponin complex in human myocardium: a functional and proteomics analysis (pag 131)
- Proteomics of Mycobacterial Pathogens (pag 132)
- Proteomic profiling of the Mycobacterium tuberculosis identifies nutrient starvation responsive toxin-antitoxin systems (pag 133)

## Five-year view

Next generation nanoLC-MS/MS-based proteomics on high-resolution, high-speed mass spectrometry platforms allows for unbiased profiling of protein (variant) expression, and may substantially expand our ability to understand the association between cancer-related genomic variation and cancer phenotypes.

In the past years, proteomics has yielded novel insights into cancer biology and a multitude of candidate biomarkers, many of which still await validation. The emerging integration of genomic and proteomic data will provide a view of the downstream consequences of genomic aberrations and may aid in pinpointing driver mutations.

Most new targeted anticancer agents inhibit the activity of crucial protein kinases for cancer biology. Phosphoproteomic measurements have the potential to determine the activity of these protein kinases and can uncover the associated cellular signaling networks. We hypothesize that phosphoproteomic measures may predict sensitivity of tumors from patients to these kinase inhibitors and thereby may provide personalized treatment strategies.

Targeted multiplexed mass spectrometry analyses will provide the consistent detection snf throughput that is needed to validate selected subsets of candidate biomarkers in larger series of clinical samples and, moreover, that this approach may find utility as diagnostic platform in the clinic.

**We look forward to continuing our discovery journey and apply and translate the findings from (phospho)proteomics to the clinical setting, and to develop robust approaches that will allow**

**for measurements in individual patients in the clinical real-time. To this end, translational research collaborations are imperative for success.**

## SCIENTIFIC OUTPUT 2012-present

## 2014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## Book chapters:

Jimenez CR, Verheul HM. Mass spectrometry-based proteomics: from cancer biology to protein biomarkers, drug targets, and clinical applications. Am Soc Clin Oncol Educ Book. 2014:e504-10. doi: 10.14694/EdBook_AM.2014.34.e504. PubMed PMID: 24857147.

## Non-refereed articles in popular science and clinical journals

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D.T. Kruger, S. C. Linn, K. Beelen, S. Sleijfer, M.P.H.M. Jansen, C.R. Jimenez, E. Boven. De Everolimus Biomarker Studie: analyse van de PI3K/Akt/mTOR route om meer inzicht te verkrijgen in de effectiviteit van everolimus in combinatie met exemestaan bij gemetastaseerd mammacarcinoom. Nederlands Tijdschrift voor de Oncologie, 2014

# EDUCATION

In the period 2012-2014 several lectures were given to bachelor, master and PhD students. We participated in the VU master course 'bioinformatics for translational medicine'; in the VUmc Master Oncology (in the courses 'Tumor Biology' and 'Innovative therapeutics'); in the AIO cursus 'Personalized Oncology of the UMCU, in the HLO course Fighting cancer of the HAN in Nijmegen en in the "NVMO nascholingscursus oncologie". Furthermore, the master course 'Biomedical Proteomics' was organized in the past 3 years for more in-depth education of students and collaborators. In addition, several VU bioinformatics master students and master oncology students performed their internship at the OPL (for a listing see page 5).

# INDICATORS of ESTEEM

## Obtained grants

| Grants PI | Title | Year of award |
|---|---|---|
| Dr. CR Jimenez, coPIs Prof. dr. S. Burgers (NKI), Dr. K. Grunberg (572K) | Response prediction for cisplatin-based treatment regimens in non-small cell lung cancer using a protein-based assay | 2015-2018 (KWF VU2014-6816) |
| Dr. CR Jimenez, coPIs Dr. N. Van Grieken, Prof. dr. HMW. Verheul (150K) | Kinome pathway activity assay by targeted mass spectrometry-based phosphoproteomics to enable personalized treatment of cancer with kinase inhibitors | 2015-2016 (CCA2014-1-15) |
| Dr. B. Carvalho*, PIs Dr. CR Jimenez* and Prof. dr. GA Meijer *Shared PI (560K) | Signaling pathways involved in colorectal adenoma-to-carcinoma progression | 2015-2019 (KWF VU2014-6813) |
| Dr. E. Giovanetti*, Dr. CR Jimenez*, Dr. M. Bijlsma (200K) | Phosphoproteomics on patient-derived tumor xenografts, to identify signaling pathways & candidate driver kinases in Pancreatic Cancer | 2014-2018 (VUmc-AMC alliance project) |
| Dr. CR Jimenez, coPIs Prof. dr. J. Jonkers (NKI), Prof. dr. PJ van Diest (UMCU) (560K) | Discovery and clinical validation of novel protein biomarkers for homologous recombination deficient breast cancer | 2013-2017 (KWF VU2013-6020) |
| Dr. CR Jimenez, coPIs Prof. dr. HMW Verheul and Dr. E Boven (55K proteomics part) | (Pharmaco)genetics and proteomics on the efficacy and/or toxicity of everolimus | 2013 (Industry-sponsored side study Novartis) |
| Dr. CR Jimenez, coPIs Prof. dr. HMW Verheul and Dr. F van Eertweegh (100K proteomics part) | Response Monitoring and Resistance Prediction with Positron Emission Tomography and Tumor Characteristics (Reposit) | 2013 (Industry-sponsored side study Roche) |
| Dr. CR Jimenez (200K) | Instrumentation grant | 2012 VUmc-CCA |
| **Grants coPI/consortium** | | |
| PIs: Prof. dr. JP Medema, Prof. dr. J. IJzermans, Prof. dr. H. van Krieken, Dr. CR Jimenez, Dr. Miriam Koopman (500K out of 3.4M Euro) | Improving clinical management of colon cancer through CONNECTION, a nation-wide Colon Cancer Registry and Stratification effort. | 2014-2019 (Alpes d'HuZes; UvA 2013-6331) |
| Coordinator Prof. dr. GA Meijer, Dr. CR Jimenez investigator (350K out of 2M Euro) | CRC Bioscreen | 2013-2015 Valorisation project of the Center of Translational Molecular Medicine |
| Coordinator Prof. dr. GA Meijer, Dr. Jimenez investigator (100K proteomics part) | Translational IT (TraIT), | 2012-2015 Consortium grant of the Center of Translational Molecular Medicine |
| Dr. RJA Fijneman, coPIs Dr. CR Jimenez and Prof. dr. GA Meijer (560K) | Tumor-specific protein biomarkers for early detection of colorectal cancer | 2013-2017 (KWF VU2013-6025) |
| PI Prof. dr. Scheltens, coPI Dr. CR Jimenez (150K) | Multiplex MS assay for early AD diagnosis | 2013-2015 (International Foundation for Alzheimer's Disease Research) |
| PIs Dr. J. Janssen, Dr. CR Jimenez and Prof. dr. G. Ossenkoppele (250K) | AML phosphoproteomics for personalized therapy | 2012-2016 (VUmc-CCA) |

## Invited lectures at (inter)national conferences

*International invited lectures Dr. Connie R Jimenez*

2014 Human Proteome Organisation (HUPO) 2014, Madrid, Spain, Invited speaker (7 okt. 2014); Title: From (colon) cancer proteomics to biomarkers, drug targets and clinical applications

2014 American Society Clinical Oncology (ASCO), Invited speaker and session chair at the educational session on Cancer Proteomics (June 2014)

2013 7th European Summerschool Advanced proteomics (9 aug. 2013, Brixen, Italy); Title: "Cancer Proteomics"

2013 HUPO2013 (sept., Japan); Title: "Label-free proteomics for biomarker discovery and validation in proximal fluids"

2012 2nd workshop Biomarkers in the early diagnosis of neurodegenerative disorders. (8 juni 2012, Assisi, Italy); Title: "New CSF biomarkers for AD: report from the cNEUPRO discovery phase"

2012 19th Arbeitstagung Micromethods in Protein Chemistry (26 June, 2012 Bochum); Title: "Cancer biomarker discovery and validation: Label-free mass spectrometry-based proteomics"

*National invited lectures Dr. Connie R Jimenez*

2014 Symposium of the Center for Personalized Cancer Treatment (9 0kt 2014, Utrecht); Title: (Phospho)Proteomics, pathways, biomarkers

2013 Netherlands Proteomics Center 10-year anniversary meeting (11 feb. 2013 Utrecht); Cancer Proteomics: Novel biomarkers for colon cancer screening and BRCA1 deficient breast cancer

2013 7 maart 2013, PAC Imagine symposium, Amsterdam; From peptidomics to proteomics, a personal view of 25 years of label-free mass spectrometry

2012 NBIC, Hot topics day (5 March. 2012, Utrecht); Peptide and protein quantification using Mass Spectrometry. "Label-free mass spectrometry-based proteomics for cancer biomarker discovery, Protein quantitation by spectral counting: A pragmatic perspective"

2012 NUBIN2012 symposium (15 juni 2012, Amsterdam); "New CSF biomarkers for AD: report from the cNEUPRO discovery phase"

*Oral presentations Dr. Connie R Jimenez*

2013 International Society of Extracllular Vesicles (ISEV) meeting (18 april, Boston); Title: "Proteomics of Exosomes Secreted by Cancer Cell lines and Primary Cells Reveals Oncogenic Signalling and Biomarker Potential"

*Invited lecture Dr. Sander R Piersma*

- 8 nov. 2014. Fall meeting Netherlands Proteomics Platform, Amsterdam. Title: 'Label-free phosphoproteomics of cancer cells"

## (Inter)national functions:

- Steering committee — Netherlands Proteomics Platform
- General Council Member — European Proteomics Association (EuPA)
- Board of Directors/ Council — Human Proteome Organisation
- Advisory Board — Dutch Techcentre for Life Sciences

## Memberships of editorial boards:

- Journal of Proteomics
- Molecular and Cellular Proteomics
- Proteomics
- Guest Editor, special issue, 2014, 'NeuroProteomics', BBA Proteins and Proteomics

## Organisation of congresses:

2014 Co-organizer 14th Fall Meeting of the Netherlands Proteomics Platform "'Implications of the draft human proteome and subproteome in health and disease', 14 nov. 2014, Rotterdam

2013 Chair and local organizer, 13th Fall Meeting of the Netherlands Proteomics Platform 'Proteomics suite of applications and new developments', 8 Nov. 2013, Amsterdam

2012 Chair and local organizer, 12st Fall Meeting of the Netherlands Proteomics Platform 'Current state-of-the-art of Proteomics in the Netherlands", 30 Nov. 2012, Amsterdam

## Media attention

1. C2W Life Sciences 2014. Interview met CR Jimenez and RJA Fijneman. Publication title “De test in het darmkanker bevolkingsonderzoek detecteert bloed in feces. Dat bloed hoeft niet per se van beginnende darmkanker te komen. Wordt het niet eens tijd voor een ‘echte’ darmkanker test?”
2. Oncologie Up-to-date 2013. Interview met CR. Jimenez, S Rottenberg, PJ Van Diest. Publication title: “Innovatief onderzoek naar eiwitten bij triple negatieve borstkanker”. Vol 5 • nummer 1.
3. Oncologie Up-to-date 2013. Interview met CR. Jimenez, RJA Fijneman, GA Meijer. Publication title: “Proteomics voor vroege detectie darmkanker”. Vol 4 • nummer 6.
4. C2W Life Sciences 2013. Interview met CRJ. Publication title “Tandem-MS brengt ziekte in kaart Met geavanceerde MS-technieken zoekt het Amsterdamse OncoProteomics Laboratory naar biomarkers”.

## Societal Impact

- Lekenppublicatie: Kankerbreed (2012, maart issue) ‘OncoProteomics voor ontdekken van biomarkers en drug targets’

## Patent applications

1. Patent 2008707 “Biomarkers”, a screening method for the diagnosis of colorectal cancer comprising identifying protein markers in (stool) samples, where the markers are selected from a large group of protein biomarkers known to be over-expressed in cases of colorectal cancer (priority date: 26th April 2012).
2. Patent P6046553PCT (WO13124740) “Biomarkers for tissue- and biofluid-based detection of BRCA1-deficient breast cancer “ (2012)
3. Patent P101698EP00 “Biomarkers for early diagnosis of Alzheimer’s disease” (2013)
4. Patent P103597EP00 ‘’Novel diagnostic markers for FTD and its subtypes’’ (2013)
5. Patent P98898 US00 ‘Bri2 as a novel biomarker for Alzheimer’s disease’ (2013)

## Collaborations

### International

- Dr. Davide Chasserini (University of Perugia, Italy)
- Prof. dr. Andrea Binaglia (University of Perugia, Italy)
- Cira Garcia de Durango Caveda (CEU San Pablo School of Medicine, Madrid, Spain)
- Prof. Carsten Korth (University of Düsseldorf, Germany)
- Prof. Steve Carr (MIT Broad Institute, Boston, USA)
- Dr, Rob Slebos and Prof. Daniel Liebler (Vanderbilt University, Nashville, USA)
- Dr. Thorsten Muller, Prof. Katrin Marcus, Prof. Helmut Meyer (Medizinische Proteom Center, Bochum, Germany)
- Dr. Frode Berven (Proteomics Unit, University of Bergen, Bergen, Norway)
- Dr. Uros Raijcevic and Simone Niclou (NorLux Neuro-Oncology Laboratory, Luxembourg)
- Dr. Okay Saydam **(Harvard Medical School, Boston, USA)**
- Dr. Ira **Skvortsov (Innsbruck Medical University, Austria)**

### National

- Prof. Jan Paul Medema, Dr. Louis Vermeulen (AMC, Amsterdam)
- Dr. Maarten Bijlsma (AMC, Amsterdam)
- Dr. Onno Kranenburg (UMCU, Utrecht)
- Dr..Petra van der Groep, Prof. Paul van Diest (UMCU, Utrecht)
- Dr. Sven Rottenberg, Dr. Jos Jonkers, Dr. Sabine Linn (NKI, Amsterdam)
- Prof. Anton Berns (NKI, Amsterdam)
- Dr. Olaf van Tellingen (NKI, Amsterdam)
- Dr. Sjaak Burgers (NKI, Amsterdam)
- Dr. Annemarie de Vries, Dr. Wendy Rodenberg (RIVM, Utrecht)
- Dr. Carlos Fitzsimons (UvA, Amsterdam)
- Dr. Connie Bezzina (AMC, Amsterdam)
- Dr. Peter van Veelen (LUMC, Leiden)

### Local

VUmc

- Many members of the Dept. Medical Oncology
- Dr. Remond Fijneman, Dr. Beatriz Carvalho, Prof. Gerrit Meijer, Dr. Katrien Grunberg, Prof Saskia de Vies, Dr. Michiel Pegtel (VUmc-Dept. Pathology)
- Prof. Egbert Smit (VUmc-Dept. Pulmonology and NKI)
- Dr. Sonja Zweegman, Dr. Gerrit-Jan Schuurhuis, Dr. Jacquline Cloos, Dr. Jeroen Janssen, Dr. Gert Ossenkoppele, Dr. Linda Smit, Dr. Paul Noordhuis (VUmc-Dept. Hematology)
- Dr. Irene Bijnsdorp (Dept. Urology)
- Prof. Ruud Brakenhoff (Dept. Otolaryngology/Head-Neck Surgery)
- Dr. Josephine Dorsman, Dr. Quinten Waisfiz, Dr. Rob Wolthuis (Dept. Clinical Genetics)
- Dr. Charlotte Teunissen, Dr. Rob Veerhuis (VUmc-Dept. Clinical Chemistry)
- Dr. Wilbert Bitter, Dr. Edith Houben (Dept. Med. Microbiology)
- Dr. Juan Garcia Vallejo (Dept. MCBI)
- Prof. Marjolein van Egmond (Dept. MCBI/Surgery)
- Dr. Marco Helder (Department of Orthopaedic Surgery)
- Dr. Wilma van den Berg, Dr. Henk Berendse (VUmc-Dept. Anatomy and Neurosciences)
- Prof. Wiesje van der Flier, Prof. Philip Scheltens (VUmc-Dept. Neurology)
- Prof. Jolanda van der Velden (Dept. Physiology, ICAR)
- Prof. Dr. O.S. Hoekstra, Drs. K.M. Duvivier (Dept. of Radiology and Nuclear Medicine)
- Dr. Christan Rausch (CCA Dry Lab)

VU

- Dr. Sanne Abeln, Prof. Jaap Herringa (Dept. Bioinformatics, FEW)
- Dr. Bert de Boer (FEW)
- Dr. Martine Smit, Dr. Marco Siderius, Dr. Rob Leurs (FEW)
- Dr. Heidi de Wit, Dr. Jan van Weering (Medical Genomics)
- Dr. Ka Wan Li, Prof. dr. Guus Smit (Dept. Molecular & Cellular Neurobiology)

# APPENDIX: PROJECT SUMMARIES

**(for overview project titles, see pages 18-20)**

**1. Proteomics of biomarker-rich (extracellular) subproteomes (secretome, exosomes, platelets)** (pag. 29- 43)

- 10 sub-projects

**2. Phosphoproteomics for insight into cancer signaling, identification of drug targets and biomarkers for patient stratification** (pag. 44- 56)

- 8 sub-projects

**3. Colorectal cancer (CRC) proteomics** (pag. 57- 72)

- 10 sub-projects

**4. Breast cancer proteomics** (pag. 73- 80)

- 5 sub-projects

**5. Lung cancer proteomics** (pag. 81- 86)

- 3 sub-projects

**6. Mining high-dimensional datasets** (pag. 87- 94)

- 4 sub-projects

**7. Other core/collaborative projects** (pag. 95-133)

- Miscellaneous cancer proteomics (pag. 95-110)
- Neuroproteomics (pag. 111-122)
- Proteomics of signaling protein complexes and perturbation of cellular state (123-133)

# Evaluation of a novel method for extracellular vesicle isolation:
## *Assessment of exosome enrichment, sensitivity and reproducibility in comparison to the standard ultracentrifugation method*

Inge de Reus, Tim Schelfhorst, Robin Beekhof, Meike de Wit, Sander R. Piersma, Thang V. Pham, Jaco C Knol, Egbert F. Smit, Henk M. W. Verheul, Connie R. Jimenez.

**Background**
Extracellular vesicles (EVs) are cell-derived entities enclosed in a lipid bilayer of plasma membrane or endocytic origin, also known as exosomes. With their their shedding into body fluids such as blood, and their cargo containing tissue- and/or disease-specific (signature) molecules, there is emerging interest in EVs as a rich source for disease biomarkers. However, in order to study EVs, they need to be isolated from biofluids. The widely used method for EV isolation makes use of (laborious and time-consuming) differential ultracentrifugation steps and requires relative high sample amounts. Therefore, a rapid and easy to use EV isolation method requiring lower sample amounts would be favorable.

**Aim**
To evaluate a novel method for extracellular vesicle isolation and exosome proteome profiling and compare it to the gold-standard differential (ultra)centrifugation method.

**Approach**
The new method (New England Peptide) employs a peptide (Vn96) that captures heatshock proteins on the vesicle surface and thereby can precipitate HSP-containing vesicles from biofluids in only a single centrifugation step (HSP-EV). We compared this isolation method to the standard ultracentrifugation (UC-EV) method using label free GeLC-MS/MS and spectral counting.
Figure 1 displays the workflow comparison.

**Methods**
Cell secretome from (~1 billion) CRC HT29 cells was collected for starting material for triplicate analysis of the two methods. The secretome was clarified and concentrated six times. For the UC-EV isolation method, 60 ml of secretome was used per replicate. For the HSP-EV isolation method, only 2 ml of clarified secretome per replicate was incubated with 50µg Vn96 peptide for 25 minutes at room temperature.EVs were collected by centrifuging at 16.000 x g for 15 minutes. Proteins in all EV fractions were dissolved and then separated on 4-12% Bis-Tris gradient gels (see figure 2). Gels were sliced in 5 bands and proteins were digested overnight with trypsin. Peptides were analyzed using label-free LC-MS/MS and spectral counting for protein quantification.

Figure 1. Overview of the method comparison workflow

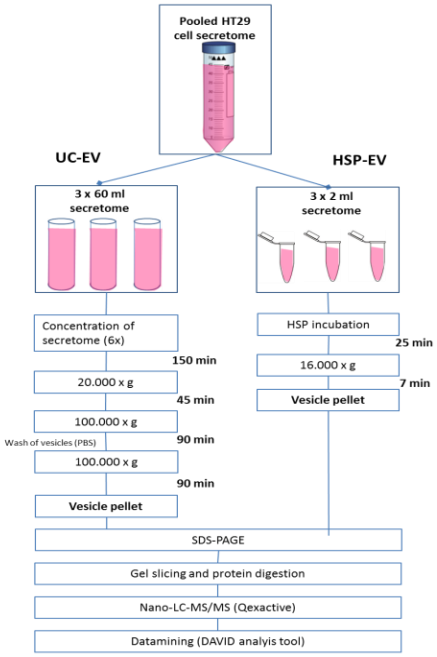
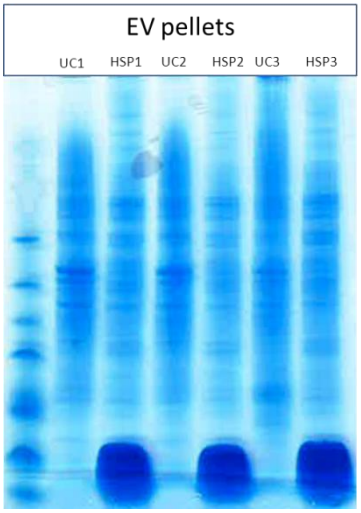



Figure 2. Coomassie stained gels with EV fractions form UC-EV and HSP-EV methods.



**Results**

Both methods yielded comparable numbers of total identified proteins (3149 and 3129 proteins, in UC-EV and HSP-EV respectively), with comparable reproducibility of protein identification (72%-75%) and quantification (21%-18%) in triplicate analyses. The majority of proteins identified in the EV fractions (2454;75%) is identified using both methods, with a substantial overlap of proteins (1116; 23%) present in the Exocarta database (www.exocarta.org) of experimentally identified exosome proteins (see figure 3). 'Classical' exosome markers as well as proteins linked to vesicle-related processes were enriched in the EV fractions obtained by either method. Analysis with DAVID GO analysis tool showed that vesicle-related terms were enriched in the EV fractions and that overall similar GO terms were found for EV samples prepared with the UC-EV and HSP-EV method.

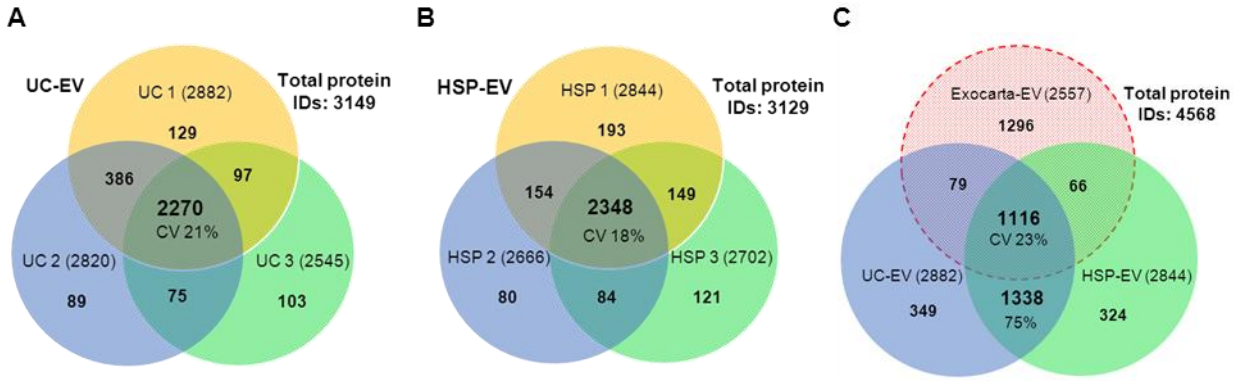



Figure 3. Overview of protein identification, quantification and reproducibility obtained with the HSP-EV and UC-EV isolation methods. A-B Venn diagrams enumerating shared and unique proteins identified in three replicate EV isolations per workflow with the UC-EV method (A) or the HSP-EV method (B). C.Overlap of identified proteins in EV fractions with both methods and the Exocarta database.

**Conclusion and outlook**

We have shown that the novel EV isolation method (HSP-EV) tested here allows for reproducible analysis of the EV proteome. In terms of identified proteins and associated gene ontologies, the HSP-EV proteome is largely comparable to the proteome of EVs purified by the standard ultracentrifugation (UC-EV) method, and is enriched for 'classical' exosome markers. Combined with the low sample input, ease-of-use, simplicity, and spectacular speed of the HSP-EV isolation method (more than ten times less time-consuming than the UC-EV method), we anticipate that this novel avenue of preparing EV fractions will greatly facilitate biomarker discovery efforts in cancer or other diseases and enable novel non-invasive diagnostic applications.

*This work has been submitted as technical brief for publication in Proteomics and is currently in review.*

# Proteomics of exosomes secreted by cancer cell lines and primary cells reveals oncogenic signaling and biomarker potential

Meike de Wit[1], Remond JA Fijneman[2] , Mehrdad Lavaei[1], Donna M. Fluitsma[3], Jaco C Knol[1], Sander R. Piersma1, Thang V. Pham[1], Renske Steenbergen[2], Gerrit A Meijer[2], Michiel Pegtel[2], Henk MW Verheul[1], Connie R Jimenez[1]

[1]Dept. Pathology, [2]Dept. Medical Oncology, [3]Dept. Mol. Cell. Biology and Immunology, VU University Medical Center

**BACKGROUND** Exosomes are 40-100 nm membrane vesicles that are released by cancer and normal cells, after the fusion of multivesicular bodies with the plasma membrane. Multiple functions have been attributed to exosomes including antigen presentation and intercellular communication. Importantly, exosomes carry tumor-specific antigens and have been identified in various biofluids. Therefore, exosomes may provide an attractive platform for biomarker discovery.

**AIM** of this study is identification of exosome core proteins and cancer-type specific proteins to obtain insight into aberrant exosomal functions in cancer and for candidate biomarker discovery.

**APPROACH** Quantitative proteomics based on Gel-nanoLC-MS/MS of exosomes released by a panel of 9 cancer cell lines and 2 normal cell types and their corresponding total cell lysates. Exosomes were harvested by differential centrifugation from a panel of 9 human cancer cell lines and 2 primary human cells.

**RESULTS** Exosome isolation by differential centrifugation was reproducible as deduced from replicate analyses. The purity of the exosome preparation was good as verified by electron microscopy and western blot. The total dataset of exosomes contained 3302 exosome proteins with 1300-1400 exosome proteins per cell line. The core exosome proteome shared by all cells comprised 343 proteins of which a subset was highly enriched in exosome relative to lysate. These proteins were associated with the terms RNA post-transcriptional modification, protein synthesis, and cell signaling among others. In cancer exosomes, we identified established tumor type-specific antigens and proteins belonging to oncogenic pathways.

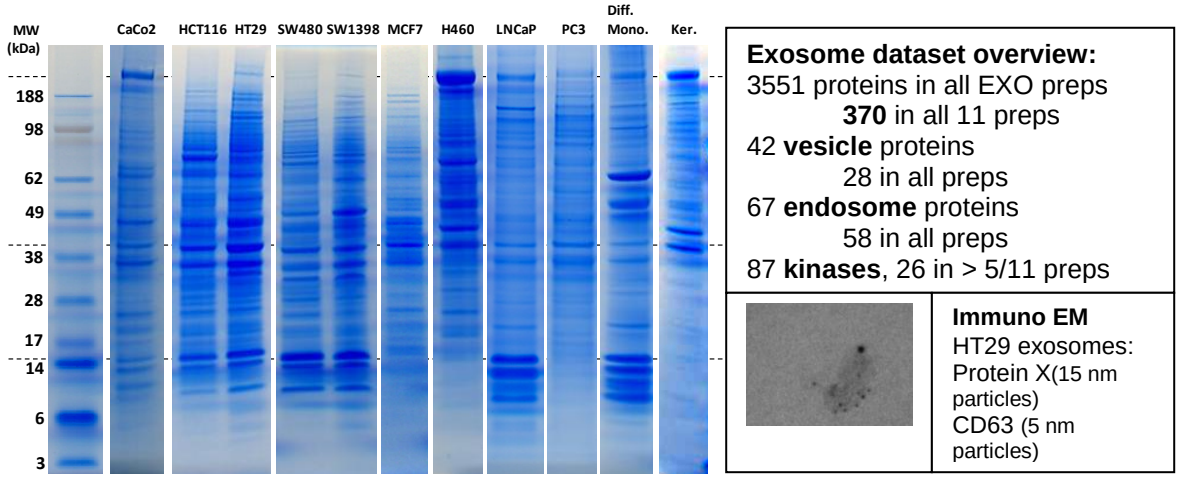



Figure Overlapping but different protein patterns in exosomes derived from different cancer cell lines and primary cells

**CONCLUSIONS & OUTLOOK** Comparative analysis of exosome versus cell lysate proteomes revealed a core exosome proteome associated with specific functions that may yield better insight into exosome biology. Cancer and tumor-type specific exosomal proteins may provide novel non-invasive biomarkers. To explore their use for early cancer detection and drug response predicition and monitoring.
in biofluids, we will employ targeted detection mehods (SRM mass spectrometry and antibodies).

*AACR 2011 abstract. This research is supported by the VUmc-Cancer Center Amsterdam.*

# Exosomal ITGA3 interferes with non-cancerous prostate cell functions and is increased in urine exosomes of metastatic prostate cancer patients

Irene V. Bijnsdorp[1], Mehrdad Lavaei[2], Sander R. Piersma[2], Albert A. Geldof[1], Connie Jimenez[2]

1. Department of Urology;2. OncoProteomics Laboratory, Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands

**Background** Cancer cells are able to change the protein expression and behavior of non-cancerous surrounding cells. Exosomes, secreted by prostate cancer (PCa) cells, may have a functional role in cancer metastasis and present a promising source for protein biomarkers. We found that the conditioned media from LNCaP and PC3 could decrease the proliferation and increase the migration and invasion capacity of prostate epithelial cells, while proliferation was increased in the stromal cells and migration and invasion were not affected. In order to study differences in the protein profile in the exosomes secreted by LNCaP and PC3 cells, proteomics analysis was performed.

**Aim** The aim of the present study was to identify which proteins in exosomes can influence non-cancerous cells, and to determine whether we can use urine exosomal proteins to identify high-risk PCa patients.

**Approach and methods** Figure 1 shows the study outline. Exosomes were isolated by ultracentrifugation from PC3 and LNCaP prostate cancer cells. Migration and invasion were studied by the transwell (invasion) assay. Proteomics was performed by LC-MS/MS and identified proteins were validated by Western blotting. Cellular uptake of fluorescent labeled PKH67-exosomes was measured by FACS.

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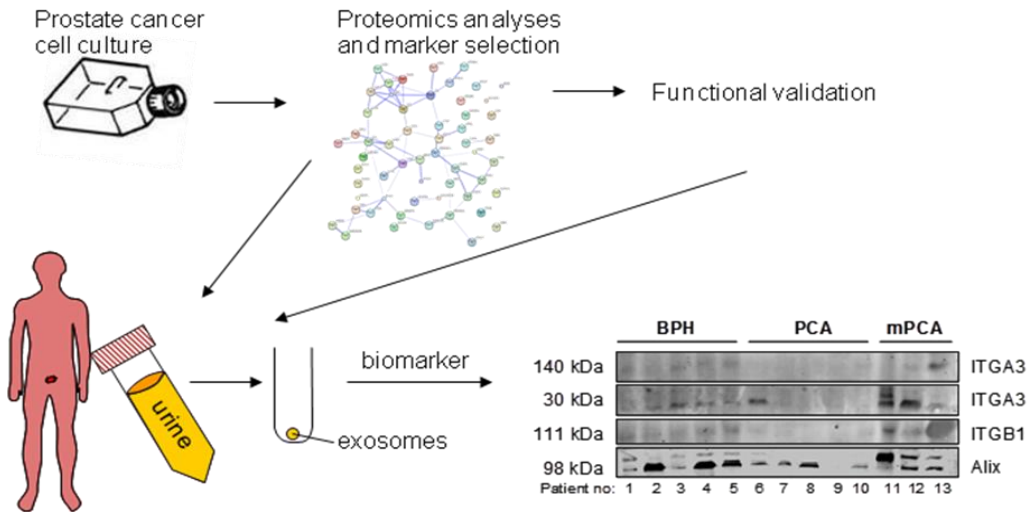



Figure 1. Study overview

**Results** In order to study differences in the protein profile in the exosomes secreted by LNCaP and PC3 cells, proteomics analysis was performed. Based on comparative protein profiling by mass spectrometry-based proteomics of LNCaP- and PC3-exosomes (details in figure 2), we selected ITGA3 and ITGB1, involved in migration/invasion, for further analyses. Inhibition of exosomal ITGA3 reduced the migration and invasion of non-cancerous prostate epithelial cells (prEC) almost completely. Cellular uptake of exosomes by prEC was higher with PC3-exosomes compared to LNCaP exosomes. Finally, ITGA3 and ITGB1 were more abundant in urine exosomes of metastatic patients ($p<0.05$), compared to benign prostate hyperplasia or PCa.

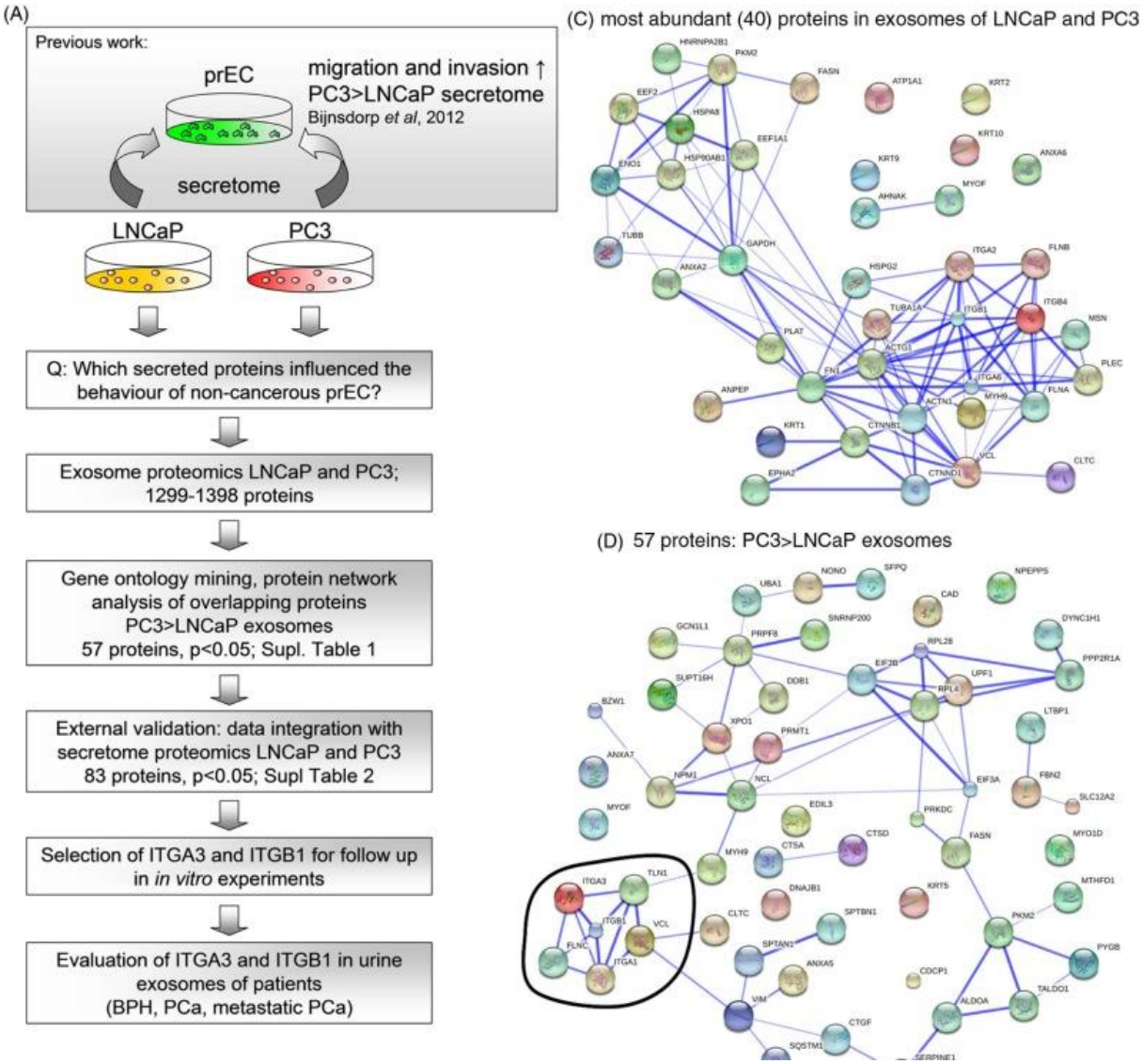



Figure 2. Summary proteomics results and regulated protein networks in PC3 vs LNCap exosomes

**Conclusions and outlook** These data indicate exosomal ITGA3 and ITGB1 may play a role in manipulating non-cancerous surrounding cells and that measurement of ITGA3 and ITGB1 in urine exosomes has the potential to identify patients with metastatic PCa in a non-invasive manner.

**Publication**

Bijnsdorp IV, Geldof AA, Lavaei M, Piersma SR, van Moorselaar RJA, Jimenez CR. ITGA3 and ITGB1 interfere with non-cancerous prostate cell functions and are increased in urine exosomes of metastatic prostate cancer patients. J Extracell Vesicles. 2013 Dec 23;2.

# Proteomics of exosomes in urine of prostate cancer patients reveals potential biomarkers

Bijnsdorp IV[1], Geldof AA[1], Piersma SR[2], Pham[2], Jimenez CR[2]

[1]Dept. Urology and [2]OncoProteomics Laboratory, VUmc

**Background** Prostate cancer is the most common cancer in males in Western countries. The diagnosis of prostate cancer is based on prostate specific antigen, T-stage and histopathological examination of tissue from biopsies. However, serum PSA has low specificity for prostate cancer, as conditions as prostatitis or benign prostate hyperplasia (BPH) can also lead to elevated PSA levels. This results in high negative biopsy rates of about 80%, when patients show a moderate elevation of PSA. In addition, for the early stage cancer patients it remains unclear which will ultimately develop aggressive cancer, and benefit from active therapy. To improve the diagnostic procedure, new biomarkers are needed, preferably through a minimally invasive method. Exosomes are small microvesicles that are released by cancer cells and can be found in urine. Exosomes contain proteins and RNAs, which are known to regulate various processes.

**Aims** To identify protein profiles of urine exosomes, and compare the profiles between patients without cancer, with those from early stage prostate cancer, and with metastatic prostate cancer.

**Approach** Quantitative proteomics was performed, based on gel-nano- LC-MS/MS of urine exosomes. Urine exosomes were collected using differential ultracentrifugation protocols, and were stored at -80˚C until further processing.

**Results** The total dataset of urine exosomes revealed > 3000 proteins. 1118 proteins were detected in all exosome samples. Several proteins were found differentially expressed between patients that did not have prostate cancer, compared to patients with prostate cancer, and compared to patients with metastasis.

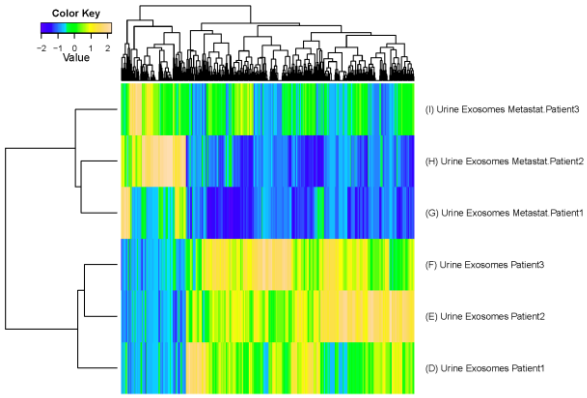



Figure – Heatmap after supervised cluster analysis of proteins, comparing patients with metastatic prostate cancer to patients with non-metastatic prostate cancer.

**Conclusion and future perspective**
Comparative analysis of exosome protein profile comparing patients without prostate cancer to patients with prostate cancer, and to patients with metastasis revealed a profile that may lead to the discovery of novel biomarkers to detect prostate cancer using a minimally invasive method. In future experiments, several proteins will be selected, which will subsequently be analyzed in an independent patient group for performance as biomarker.

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

Anna Wojtuszkiewicz, Gerrit J. Schuurhuis, Floortje L. Kessler, Sander R. Piersma, Jaco C. Knol, Thang V. Pham, Gerrit Jansen, Yehuda G. Assaraf, Gert J.L. Kaspers, Sonja Zweegman, Jacqueline Cloos*, Connie R. Jimenez*. **Shared senior authors.* VUmc Dept. Hematology and OPL.

## Background

Disturbances in apoptosis regulation are known to impact chemotherapy resistance and consequently refractoriness and relapse of acute myeloid leukemia (AML). We previously showed that apoptosis-resistant protein profile of AML blasts at diagnosis is associated with shorter disease-free survival. Specifically, by flow cytometry, we measured the expression of BCL-2, BCL-XL, MCL-1 and BAX in leukemic cells and combined these parameters to define their anti-apoptosis index (AAI). Interestingly, the AAI of normal lymphocytes in the AML patients corresponded to the AAI of AML blasts obtained from the same patient, reaching values far outside the normal AAI range of lymphocytes. In addition, the AAI in both cell types displayed parallel changes during the course of therapy. This points to a role of microenvironment in regulation of apoptosis in bone marrow cells of AML patients.

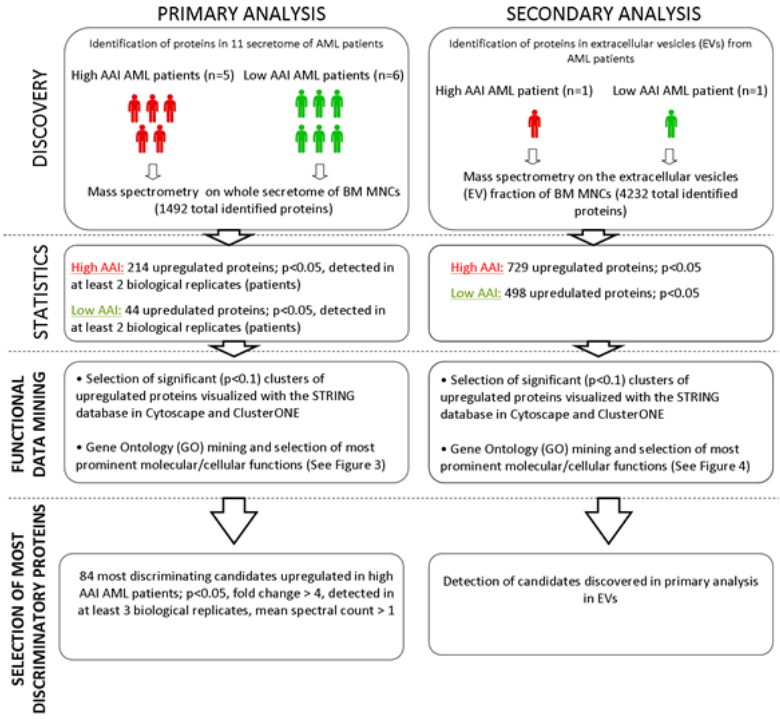
## Aim

The aim of the current study was to assess if apoptosis-resistant AML cells are able to regulate the AAI of apoptosis-sensitive cells by influencing the microenvironment, as well as to perform molecular dissection of microenvironment, to identify novel proteins that regulate apoptosis.

## Approach

We first assessed the ability of factors released from apoptosis-resistant AML blasts to modulate apoptosis-related protein expression in sensitive AML blasts. To this end, Bcl-2 upregulation was determined in apoptosis-sensitive AML blasts upon contact culture with apoptosis-resistant AML blasts. To characterize the AML microenvironment, patient samples were selected dysplaying either apoptosis-resistant (high AAI; n=5) or apoptosis-sensitive profile (low AAI; n=6). The blasts were cultured in serum free medium for 18 hours before harvesting the conditioned medium. Using mass spectrometry-based proteomics, comparative analysis was performed on secretomes derived from apoptosis-resistant and apoptosis-sensitive AML blasts (Figure 1). On a subset, we also investigated the extracellular vesicle (EV) fraction of proteins from a high AAI patient with those of low AAI patient.

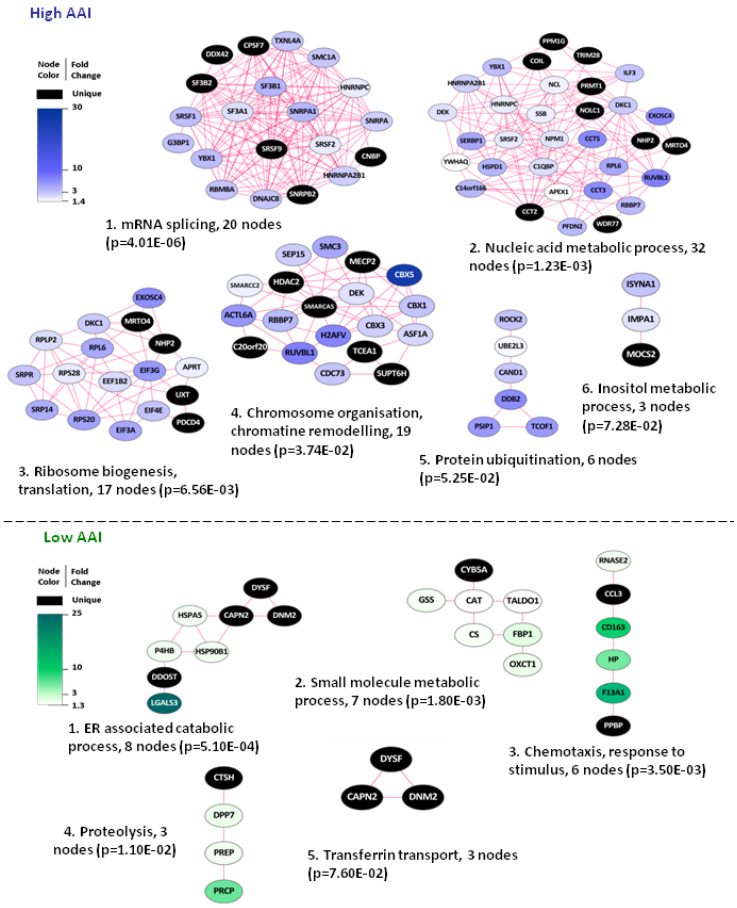
**Figure 1.** Overview of proteomics analysis performed on the whole secretome and extracellular vesicles of high AAI and low AAI leukemic blasts of AML patients.



## Results

First, we showed that apoptosis-resistant AML blasts (high AAI) release factors that modulate sensitive AML blasts (low AAI) to upregulate Bcl-2 and become apoptosis-resistant. 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 (1.7-fold, p=0.0067 and p=1.0 respectively). Strikingly, we found that the major functional protein clusters upregulated in secretomes of the apoptosis-resistant AML were involved in mRNA splicing, protein translation and chromatin remodeling/chromosome organization (Figure 2). We further compared protein profiles of EV fraction of a high AAI patient to those of a low AAI patient. Proteomic analysis of these fractions of the conditioned medium showed that the functional protein networks found in the whole secretome are well-represented in EVs that are enriched for exosome markers.

**Figure 2.** Functional clusters of proteins upregulated in the whole secretome of high AAI and low AAI primary AML cells.




## Conclusions and outlook

Transfer of functional proteins between cells by extracellular vesicles is a well documented phenomenon. Therefore, it is conceivable that the regulatory protein networks detected in the vesicles excreted by AML blasts are involved in regulation of apoptosis-related proteins in recipient AML blasts and other cells residing in the bone marrow, thereby contributing to therapy 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.

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1. A. Wojtuszkiewicz, G. J. Schuurhuis, F. L. Kessler, S. R. Piersma, J. C. Knol, T. V. Pham, G. Jansen, 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 (manuscript in revision for MCP).



*This research was supported by the VUmc Cancer Center Amsterdam*

# Proteomic analysis of cerebrospinal fluid extracellular vesicles: a comprehensive dataset

Davide Chiasserini[1], Jan R.T. van Weering[2], Sander R. Piersma[1], Thang V. Pham[1], Arjan Malekzadeh[3], Charlotte E. Teunissen[3], Heidi de Wit[2], Connie R. Jiménez[1].

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**Background**: Neurons can release a variety of extracellular vesicles (EVs) from their soma and dendrites. These EVs may have different origin, composition, morphology and possibly function than synaptic vesicles. Exosomes are 40 -100 nm EVs of endosomal origin and have recently drawn much interest for their putative roles in several human diseases, including neurodegenerative diseases .
Aim: A detailed analysis of the protein composition of CSF EVs including exosomes, using differential centrifugation coupled to high sensitivity and resolution mass spectrometry-based proteomics in order to shed light into brain (dys)function and provide the basis for biomarkers studies in neurological diseases.

**Approach**: Two independent pools (CSF1 and CSF2) of 6 ml of CSF were used for the proteomic analysis. EVs isolation was performed as previously reported [1]. Briefly, about 6 mL of CSF was centrifuged at 500 g for 10 min to remove any intact cells, the supernatant was carefully collected and spun at 2,000 g spin for 15 min and finally a t 20,000 g for 45 min. The pellet was washed and solubilized in sample buffer (P20) .The media was transferred to ultracentrifuge tubes and centrifuged at 100,000 g for 2 hours in a ultracentrifuge using a 40Ti rotor. The soluble CSF (supernatant, SN) was removed and the pellets containing EVs were resuspended in PBS, washed and centrifuged at 100,000 g for 2 hs to collect the final EV pellets (P100). Pellets were dissolved in sample buffer for proteomics and western blot experiments. SN fraction (~5 mL) was concentrated using 3 kDa filters and dissolved in sample buffer. EV Proteomics P100, P20 and SN fractions (30 ug) were loaded on gradient gels. The gels were stained with Coomassie brilliant blue G-250, in-gel digested and analyzed by nanoLC-MS/MS [1].

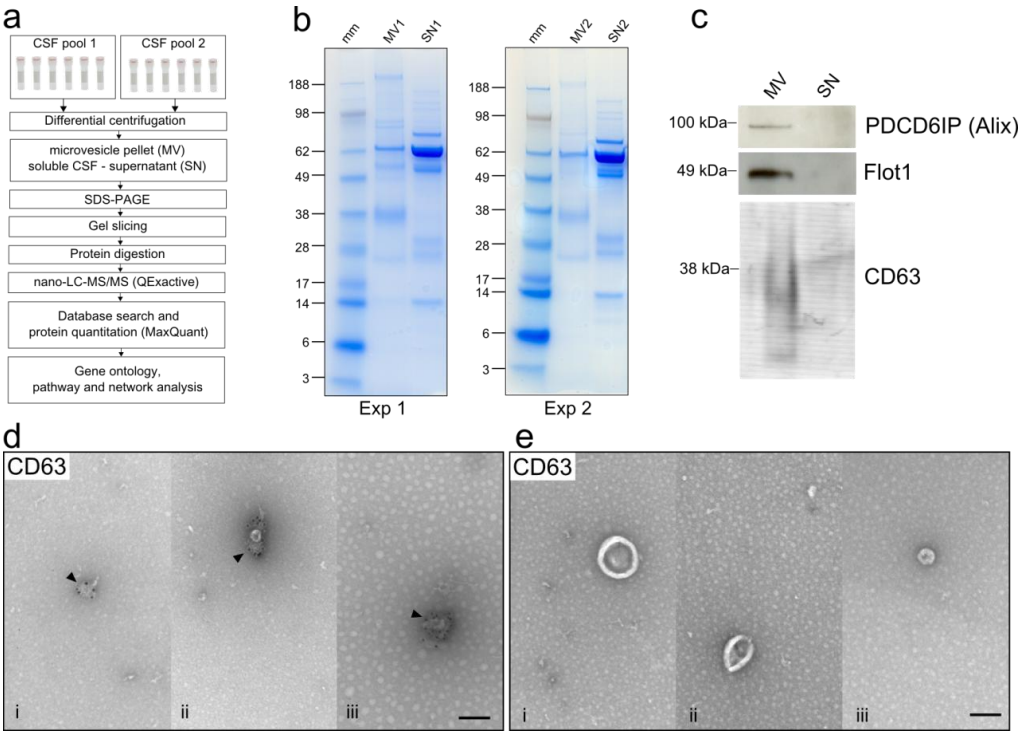

Figure 1. Characterization of CSF EVs. A. Study outline. B. SDS-page protein profile of EV isolated from CSF showing a different pattern in P100 and P20 fractions. C. Enrichment in exosomal markers Alix (PDCD6IP) and flotillin 1 in P100 EVs. D. Immuno-electron microscopy of P100 fraction showed the presence of CD63 positive and negative vesicles

**Results**: We characterized the protein composition of EVs isolated from CSF at different centrifugation speed, 20,000 (P20) and 100,000 x g (P100). The two EV proteomes exhibited an overlap of 50% in the number of identified proteins. The proteome of P100 vesicles was similar to that of exosomes as reported in literature. Interestingly P100 vesicles were enriched in proteins involved in RNA splicing, protein folding and ATP ase activity compared to the P20 fraction. Lower speed centrifugation of CSF may enrich also for membrane debris particles as shown by the higher number of transmembrane proteins in the P20 vesicles. Fig. 2 shows an schematic overview of P100 identified proteins.

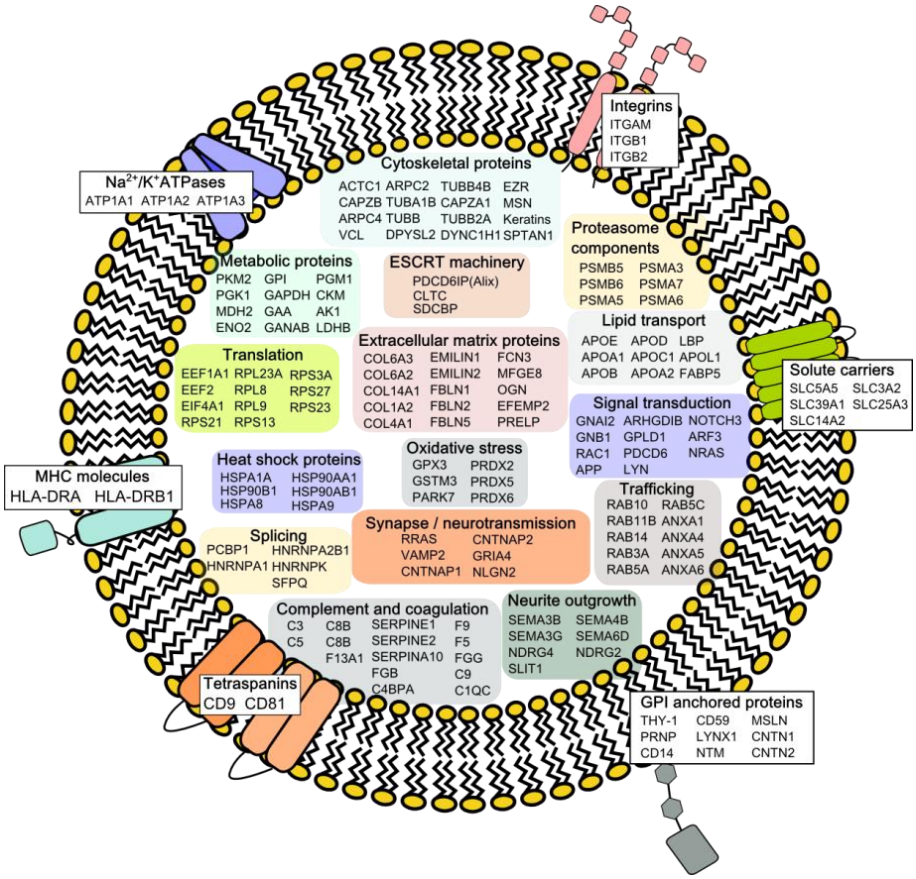


Figure 2. Schematic overview of CSF EV protein composition in the P100 fraction

**Conclusions and outlook:** Mining the proteome of different EV fractions of CSF may disclose the full biomarker potential of this biological fluid, especially for neurodegenerative diseases.

**Reference**

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

**Acknowledgements:** This research is supported by the EU kp7 MEFOPA project

# Analysis of AKT and ERK1/2 protein kinases in extracellular vesicles isolated from blood of patients with cancer

Van der Mijn JC[1], Sol N[2], Mellema W[3], Jimenez CR[1], Piersma SR[1], Dekker H[1], Schutte LM[1], Smit EF[3], Broxterman HJ[1], Skog J[4], Tannous BA[4], Wurdinger T[5], Verheul HM[1].

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**Background**:
Extracellular vesicles (EVs) are small nanometre-sized vesicles that are circulating in blood. They are released by multiple cells, including tumour cells. We hypothesized that circulating EVs contain protein kinases that may be assessed as biomarkers during treatment with tyrosine kinase inhibitors.

**Methods**:
EVs released by U87 glioma cells, H3255 and H1650 non-small-cell lung cancer (NSCLC) cells were profiled by tandem mass spectrometry. Total AKT/protein kinase B and extracellular signal regulated kinase 1/2 (ERK1/2) levels as well as their relative phosphorylation were measured by western blot in isogenic U87 cells with or without mutant epidermal growth factor receptor (EGFRvIII) and their corresponding EVs. To assess biomarker potential, plasma samples from 24 healthy volunteers and 42 patients with cancer were used.

**Results**:
In total, 130 different protein kinases were found to be released in EVs including multiple drug targets, such as mammalian target of rapamycin (mTOR), AKT, ERK1/2, AXL and EGFR. Overexpression of EGFRvIII in U87 cells results in increased phosphorylation of EGFR, AKT and ERK1/2 in cells and EVs, whereas a decreased phosphorylation was noted upon treatment with the EGFR inhibitor erlotinib. EV samples derived from patients with cancer contained significantly more protein ($p=0.0067$) compared to healthy donors. Phosphorylation of AKT and ERK1/2 in plasma EVs from both healthy donors and patients with cancer was relatively low compared to levels in cancer cells. Preliminary analysis of total AKT and ERK1/2 levels in plasma EVs from patients with NSCLC before and after sorafenib/metformin treatment ($n=12$) shows a significant decrease in AKT levels among patients with a favourable treatment response ($p<0.005$).

**Conclusions**:
Phosphorylation of protein kinases in EVs reflects their phosphorylation in tumour cells. Total AKT protein levels may allow monitoring of kinase inhibitor responses in patients with cancer.

# Surface proteomic analysis of osteosarcoma identifies EPHA2 as receptor for targeted drug delivery

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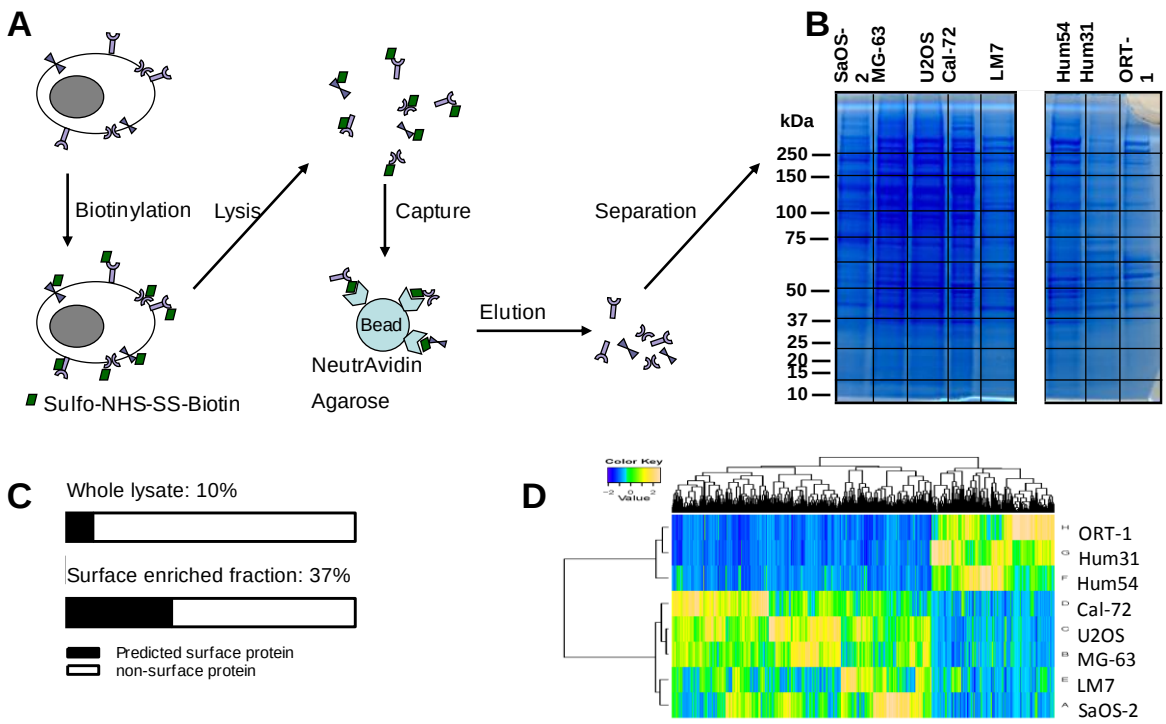
**Background** Osteosarcoma (OS) is the most common bone tumour in children and adolescents. The treatment currently consists of a combination of multi-agent chemotherapy and radical surgery when feasible. Despite this aggressive regimen, the survival outcomes are unsatisfactory, especially for patients with metastatic disease or patients with a poor response to induction chemotherapy. At present, 5-year survival rates are approximately 65% for localised and 20% for metastatic OS. By employing the targeted delivery of drugs, higher chemotherapy doses can be achieved at the site of the tumour while sparing healthy tissues. The identification of druggable targets or targeting ligands is an essential step in the design of targeted therapy for OS.

**Aim** Identification of protein targets on osteosarcoma cells that can serve as a receptor for targeted drug delivery, ultimately increasing the efficacy of current therapy regimens.

**Approach** Cell surface biotinylation of five OS cell lines and three human primary osteoblasts (OBs) for cell surface protein isolation coupled to in-depth proteomics using Gel-nanoLC-MS/MS.

**Methods** We conducted a comparative analysis of the surface proteome of human OS cells and osteoblasts using cell surface biotinylation combined with nano-liquid chromatography - tandem mass spectrometry-based proteomics to identify surface proteins specifically upregulated on OS cells. This approach generated an extensive data set from which we selected a candidate to study for its suitability as receptor for targeted treatment delivery to OS. First, surface expression of the ephrin type-A receptor 2 (EPHA2) receptor was confirmed using FACS analysis. Ephrin type-A receptor 2 expression in human tumour tissue was tested using immunohistochemistry. Receptor targeting and internalisation studies were conducted to assess intracellular uptake of targeted modalities via EPHA2. Finally, tissue micro arrays containing cores of human OS tissue were stained using immunohistochemistry and EPHA2 staining was correlated to clinical outcome measures.

**Results** In total, 2841 proteins were identified. 684 proteins were significantly upregulated in OS compared to the OBs ($p < 0.05$); 151 proteins were verified as being cell surface proteins, deriving from their entries in the Uniprot Knowledgebase. Putative receptors for targeted drug delivery to OS were selected based on their consistent expression on tumour cell lines (all 5 OS cell lines), highly differential regulation (> 10-fold up) and abundance (> 5 spectral counts per OS cell line)., resulting in 43 candidate proteins. The EPHA2 receptor was the most abundant surface protein in our dataset, thus, we chose to further investigate this protein for its suitability as a targeting receptor for drug delivery.
FACS analysis confirmed surface expression and overexpression of EPHA2 on OS cell lines compared to OBs ($p < 0.01$). Immunohistochemical staining of 18 archival tumour samples and 10 normal bone sections showed that EPHA2 is expressed in the majority of human OS, both primary and metastatic lesions and only scarcely expressed on normal bone, indicating clinical relevance of this receptor in OS. Finally, all OS cell lines were readily infected with AdYSA whereas the OBs remained unaffected ($p < 0.05$). EPHA2 receptor blocking by a synthetic peptide significantly reduced AdYSA uptake in all OS cell lines ($p < 0.01$) suggesting that uptake of AdYSA is specifically mediated by EPHA2. Patients with EPHA2-positive tumours showed a trend toward inferior overall survival.




**Conclusions** Cell surface biotinylation combined with nanoLC-MS/MS succesfully identified receptors for targeted drug delivery in OS. EPHA2 was abundantly expressed on the surface of all OS cells investigated. We validated the potential of drug targeting via this receptor, indicating that EPHA2 can be considered a promising receptor for targeted drug delivery to OS, ultimately improving the therapy efficacy of current regimens for this tumour.

*This research was supported by the VUmc Cancer Center Amsterdam*

# Platelet proteomics for cancer biomarker discovery, study 1

M. Walraven[1], Sander R. Piersma[2], Inge de Reus[2], Thang V Pham[2], Henk J. Broxterman[1], Henk M.W. Verheul[1]*, Connie R. Jiménez[2]* * Shared senior authors

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**Background** Platelets are anucleated fragments originating from the processing of megakaryocytes and perform essential roles in normal thrombus formation and coagulation, but also in pathological thrombosis, inflammation and atherosclerosis. More recently, based on findings that platelets enhance endothelial cell proliferation and because of their localisation at sites of tumor microvessels, the hypothesis was put forward that platelets might trigger new blood vessel formation or angiogenesis in tumors. In addition, the potential uptake of tumor-derived proteins implicates that the platelet proteome in the context of cancer may provide a new source of biomarkers.

**AIM** Application of mass spectrometry-based proteomics for platelet protein profiling and candidate biomarker discovery for (early) cancer detection and drug response.

**Results** Recently, we established a reproducible protocol for platelet releasate proteomics to identify platelet contributions in serum, which detection in whole serum would be hampered where the secreted platelet contents are present in a high background of major plasma proteins. We reported the activated platelet releaste proteome comprising a total of 716 identified proteins with 225 proteins present in the releasate of 3/3 volunteers (Piersma et al., Journal of Proteomics, 2009). Meta-analysis revealed 55% overlap with existing datasets of platelet $\alpha$-granules and microparticles, and 45% novel platelet releaste proteins.
We recently extended these observations to a pilot analysis of platelet releasate and platelet lysate of control subjects and 4 cancer patients (hepatocellulair carcinoom, oesofaguscarcinoom, pancreascarcinoom, tonsilcarcinoom). The results are very promising, in both platelet lysate and releasate multiple significantly regulated proteins were found, many of the absent in one group and present in all subjects of the other group.

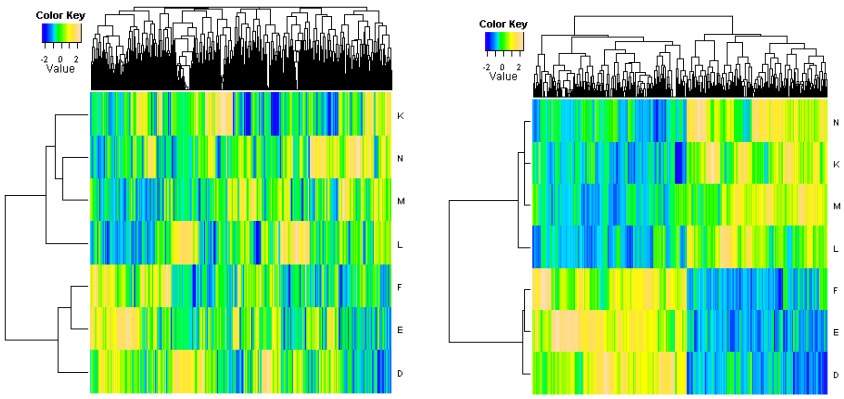



Figure. Heat maps of the platelet proteome in cancer patients and control subjects. Un-supervised (left) and supervised (right) cluster analysis using significantly regulated ($p < 0.05$) and all platelet proteins, respectively. In each case two clusters are obtained with a perfect separation of controls (samples labeled F, E, and D) and cancer patients (samples labeled K, L, M, N; **hepatocellulair carcinoom, oesofaguscarcinoom, pancreascarcinoom, tonsilcarcinoom**) (Jimenez and Verheul, unpublished data).

**Outlook** Together these results underscore the feasibility and power of a state-of-the-art mass spectrometry-based proteomics approach to study the composition of the soluble and microparticulate proteome released by platelet activation. Platelet proteomics will be extended to the analysis of patients in clinical trials with targeted anti-cancer agents to explore the potential for drug response prediction and monitoring

**This research is supported by the VUmc-Cancer Center Amsterdam and Aegon.**

# Platelet proteomics for cancer biomarker discovery, study 2

M. Walraven[1], Sander R. Piersma[2], Thang V Pham[2], Henk Dekker[1], Henk J. Broxterman[1], Henk M.W. Verheul[1]*, Connie R. Jiménez[2]* * Shared senior authors

[1]Angiogenesis Laboratory and [2]OncoProteomics Laboratory, Dept. Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands;

**Background** In platelet proteomics study two we expand on the results of study one (see above) and included more patient samples including pre-treatment and on-treatment samples.

**Patients and Methods** Platelets were isolated from patients with advanced stage of cancer. The cohort of patients consisted of: colon carcinoma (3 patients), rectum carcinoma (1 patient), anal cancer (1 patient), esophageal cancer (2 patients), pancreatic cancer (1 patient), gastric cancer (1 patient), hepatocellular cancer (1 patient), tonsil carcinoma (1 patient), tongue carcinoma (2 patients) and primitive neuroectodermal tumor (1 patient).

Blood was collected by free-flow after which platelets were isolated by a couple of consecutive centrifugational steps, including washing steps. From one part of the platelets a pellet was obtained of resting platelets, from another part releasate of activated platelets (by TRAP) was obtained. Both were resolved in sample buffer to analyse by mass spectometry-based proteomics. Here we describe the results of the pellet analysis.

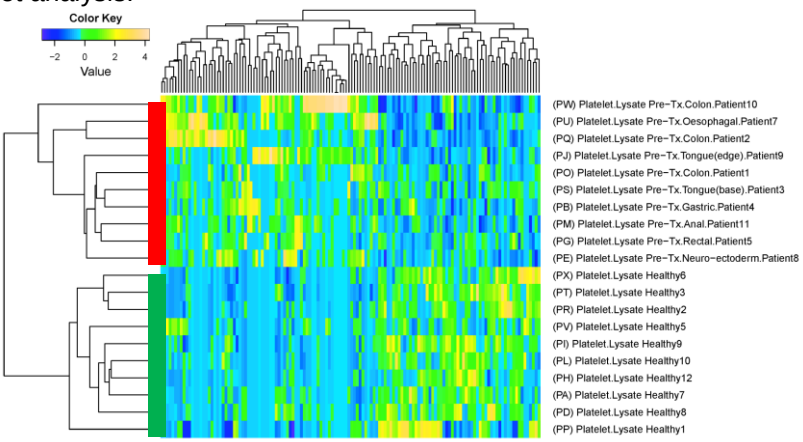



Fig. 1. Heat map visualizing differential platelet proteins in the comparison cancer (red bar)-control (green bar)

**Results** In total 4200 platelet proteins were identified across all platelet samples of which 137 proteins were significantly regulated ($p < 0.05$) with 73 proteins upregulated in cancer platelets and 36 proteins upregulated in control platelets (including a Fc> 1.5 cut-off). Cancer platelet associated proteins are involved in 'Defense response', 'Immune system Process', 'Regulation of cytokine production' and 'Response to external stimulus'. Control platelet associated proteins are involved in 'Cellular component movement', 'Cell migration', and 'Cell motility'. Interestingly, platelet proteomics of cancer patients before and during treatment were also very discriminating (fig 2 below).

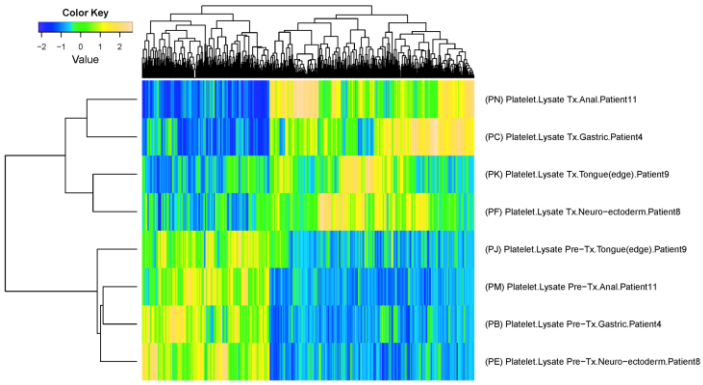



Fig. 2. Heat map visualizing clustering of differential platelet proteins in the comparison pre- vs on-treatement.

**Conclusions and outlook**
The platelet proteome harbors potential as liquid biopsy indicating the presence of cancer and treatment-induced alterations. Further studies are warranted to explore a potential link with response to therapy

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

Johannes C. van der Mijn#, Mariette Labots#, Sander R. Piersma, Thang V. Pham, Jaco C. Knol, Henk J. Broxterman, Henk M. Verheul#, Connie R. Jiménez#* # equal contributions

[1]OncoProteomics Laboratory, [2]Angiogenesis Laboratory, Department of Medical Oncology, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, the Netherlands

**Introduction and objectives** Mass spectrometry based phosphoproteomics emerged in the past decade as advantageous approach for the analysis of tyrosine phosphorylation on proteins and receptor tyrosine kinase (RTK) signaling. For comprehensive analysis, immunoaffinity purification is performed, requiring a phosphotyrosine antibody. Here we compared the performance of two antibodies for label-free phosphotyrosine-based phosphoproteomics. The best performing antibody was applied to analyze erlotinib effects on EGFR signaling in the U87 glioma cell line with or without an activating mutation of the epidermal growth factor receptor (EGFRvIII).

**Aim** The aim of this project is to compare antibody-based enrichment for phosphotyrosine-based phosphoproteomics using cancer cell lines and application to glioblastoma U87 EGFR wild-type and mutant cells.

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

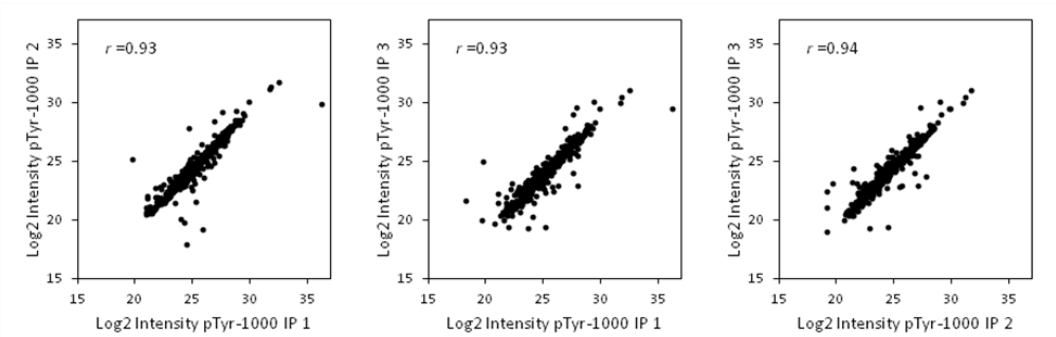
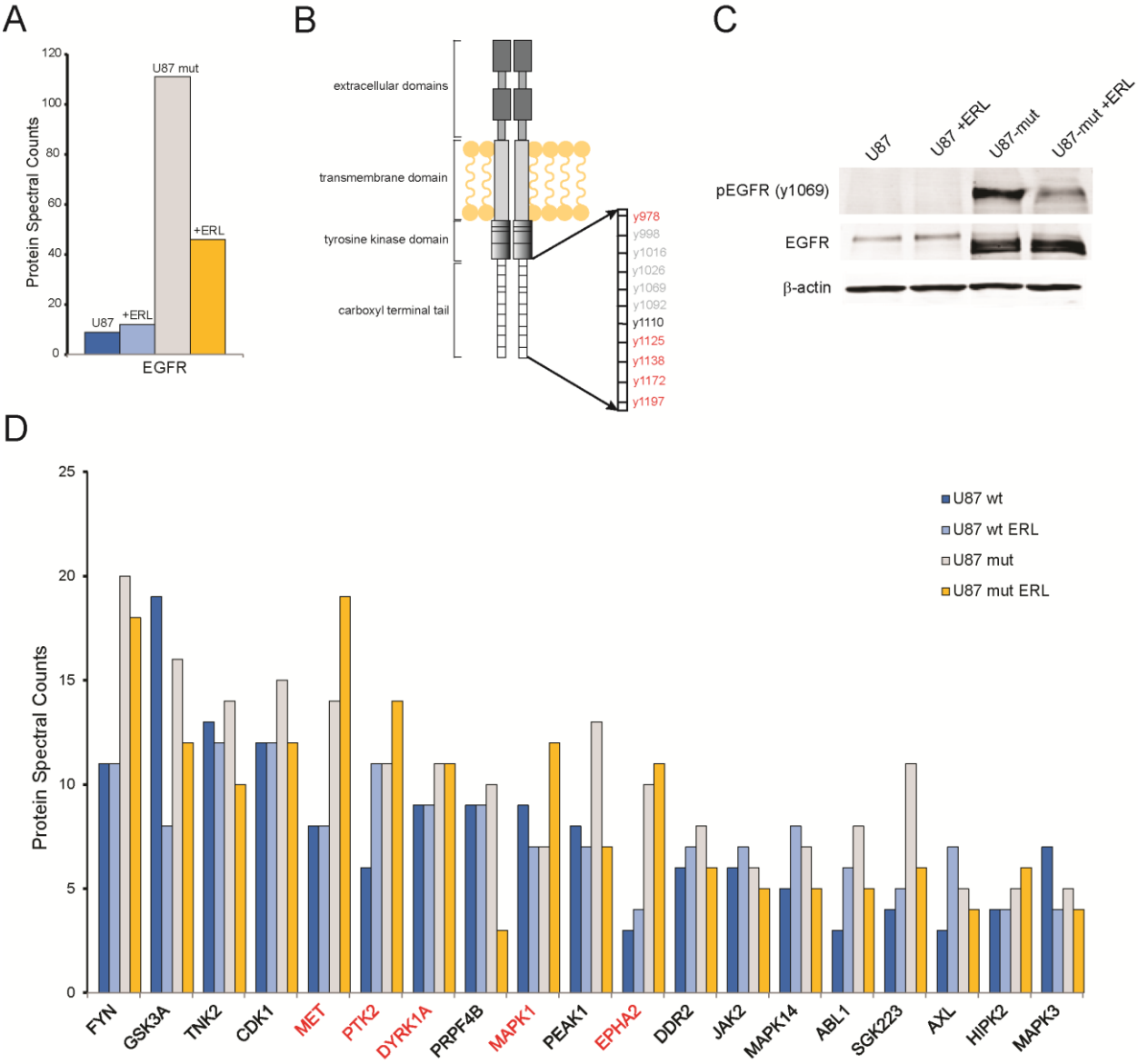



Figure 1. Correlation of log2 phosphopeptide Intensities for replicate pTyr-1000 IPs (n=3). Pearson correlation r calculated on log2 transformed Intensities of peptides detected in both replicate IPs (overlap).

**Results and discussion** 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. Most (97%) 4G10-captured phosphopeptides were also identified by P-Tyr-1000.
Application of P-Tyr-1000 in a label-free phosphoproteomics workflow to U87 +/- EGFRvIII mutated cancer cells showed a 59% reduction in EGFR phosphorylation upon exposure to a specific small molecule EGFR-inhibitor. Reduced EGFR phosphorylation at y978, y1125, y1138, y1172, y1197 and increased phosphorylation at y1110 was observed. In cells overexpressing EGFRvIII, enhanced phosphorylation of FYN, MET, PTK2, DYRK1A, MAPK1 and EPHA2 was found. Our results implicate these molecules as potential targets for future testing in order to overcome resistance to EGFR inhibitors.

**Conclusions** The P-Tyr-1000 phosphotyrosine antibody performs superiorly when compared to 4G10 antibody for capture of tyrosine-phosphorylated peptides by label-free phosphoproteomics. This workflow allows insight into activated cancer signaling patwhays and evaluation of drug target phosphorylation and may give insights in the pharmacodynamic effects of tyrosine kinase inhibitors.




**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 10 mg. Furthermore, application of the P-Tyr-1000 antibody in a standardized phosphoproteomics workflow allows quantitation of drug target inhibition and provides insights in alternative signaling pathways in cancer cells.

**Publications**

This work has been submitted to Journal of Proteomics special issue HUPO 2014

# Phosphoproteomics of a panel of AML cell lines

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[2]Department of Hematology, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, the Netherlands; **Shared senior authors*

**Background** Acute myeloid leukemia (AML) is a malignant hematopoietic stem cell disease caused by a differentiation block at the level of immature progenitors leading to massive accumulation of malignant cells in the bone marrow and suppression of normal hematopoiesis. The disease is highly heterogeneous with 2 year survival rates, after intensive chemotherapy regimens depending on cytogenetic and molecular risk factors, varying between 70% for patients with good risk and less than 10% in those with poor prognostic features.
In recent years clinical success has been achieved in the treatment of several cancer types with kinase inhibitors (KIs) and they may prove to be a valuable addition to standard therapy in AML. However, a proper rationale to select a suitable KI is still lacking. Phosphoproteomics based on tandem mass spectrometry (MS/MS) is a powerful approach for unbiased, global profiling of protein phosphorylation, and could identify aberrancies in signal transduction pathways and associated key kinases that may be suitable for targeted treatment with KIs.

**Aim** The aim of this study is to identify candidate driver kinases and signaling routes that may be targets for kinase inhibitor treatment by analyzing 16 selected AML cell lines.

**Approach** To get an impression of phosphorylation in an heterogeneous disease such as AML, a cell line panel of 16 AML cell lines (EOL-1, HEL, HL-60, Kasumi-1, Kasumi-3, Kasumi-6, KG-1, KG-1a, ME-1, ML2, MM6, MOLM-13, MV-4-11, NB-4, OCI-AML3, THP1) was characterized on both the proteomic (one-shot analysis) and phosphoproteomic (pTyr) level. Phospho-Tyrosine containing peptides were captured from AML cell line lysates, containing 10 mg of protein, using the pY1000 antibody in an immunoprecipitation (IP) experiment following our in-house protocol. During analysis we focus on identifying key kinases of which some will be validated in a kinase inhibition drug experiment.

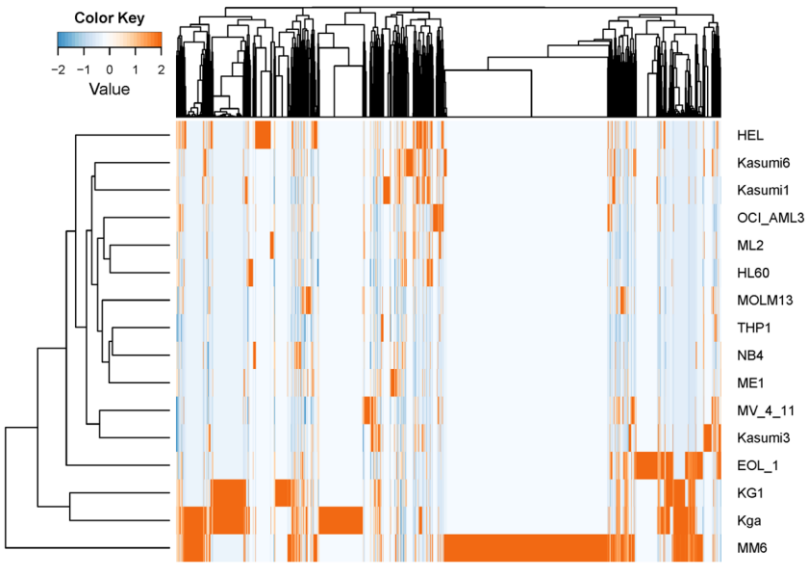


Figure 1. Unsupervised clustering using phosphopeptide intensities reveals heterogeneous phosphorylatioin patterns that do not cluster according to mutation status or Fab classification

**Results** The whole phosphoproteome dataset of 16 AML cell lines contained 4853 identified phosphopeptides corresponding to 2280 phosphoproteins, including 138 phosphokinases. Unsupervised cluster analysis using all data indicated two clusters based on amount of

phosphopeptides detected; one main cluster with relatively low phosphorylation levels and a more dispersed cluster containing the cell lines with high phosphorylation levels. Clustering was not found to be related to FAB classification of the cell lines, or related to mutational status.
Ranking of phosphokinases identified in each of the 16 AML cell lines confirmed FLT3, PDGFRa, JAK2 and c-KIT as hyperphosphorylated kinases in AML cells with mutations in these genes (Fig. 2). Interestingly, AML cell lines without known kinase-related genomic aberrations also showed high activation of several kinases which could be potential KI targets. Current data analysis will link phosphokinases to downstream phosphoprotein substrates and pathways in order to prioritize (combinations of) candidate driver kinases for functional perturbation experiments. This analysis is still ongoing.

**Conclusions and outlook** In this experiment we show that we are able to detect key activated kinases already known to be involved in AML, FLT3, PDGFRa, JAK2, and c-KIT. Furthermore, we identified some interesting candidate kinases for treatment, also for the cell lines not harboring any known kinase mutation. Further functional analysis will be performed to confirm selected candidate driver kinases as possible targets for treatment with KIs.

*This research was supported by the VUmc Cancer Center Amsterdam*

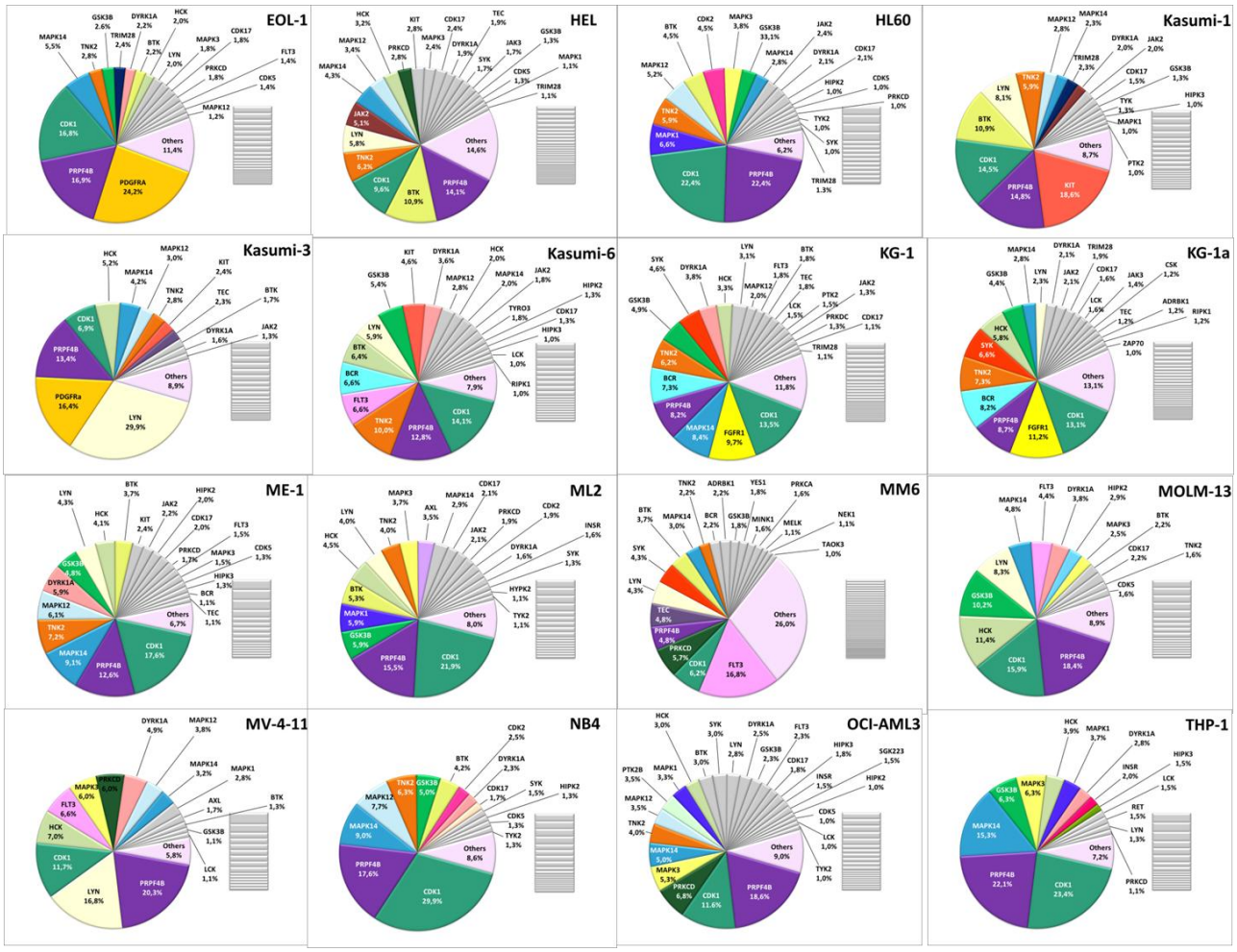


Figure 2. Phospho kinase ranking based on spectral counting of all cell lines. Colors correspond to a single kinase across all pie charts.

## Phosphoproteomics for therapy response prediction in pancreatic cancer

**Tessa Y.S. Le Large[1], Maarten F. Bijlsma[2], Mariette Labots[1], Marc G. Besselink[2], Geert Kazemier[3], Nicole C.T. van Grieken[4], Hanneke van Laarhoven[2], Elisa Giovannetti[1], Connie Jimenez[1]**

**[1]Dept. Medical Oncology, VU University Medical Center, [2]AMC, [3]Dept. Surgey, [4]Dept. Pathology, VU University Medical Center, VU University Medical Center, Amsterdam, The Netherlands**

**Background**
**Pancreatic cancer is a disease with a very poor prognosis, with a 5-year survival of 5 %. Most patients present with locally advanced or metastatic disease, at which point only palliative chemotherapy is an option. Unfortunately, due to strong inter- and intra-tumor heterogeneity these tumors are very chemoresistant. Current treatment regimens (FOLFIRINOX or Gemcitabine) result in a survival benefit of respectively 11 and 6 months. New treatment options are warranted.**
**Phosphorylation of proteins is commonly deregulated in several cancer types. Using phosphoproteomics, it is possible to identify activated proteins and it can give an insight in aberrant functional pathways. These profiles can shed light on the prediction of prognosis and therapy resistance of patients with pancreatic cancer and can point out new drug targets for personalized treatment.**

**Aims**
**To employ phosphoproteomics to understand the mechanisms underlying drug sensitivity/ resistance in pancreatic cancer and identify differentially activated pathways and driver kinases for treatment targets.**

**Methods**
**Comparative proteomic and phosphoproteomics analysis will be performed on pancreatic cancer cell lines, primary cell cultures with adjacent xenograft material, and patient tumor tissue. At present there is a library of 15 existing cell lines and 8 primary cultures available. During the next years tissue will be collected in the biobank of the participating centers, VUmc and the AMC. Signaling pathways and driver kinases will be identified with phosphoproteomics. Lysates from cell culture or tissue will be digested to peptides using trypsin. Phosphopeptides will be captured using titaniumoxide and anti-phosphotyrosine antibody coated beads, and analyzed subsequently by high resolution mass spectrometry. Differentially activated pathways will be analyzed with ingenuity pathway analysis and candidate driver kinases will be identified. This information will be integrated with existing genomic data.**
**The functional role of candidate driver kinases will be evaluated using lentiviral silencing experiments and drug perturbation experiments. Furthermore, existing xenograft models can be used to confirm drug activity in vivo. Also the expression of identified driver kinases will be validated in tissue microarrays of patients with pancreatic cancer.**

**Outlook**
**We expect to get an insight in the aberrant functional pathways in pancreatic cancer. Furthermore, we expect to find subsets with different phosphoprofiles and specific driver kinases. With these findings, we will functionally test new drug targets and combinations towards personalized medicine.**

*This research is a VUmc-AMC alliance project supported by VUmc-AMC*

# Phosphoproteomics for therapy response prediction in solid tumors with a focus on colorectal cancer

PhD project Robin Beekhof

Supervision: Connie R. Jimenez, Mariette Labots, Henk M.W. Verheul
Oncoproteomics laboratory, Department of Medical Oncology, VU University Medical Centre Amsterdam

Collaboration: Dr. Livio Trusolino and Dr. Andrea Bertotti, Department of Oncology, University of Torino Medical School

**Background** Biological processes such as cell growth and survival are regulated by interwoven signaling pathways that are deranged in cancer. Protein kinases are important regulators and mediators in these networks. They modify the activity of other proteins by adding a phosphate (phosphorylation). Aberrant kinase activity underlies deregulated signalling in cancer development and progression.
Multiple protein kinase inhibitors have recently been developed and approved for clinical use in cancer patients. Despite extensive knowledge of cancer genomes and cancer biology, it is incompletely understood why these inhibitors are only effective in a minority of patients. Moreover, even in initially responsive patients the effectiveness of these drugs is limited, as patients invariably develop therapy resistance. Both intrinsic resistance and escape from kinase inhibition can result from activation of redundant signalling pathways or activated mutated pathway components downstream of the targeted kinase. These changes enable a tumor to bypass the drug target.
To accurately predict phosphoprotein targets in complex, crosstalking cellular signaling networks in cancer, a post-genomic approach is urgently needed, that captures cellular protein activation states on a global scale. In particular, mass spectrometry-based phosphoproteomics is advantageous as it allows comprehensive protein phosphorylation profiling.

**Aim** In this project, phosphoproteomics will be employed to uncover aberrantly active signaling pathways and drug targets in tumors of patients with colorectal cancer, a disease with high incidence and mortality.

**Approach** State-of-the-art genomically characterized patient-derived xenograft models characterized for cetuxumab response (both in human and mouse) will be used to discover phosphoproteome profiles associated with cetuximab sensitivity and resistance and identify new drug targets. The PDX models include sensitive models that are KRAS, NRAS, BRAF, and PIK3CA wilt-type, resistant models that are mutant for KRAS, NRAS or BRaf, resistant models that are quadrupole wild-type and resistant models that are Her2 or Met amplified. Group comparison of these untreated PDX tumors will reveal predictive phosphoproteome profiles associated with cetuximab resistance in Ras mutant and Ras wild-type cancers.
Model systems in vitro and in vivo will be employed to investigate novel (combination) treatments targeting selected activated “driver” protein kinases in wild-type resistant tumors. Analysis of patient biopsies obtained in the IMPACT-CRC trial will validate the predictive value of the phosphoproteome profiles.

Other planned studies in solid tumors include renal tumors characterized for sunitinib response and pre- and on-treatment biopsies of solid tumors of patients receiving various anti-kinase inhibitors.

**Outlook** The proposed studies will yield a phosphoproteome-based approach to reveal an individual tumor’s Achilles' heel(s), and elucidate potential resistance mechanisms. The studies in CRC will show the value of phosphoproteomics for identifying major kinase-driven cancer pathways, potential therapeutic (combination) strategies and a predictive signature for cetuximab response.

# Phosphoproteomics analysis of renal cell cancer cells exposed to sunitinib reveals targets for new combination treatment

Johannes C. van der Mijn[1-2], Henk J. Broxterman[1], Jaco C. Knol[1], Sander R. Piersma[1], Richard R. De Haas[1], Henk Dekker[1], Thang V. Pham[1], Victor W. Van Beusechem[1], Balazs Halmos[3], James W. Mier[2], Connie R. Jiménez[1,#], Henk M.W. Verheul[1,4,# #] Shared senior authors

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**Background** The tyrosine kinase inhibitor sunitinib is registered as first line treatment for patients with advanced renal cell cancer (RCC). *In vitro* data on the interaction with recombinant kinases suggest that sunitinib may inhibit multiple kinases, including Axl which is highly expressed in renal cell cancer. **Aim** We investigated the effect of sunitinib on global tyrosine phosphorylation in tumor cells to increase our understanding of its mechanism of action.

**Methods** The MTT assay was used to determine the effect of sunitinib on proliferation of a panel of cell lines, including 786-O RCC and HCC827-ER3 NSCLC cells. Global tyrosine phosphorylation in 786-O cells was measured by mass spectrometry-based phosphoproteomics with or without preincubation with sunitinib. Selected phosphorylation sites in Axl, FAK and p38α were validated by Western Blots.

**Results** Sunitinib inhibited tumor cell proliferation of 786-O and HCC827 cells upon 96 hour exposure for 50% at a concentration (IC50) of 2 and 5 µM, respectively. Global phosphoproteomics analysis of 786-O cells yielded 1519 phosphopeptides, corresponding to 675 unique phosphorylated proteins. Two hour incubation with sunitinib at its IC50 downregulated 86 phosphopeptides, including CDK5, DYRK3, DYRK4, G6PD, PKM and LDH-A. Upregulation was observed of 94 phosphopeptides including peptides derived from Axl, FAK, EPHA2 and p38α. Induction of Axl- (y702), FAK- (y576) and p38α (y182) phosphorylation was confirmed by Western Blot in 786-O, A498 and HCC827-ER3 cells. Subsequent gene ontology analysis of the modulated proteins, highlighted activation of receptor tyrosine kinase, integrin / focal adhesion kinase (FAK) signaling by sunitinib.

**Conclusions** Sunitinib inhibits tumor cell proliferation and activity of multiple kinases, but also induces Axl and FAK signaling. Activation of protein kinase signaling by sunitinib sheds new light on its mechanism of action and provides potential leads for new combination treatment strategies.

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

Sander R. Piersma[1], Inge de Reus[1], Bharath Sampadi[1], Mariette Labots[2], Jaco C. Knol[1], Thang V. Pham[1], Henk MW Verheul[2]; Connie R. Jimenez[1]

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## Background

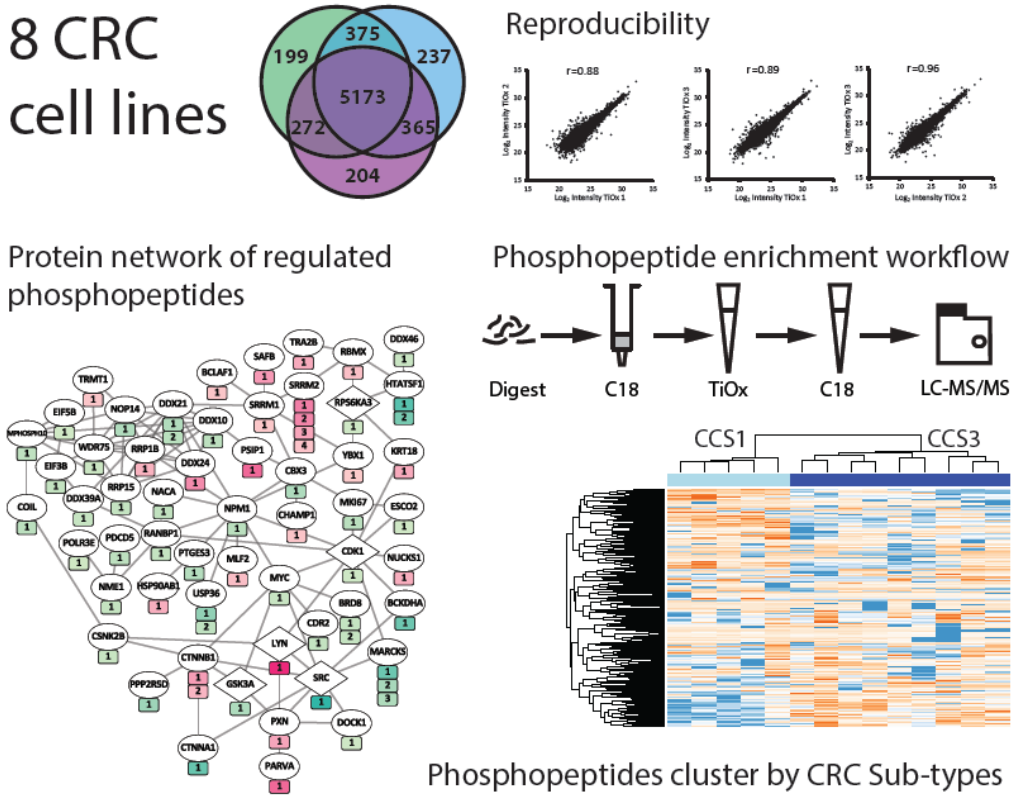
Robust phosphopeptide enrichment methods are required to profile signaling networks in cancer cell lines and tissues with minimal fractionation. With recent instrument developments thousands of unique phosphopeptides can be detected by single-shot LC-MS/MS. However, successful phosphoproteomics experiments still rely on efficient phosphopeptide enrichment from a tryptic digest prior to LC-MS/MS analysis.

## Aims

Performance assessment of single-shot LC-MS/MS label-free phosphoproteomics using TiOx-based phosphopeptide enrichment and application to a panel of 8 colorectal cancer cell lines representing 3 CRC subtypes.

## Approach

Our preferred method for global phosphopeptide enrichment is based on titanium dioxide particles (TiOx), since TiOx does not show bias towards phosphorylated amino acid, sequence context, peptide length or hydrophobicity. To minimize binding of non-phosphorylated peptides lactic acid was used as described by Ishihama and coworkers, this method has been termed HAMMOC (hydroxyl acid modified metal affinity chromatography). We assessed the performance of HAMMOC combined with single shot label-free quantitation using a state-of-the-art Q Exactive instrument. As input we used 500 µg HCT 116 colorectal cancer (CRC) cell line digests, an amount compatible with tumor tissue needle biopsies, and performed replicate enrichments.
After method assessment we applied the same workflow to a panel of 8 CRC cell lines representing 3 CRC prognostic subtypes.



**Results**
Single-shot LC-MS/MS label-free phosphopeptide analysis of triplicate TiOx-based enrichments yields a phosphopeptide identification reproducibility of 75.8%, a depth of identification of 6014-6150 phosphopeptides and reproducibility of label-free quantification (CV) of 17.8%. Pearson correlations of replicate analyses ranged from 0.87-0.98 indicating good quantitative reproducibility. The ratio of identified phosphopeptides to non-phosphopeptides ranged from 79.0-84.4% indicating efficient phosphopeptide enrichment.

Having established a robust and reproducible global phosphopeptide enrichment procedure, the phosphorylation patterns of a panel of CRC cell lines were profiled. These CRC cell lines represent 3 recently reported CRC prognostic subtypes (CCS1, CCS2 and CCS3) identified by large-scale transcriptome analysis. To assess the baseline signaling in colorectal cancer cell lines we cultured 8 CRC cell lines in triplicate: HCT116, RKO, SW480, SW1398, Colo205, DLD1, HT29 and CaCo2. Phosphopeptides were enriched by TiOx.
In total, 12931 phosphopeptides were identified(14350 peptides identified in total, 90.4% phosphopeptide enrichment) with individual samples ranging from 7249-8231 phosphopeptides. The depth of phosphopeptide identification was slightly better than in the workflow assessment however reproducibility of identification (54.5%-63.3%) and quantification (CV 50.5%-55.2% for 3/3) was lower, indicating biological variation across cell line samples. After statistical analysis, protein-protein interaction networks, gene-ontology enrichment analysis and literature mining were performed. We focused on phosphopeptides that are differential ($p<0.05$, $|\text{fold-change}| >1.5$) between CCS1-type cell lines (chromosomal instable subtype) and CCS3-type cell lines (more aggressive, poor prognosis subtype). Differential phosphopeptides can be linked to proteins that are associated with proliferation (increased in CCS1) and proteins that are associated with epithelial-to-mesenchymal transition (EMT) (increased in CCS3). Moreover, (alternative) mRNA splicing is the top biological process term for our differential data set, and may represent an additional mechanism that underlies phenotypic differences between more (CCS3) and less (CCS1) aggressive CRC cells.

**Conclusions and outlook**
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 a substantial series of biological samples (8 cell lines, 3 biological replicates) at sample input levels compatible with clinical samples such as tumor biopsies. Our phosphoproteomics analysis of a panel of CRC cell lines warrants further studies in a larger panel and CRC tumors to confirm and extend the findings of potential subtype markers and drug targets.

**Publications**
This work has been submitted to Journal of Proteomics special issue HUPO 2014

**Acknowledgements:** This research is supported by the VUmc Cancer Center Amsterdam

# Is less enough? Scaling down protein input for phosphoproteomics based treatment selection in patients with advanced solid tumors

Mariette Labots[1], Johannes C. van der Mijn[1], Sander R. Piersma[2], Richard R. de Haas[1], Inge de Reus[2], Jaco C. Knol[2], Thang V. Pham[2], Nicole C.T. van Grieken[3], Gerrit A. Meijer[3], Henk M.W. Verheul[1], Connie R. Jiménez[2]

[1]Department of Medical Oncology, VU University Medical Center [2]OncoProteomics Laboratory, Department of Medical Oncology, VU University Medical Center, [3] Department of Pathology, VU University Medical Center

## Introduction and objectives

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 phospho-proteome. We have assessed reproducibility and tumor heterogeneity using limited input material, including needle biopsies from patients.

## Aims

- 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

Phosphopeptide immunoprecipitation (IP) using P-Tyr-1000 anti-phosphotyrosine-coated beads (PTMScan®, CST) was performed using 1, 5 and 10 mg protein from lysates of the colorectal cancer (CRC) cell line HCT116 and 3 patient-derived tumors (n=3 per protein input level). In addition, 14-gauge core needle biopsies from 3 additional tumorswere analyzed. After measurement by LC-MS/MS, MaxQuant database searching and analysis were applied for phosphopeptide identification (ID), ion-intensity-based quantification and phosphosite localization.

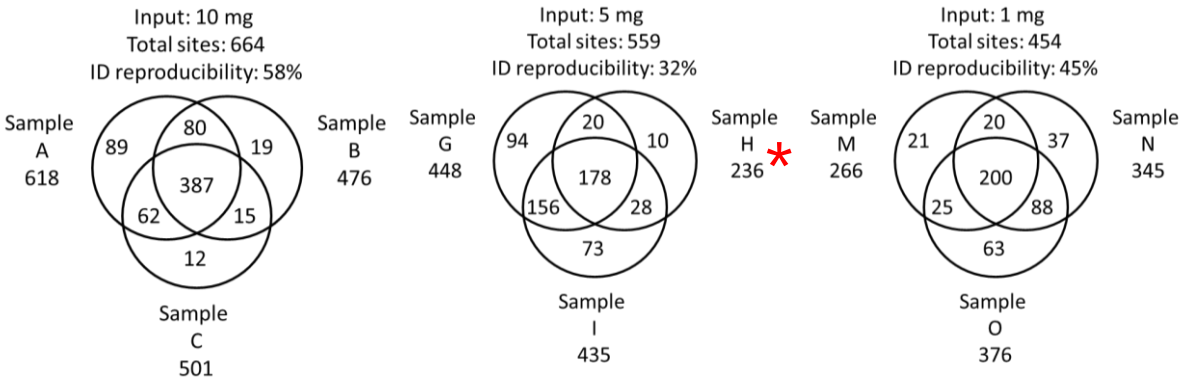



Figure. Numbers of identified pTyr containing phosphopeptides in replicate analyses using 10 mg, 5 mg and 1 mg of protein input from HCT116 cancer cell line samples. The asterisk indicates a sample where the ID propagation between samples did not function well. Overall the results indicate that phosphoproteomics is feasible down to the 1 mg protein level.

## Results and discussion

Phosphoproteomics of cell line replicates at 1, 5 and 10 mg protein input yielded a total of 454, 559 and 664 unique phosphosites, and a median number of 345 (range:266-376), 435 (236-448) and 501 (476-618) per replicate, respectively. The ID-reproducibility was 45% for 1 mg and 58% for 10 mg protein replicates. In tissue, a median total of 622 (427-754) unique phosphosites were identified per tumor, with a median number of 190 (91-353), 404 (199-512) and 546 (252-638) per sample using 1, 5 and 10 mg protein, respectively. Detected tyrosine-phosphorylated protein kinases include EGFR, SRC, FAK and ERK and many others (~ 80 different protein kinases).

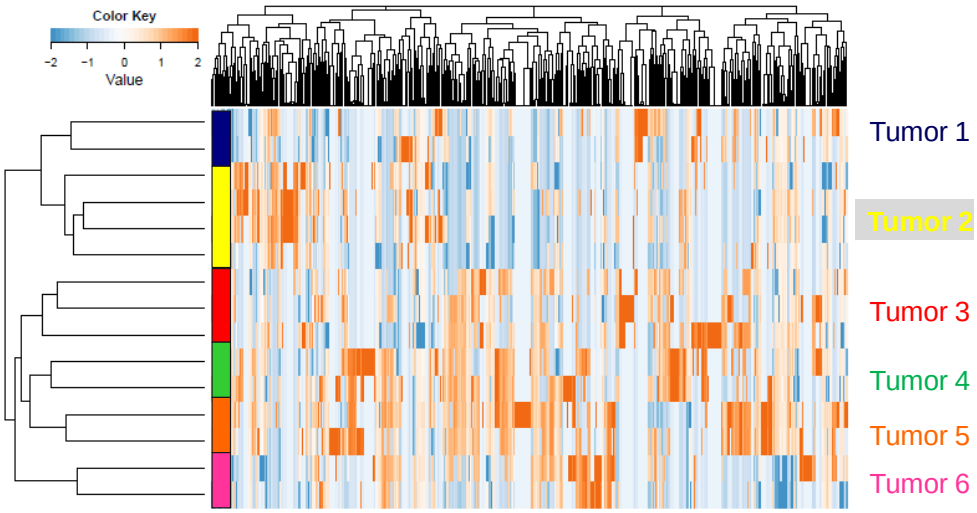



Figure 2. Unsupervised clustering based on phosphopeptide intensities shows that Intra-tumor heterogeneity is smaller than inter-tumor heterogeneity in CRC needle biopsies. Multiple 14G needle biopsies were collected from 6 CRC resection specimen and profiled using the down-scaled pTyr-based phoshoproteomics workflow at 1 mg

## 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 indicating that 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

**Outlook** Phosphotyrosine enrichment opens a window on low-abundance RTK signaling events and can be reproducibly performed at low protein input levels, enabling phosphoproteomics in clinical biopsies.

**Manuscript**

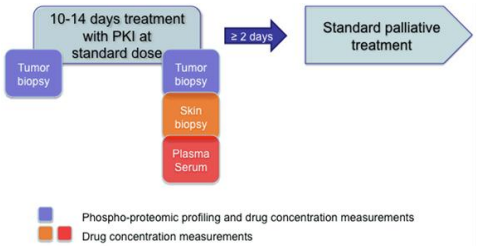
Mariette Labots*, Johannes C. van der Mijn*, Sander R. Piersma, Thang V. Pham, Richard R. de Goeij – de Haas, Inge de Reus, Henk J. Broxterman, Nicole C.T. van Grieken, Henk M.W. Verheul*, Connie R. Jiménez*. Phosphoproteomics scaled-down to tumor biopsies for future treatment selection

*Manuscript in preparation, to be submitted to Br J Cancer.*

# Tumor concentrations of kinase inhibitors in correlation with pre- vs on-treatment profiling of patient derived tumor samples

**Background** Emerging evidence shows that kinase inhibitors are selective for target receptors at low concentrations, but that these drugs act promiscuously at higher concentrations in terms of affinity to other kinases (Karaman et al., Nat Biotech 2008). Lack of selectivity may be relevant for their antitumor activity and for the development of kinase inhibitor treatment selection tools. We hypothesize that intratumoral drug concentrations will be correlated to changes of (phospho)proteomic and kinase activity profiles.
To date, literature on intratumoral kinase inhibitor concentrations is only scarcely available. One study reported a median intratumoral erlotinib concentration of 2.9 µM (range 0.3-4.9 µM ±2.1) in 4 aerodigestive tract cancer patients upon 9 days of treatment (Petty 2004). Plasma concentrations do not seem to provide a good estimation of intratumoral concentrations, as our preliminary data indicate that during sunitinib treatment of mice and cancer patients, achieved intratumoral concentrations are much higher than plasma concentrations

**Aims and approach** To correlate intra-tumoral drug concentrations to phosphoproteome profiles. To this end we will determine 1. tumor vs blood concentrations and 2. pre- to on-treatment phosphoproteomics profiling of tumor biopsies.
A pilot study in patients with advanced solid tumors (ClinicalTrials.gov identifier NCT01636908) was performed. Prior to standard palliative systemic treatment, patients were allocated to standard-dose kinase inhibitor treatment (N=5 per drug; sunitinib, sorafenib, everolimus, erlotinib, dasatinib, vemurafenib, pazopanib, regorafenib) for 10-14 days. Plasma, tumor and skin biopsies are collected within 24 hours of last dose. Sample kinase inhibitor concentrations were determined by liquid chromatography – tandem mass spectrometry (LC-MS/MS); tissue concentrations in pg/mg were converted to molarity for comparison with previously obtained preclinical sensitivity data.




| Drug cohort | Primary tumor | Tumor biopsies from patients, N = pre & on | pre *or* on | Skin biopsy N = |
|---|---|---|---|---|
| **Sunitinib** | 2 CRC, RCC, NSCLC, sarcoma | 5 | | 3 |
| **Dasatinib** | 2 CRC, esophageal, prostate, melanoma | 5 | | 4 |
| **Sorafenib** | 4 HCC, melanoma, thyroid, germ cell | 5 | 2 | 5 |
| **Erlotinib** | 2 pancreatic, esophageal, CRC, hypopharyngeal, NET | 5 | 1 | 5 |
| **Vemurafenib** | 7 Melanoma | 5 | 2 | 7 |
| **Everolimus** | 3 RCC, 3 NET | 5 | 1 | 4 |

Figure 1A.

**Results** Lack of selectivity may be relevant for their antitumor activity and for the development of kinase inhibitor treatment selection tools. To obtain more insight in their clinical mechanism of action, we aimed to determine kinase inhibitor tumor, skin and plasma concentrations in patients after 2 weeks of treatment and relate these concentrations to cell line sensitivity data. b) Tumor, skin and plasma concentrations of kinase inhibitors
Since August 2011 43 patients were screened for inclusion in the trial [Figure A]. 77% (33/43) of included and 87% (33/38) of actually treated patients are evaluable for the primary endpoint of this study. So far, inclusion of 6 cohorts (sunitinib, sorafenib, erlotinib, dasatinib, everolimus and vemurafenib), in which 5 patients completed pre- and on-treatment tumor biopsies, has been completed [Figure A]. Preliminary drug concentration results for tumor tissue, skin and plasma/serum are available; on average, skin concentrations were 2x lower than tumor concentrations. Sorafenib and erlotinib plasma concentrations were in the range of tumor concentrations while sunitinib and dasatinib plasma concentrations were at least 14-fold lower than in tumors [Figure B and C].

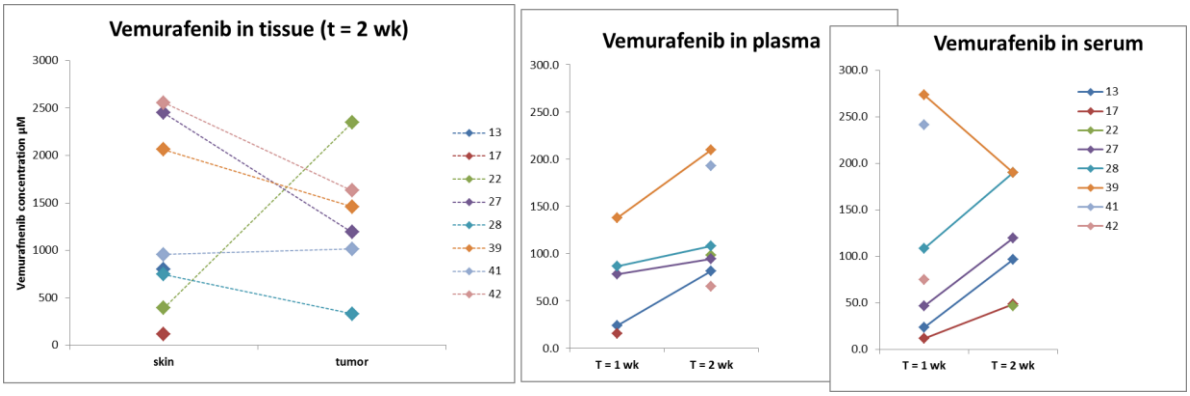



Figure 1B.

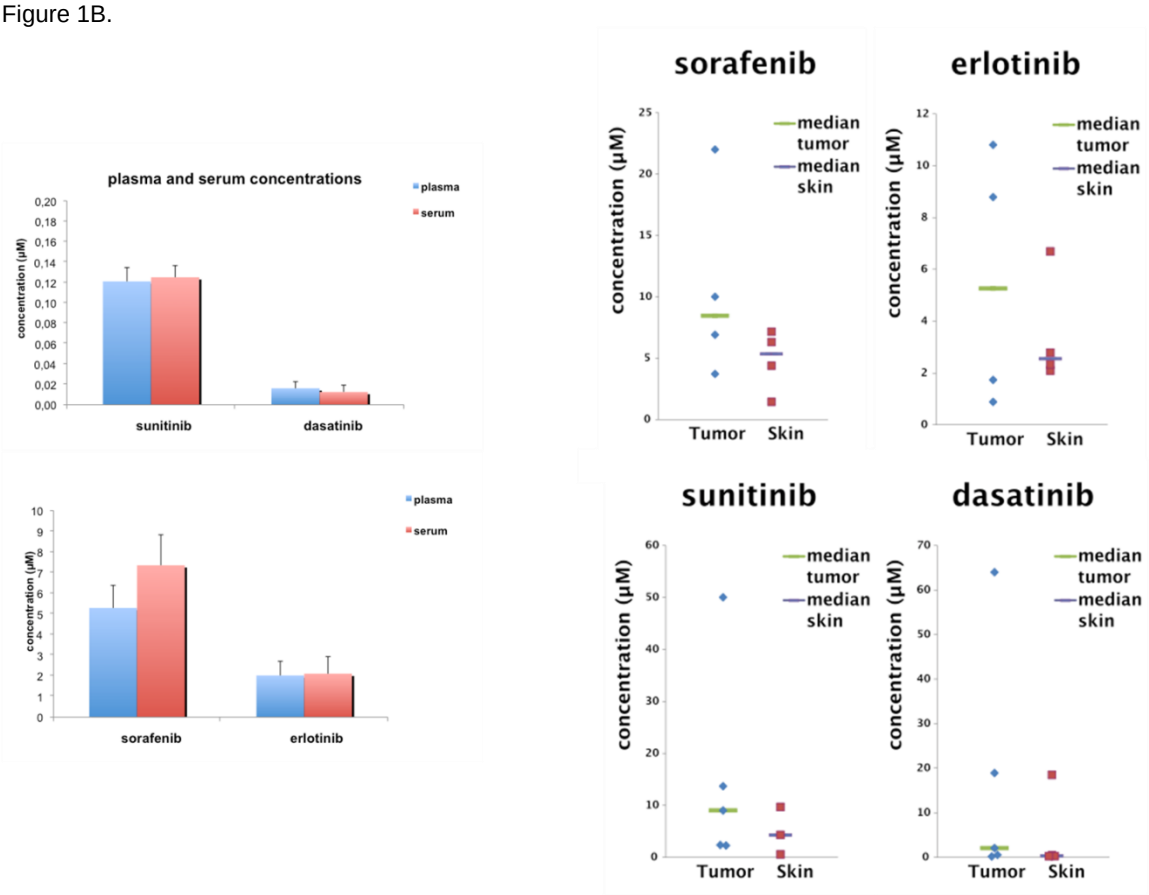



Figure 1C

**Conclusions and outlook**

Kinase inhibitor tumor concentrations may vary considerably from plasma concentrations, but are in the IC50 range of cancer cells in vitro (IC50-data not discussed in detail here). These results are indicative for the inhibitory concentrations of protein kinase inhibitors in patient tumors and should be considered for the development of individualized treatment strategies.

Currently, pre- to on-treatment phosphoproteomics analyses are being performed in tumor biopsies obtained from patients undergoing treatment with kinase inhibitors. We expect that results from these analyses as well as our patient-centered approach in general will provide us with unique and clinically applicable data in the coming years.

# Stool proteomics reveals novel candidate biomarkers for colorectal cancer screening

Linda JW Bosch[1*], Meike de Wit[2*], Gideon Oudgenoeg[2], Annemieke C Hiemstra[1], Sandra Mongera[1], Sander R Piersma[2], Thang V Pham[2], Jochim S Terhaar sive Droste[3], Frank A Oort[3], Sietze T van Turenhout[3], Ilhame Ben Larbi[3], Chris JJ Mulder[3], Beatriz Carvalho[1], Remond JA Fijneman[1], Connie R Jimenez[2#] Gerrit A Meijer[1#] **These authors have contributed equally to this work #Shared senior authors*

[1]Department of Pathology, [2]Medical Oncology and [3]Gastroenterology and Hepatology, VU University Medical Center, Amsterdam, The Netherlands

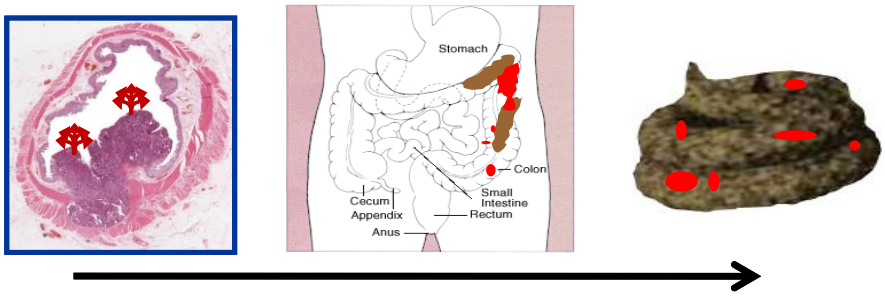
**Background**

Secondary prevention of colorectal cancer (CRC) through early detection by (population-wide) screening programs can save many lives. The widely used immunochemical fecal occult blood test (fecal immunochemical test; FIT) is based on the detection of small traces of the blood protein hemoglobin and is used to test for the putative presence of a neoplastic lesion in a non-invasive manner. Although beneficial in its current format, the FIT characteristics (sensitivity and specificity) leave room for improvement, emphasizing the need for novel tumor-specific biomarkers.

**Aim and approach**

The aim of the present study was to identify protein biomarkers for stool testing that perform better than hemoglobin, or complement hemoglobin, in order to increase diagnostic accuracy for the early detection of CRC.

For biomarker discovery, stool samples were obtained from 10 subjects without any signs of colorectal neoplasia (controls) and from 12 CRC patients (cases), as determined by colonoscopy and subsequently confirmed by histopathological evaluation in the CRC cases. Findings were validated by mass spectrometry (Q-Exactive) in an independent series of 292 stool samples obtained from control subjects (n=109) and subjects with adenomas (n=55), advanced adenomas (n=53), or CRCs (n=75). Proteins were isolated and subsequently analyzed by in-depth proteomics using gel electrophoresis, peptide extraction and nano Liquid Chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS). Spectral counts mapped to known peptides were used to quantify presence of human proteins. Quantities of proteins in samples from cases and controls were compared using the beta-binominal test.




Stool as direct link to the colon

**Results**

In total 830 human proteins were identified and levels for 221 of these differed significantly between stool samples from CRC patients and control subjects. Of these, 134 proteins were significantly enriched in CRC. Unsupervised hierarchical cluster analysis using all 830 proteins as input, revealed two clusters, one containing nine CRC stool samples and the other containing all ten control stool samples together with three CRC stool samples. In total 830 human proteins were identified in the discovery set, of which 134 were significantly enriched in CRC.

Analysis of the validation stool samples by single-shot nanoLC-MS/MS indicated that more than half of these markers are significantly more abundant in CRC samples compared to controls. A subset of these markers has more discrimination power as compared to the gold standard protein hemoglobin and several of these are complementary.

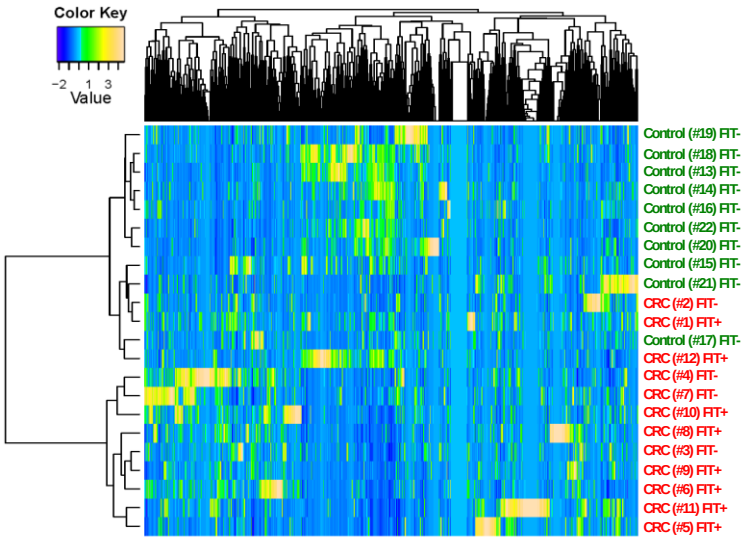



Fig. 1 Unsupervised analysis using all human stool proteins in the discovery set reveals distinct protein profiles associated with CRC cases and controls.

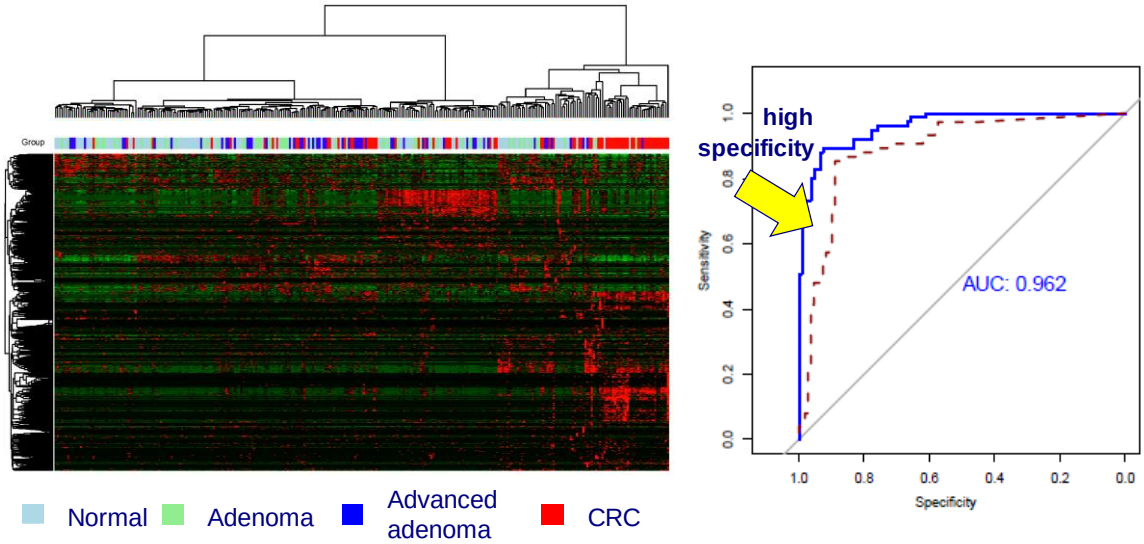



Fig. 2 Unsupervised analysis using all human stool proteins in the validation set of 292 samples (left). Right panel: example performance of one of the validated stool markers (solid blue line) relative to hemoglobin (dotted line).

**Conclusions and outlook**

Proteome profiling on stool revealed 134 proteins significantly enriched in CRC compared to control stool samples and about half of these were validated in an independent set of 292 stool samples. The most promising validated stool markers are tested in FIT samples using immunochemical assays. The first pilots show promising results.

*This research was supported by the VUmc Cancer Center Amsterdam and CTMM-DeCoDe and CTMM CRC-Bioscreen.*

*This work was presented many meetings including the AACR2012 meeting.*

# Proteomic Profiling of Colorectal Adenoma-to-Carcinoma Progression on FFPE Material

Meike de Wit1, Sofia Koustoulidou1, Thang V. Pham1, Sander R. Piersma1, Sandra Mongera2, Remond J.A .Fijneman2, Gerrit A. Meijer2, Beatriz Carvalho2, Connie R. Jimenez1.

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**Background:** Colorectal cancer (CRC) develops in a multi-step manner from a pre-malignant adenoma into a carcinoma. Only 5% of these pre-malignant adenomas will progress to cancer [1], a process mainly driven by genomic aberrations of which 85% is chromosomal instability (CIN) and 15% microsatellite instability (MSI). More specifically, gains in chromosome 20q, 13q and 8q are strongly associated with advanced lesions including adenomas. This led to the hypothesis that adenomas which have acquired these aberrations may progress to malignancy; these adenomas are therefore classified as high-risk adenomas. Detection of colorectal neoplasia in an early stage has the most impact on cancer incidence and mortality; therefore, biomarkers that can discriminate the high-risk adenomas from low risk or carcinomas would be of great clinical value. Using previously characterized formalin fixated paraffin embedded (FFPE) tissue, we selected high-risk and low-risk adenomas for proteomic profiling.

**Aim:** Firstly, to identify protein biomarkers that distinguish low-risk adenomas from high-risk adenomas or high-risk adenomas from carcinomas. Secondly, to gain insight in the molecular changes that occur during adenoma-to-carcinoma progression on the protein level.

**Approach:** 10 low-risk adenomas, 9 high-risk adenomas, 10 CIN carcinomas and 7 MSI carcinomas were selected from the tissue archive of the department of Pathology at the VU University Medical Centre (Amsterdam, The Netherlands). FFPE samples were sliced in a microtome (Five sections of 10µm each) and examined by a pathologist to confirm a tumor content of at least 70% cancer cells. Following deparaffinization and staining with haematoxylin the slides were then manually microdissected with a scalpel and lysed in NuPAGE® LDS Sample Buffer with 0.1M DTT at 99oC for 1h. Approximately 10µl of buffer was used per milligram of tissue. These protein extracts were subjected to in-depth proteome profiling by a GeLC-MS/MS workflow. Quantitative comparisons were based on label-free spectral counting, p-values were calculated using the paired Beta-Binomial test, and the threshold for significance was set to $p<0.05$.

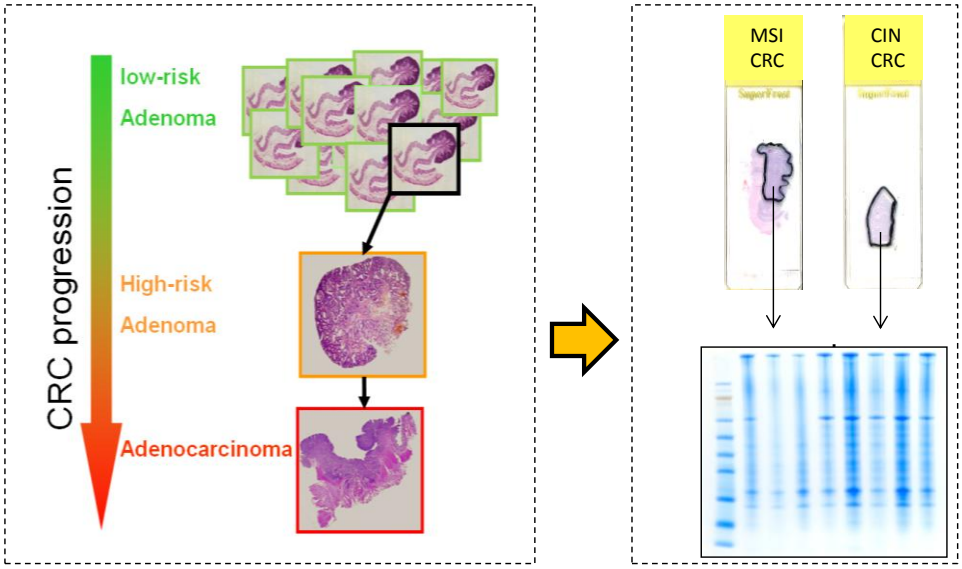


Fig. 1. Colorectal cancer (CRC) develops from an adenoma, 5% of which will progress to cancer. Adenomas are classified as high-risk adenomas when gains in chromosome 20q, 13q and 8q are present. Samples in cluded in the proteomics analysis:10 low-risk adenomas, 9 high-risk adenomas, 10 CIN carcinomas and 7 MSI carcinomas.

**Results:** A total of 3670 proteins were identified in these 36 samples. Of these, 2879 (78%) were identified in all sample types. Furthermore 87% of proteins overlapped between MSI CRC samples and CIN CRC samples and 91% between high-risk adenomas and CIN CRC samples, suggesting that the biological variation between CIN and MSI is higher than between (CIN) adenomas and CRC. Unsupervised hierarchical cluster analysis using all 3670 proteins as input, revealed three main clusters, one containing only CRC samples (N=7), one enriched for adenoma samples (N=12) and with only a few carcinomas (N=2) and a third cluster with a mixture of adenomas (N=7) and carcinomas (N=8). Beta-binomial statistics revealed 126 proteins that were significantly more present in high-risk versus low-risk adenomas, 147 proteins were significantly more present in carcinomas versus adenomas and 134 proteins were significantly enriched in high-risk adenomas and carcinomas combined compared to low-risk adenomas.

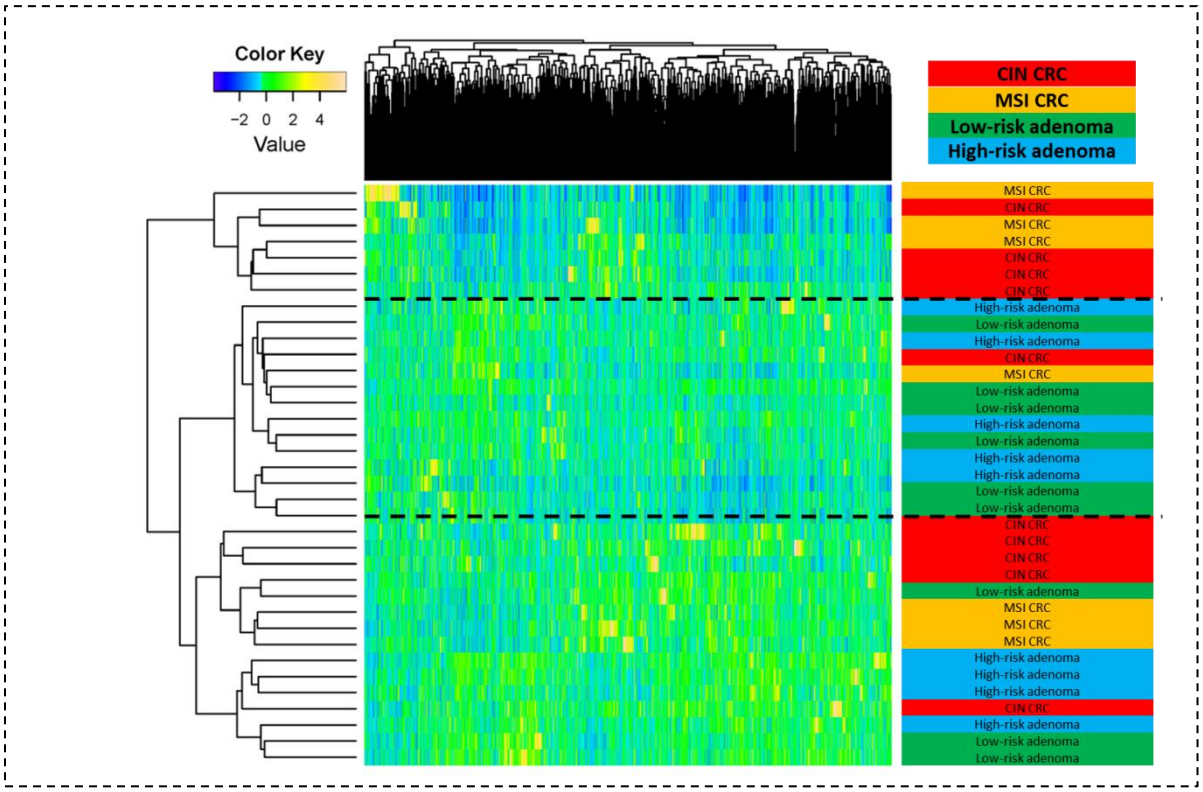


Fig 2. Unsupervised cluster analysis using all proteins identified in all FFPE samples. 3670 proteins were identified, 2879 (78%) were identified in all sample types. 126 proteins were significantly more present in high-risk versus low-risk adenomas,147 proteins were significantly more present in CRC versus adenomas and 134 proteins were enriched in high-risk adenomas and CRC versus low-risk adenomas.

**Conclusion and outlook:** We conclude that FFPE proteome profiling of human colon tissue is a powerful strategy to discover novel candidate biomarkers for CRC. We identified 3670 proteins that include several proteins that potentially could be used for discrimination between high-risk lesions and low-risk lesions. Further data analysis is currently ongoing to investigate altered biological processes and to select the most promising candidate protein markers for follow-up.

**Publication iculding CRC FFPE proteomics**

Piersma SR, Warmoes MO, de Wit M, de Reus I, Knol JC, Jiménez CR. Whole gel processing procedure for GeLC-MS/MS based proteomics. Proteome Sci. 2013 Apr 23;11(1):17.

# Colorectal cancer candidate biomarkers identified by tissue secretome proteome profiling

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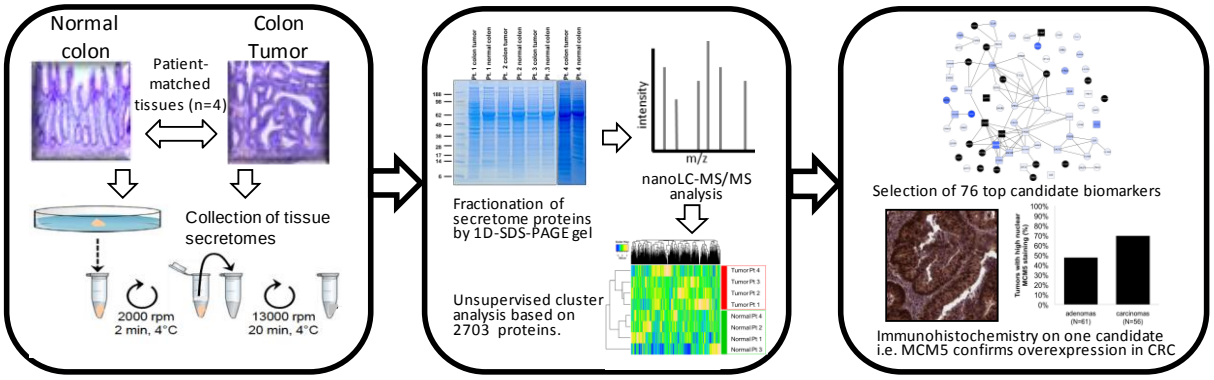
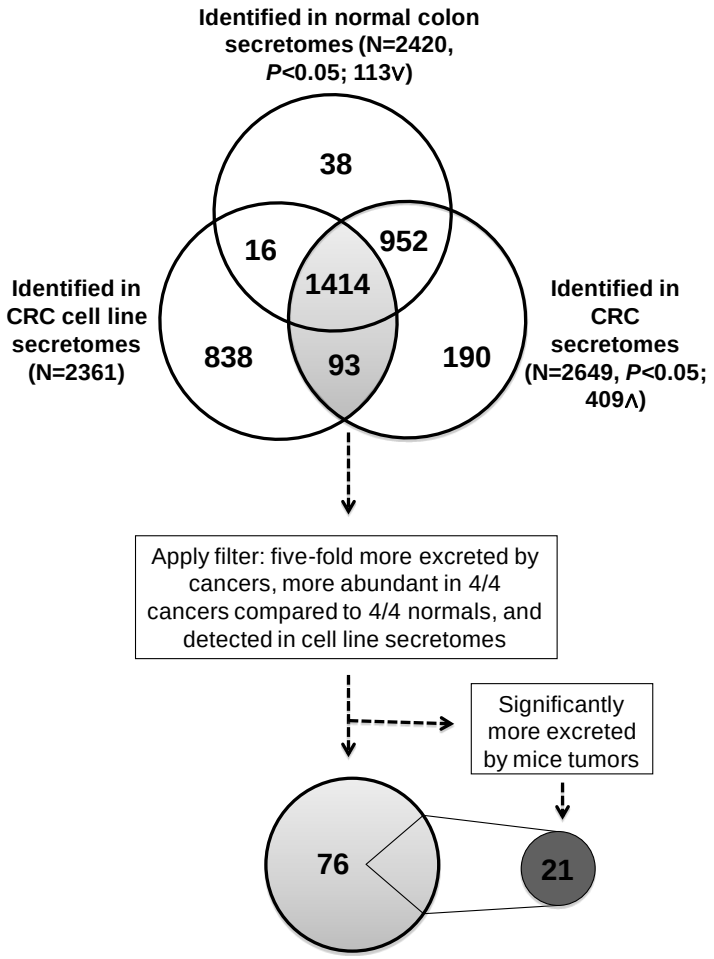
**Background:** Colorectal cancer (CRC) is a major health problem. Biomarkers associated with molecular changes in cancer cells can aid early detection, diagnosis, prognosis, therapy selection, and disease monitoring. Tumor tissue secretomes are a rich source of candidate biomarkers. The aim of this study was to identify CRC protein biomarkers in tissue secretomes.




**Approach**: Proximal fluids or secretomes are a rich source of candidate biomarkers as they contain high concentrations of tissue-derived proteins. In this study we analyzed secretomes of tissues from the FabplCre;Apc(15lox/+) mouse model that represents early-stage development of human sporadic CRC, secretomes of four pairs of fresh human CRC tissue and patient-matched normal colon tissue samples, and secretomes of five CRC cell lines by GeLC-MS/MS. Data analysis was based on label-free spectral counting, Ingenuity Pathway Analysis, Secretome/SignalP, STRING and Cytoscape.

**Results:** 2703 proteins were identified in the human tissue secretomes, of which 409 proteins were significantly more present in CRC samples than in controls. Biomarker selection of 76 candidates was based on consistent and abundant over-representation in cancer- compared to control-secretomes, and presumed neoplastic origin based upon overlap analysis with the cell line dataset. These proteins are linked to specific molecular functions: 1; DNA replication, 2; RNA metabolic process, 3; RNA splicing, 4; cytoskeleton polarity, 5; extracellular structure organization, 6; mitochondrial fragmentation. Furthermore, overlap analysis with the proteins identified in the mouse tissues revealed 21 biomarkers suited for early detection of CRC including a cluster of DNA replication linked MCM proteins.
Immunohistochemistry confirmed overexpression in CRC of one candidate marker (MCM5). By ELISA analysis we verified that protein levels of CHI3L1 were significantly increased in sera from patients with adenomas and advanced adenomas compared with control individuals, in contrast to the CRC biomarker CEA.




**Conclusion:** We identified promising biomarker proteins that have potential to be used for development of blood- or stool-based assays to support clinical management of CRC. Further studies are required to validate clinical applicability of these candidate biomarkers.

*This research is supported by an Aegon International Scholarship in Oncology, and by the VUmc-Cancer Center Amsterdam.*

**Publications**

de Wit M, Kant H, Piersma SR, Pham TV, Mongera S, van Berkel MP, Boven E, Pontén F, Meijer GA, Jimenez CR, Fijneman RJ. Colorectal cancer candidate biomarkers identified by tissue secretome proteome profiling. J Proteomics. 2014 Mar 17;99:26-39.

Schaaij-Visser TB, de Wit M, Lam SW, Jiménez CR. The cancer secretome, current status and opportunities in the lung, breast and colorectal cancer context. Biochim Biophys Acta. 2013 Nov;1834(11):2242-58.

de Wit M, Fijneman RJ, Verheul HM, Meijer GA, Jimenez CR. Proteomics in colorectal cancer translational research: biomarker discovery for clinical applications. Clin Biochem. 2013 Apr;46(6):466-79.

Fijneman RJ, de Wit M, Pourghiasian M, Piersma SR, Pham TV, Warmoes MO, Lavaei M, Piso C, Smit F, Delis-van Diemen PM, van Turenhout ST, Terhaar sive Droste JS, Mulder CJ, Blankenstein MA, Robanus-Maandag EC, Smits R, Fodde R, van Hinsbergh VW, Meijer GA, Jimenez CR. Proximal fluid proteome profiling of mouse colon tumors reveals biomarkers for early diagnosis of human colorectal cancer.Clin Cancer Res. 2012 May 1;18(9):2613-24.

*Colorectal cancer proteomics, sub-project 4*

# Chromatin-associated proteins in colorectal adenoma and carcinoma tissues

Jaco C Knol[1], Jakob Albrethsen[1], Thang V. Pham[1], Sander R. Piersma[1], Sandra Mongera[2], Beatriz Carvalho[2], Meike de Wit[2], Remond JA Fijneman[2], Gerrit A Meijer[2], and Connie R Jimenez[1]

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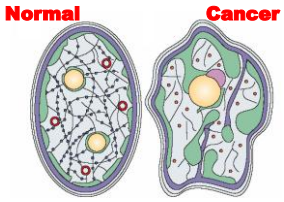
**Background** Altered nuclear and genomic structure and function are hallmarks of cancer cells. Research into nuclear proteins in human tissues could uncover novel molecular processes in cancer. Here, we examine biochemical tissue fractions containing chromatin-binding (CB) proteins in the context of colorectal cancer (CRC) progression.




**Method** CB protein-containing fractions were biochemically extracted from human colorectal tissues, including carcinomas with chromosomal instability (CIN), carcinomas with microsatellite instability (MIN), and adenomas. The CB proteins were subjected to label-free LC-MS/MS and the data were analyzed by bioinformatics.

**Results** Over 1700 proteins were identified in the CB fraction from colonic tissues, including 938 proteins associated with nuclear annotation. Of the latter, 169 proteins were differential between adenomas and carcinomas. In this adenoma-versus-carcinoma comparison, apart from specific changes in components of the splicing and protein translational machineries, we also identified significant changes in several proteins associated with chromatin-directed functions. Furthermore, several key cell cycle proteins as well as those involved in cellular stress were increased, whereas specific components of chromosome segregation and DNA recombination/repair systems were decreased.

**Conclusions** Our study identifies proteomic changes at the subnuclear level that are associated with adenomas and CRC that may be further investigated.

*This research is supported by the VUmc-Cancer Center Amsterdam.*

**Publication**

Knol JC, de Wit M, Albrethsen J, Piersma SR, Pham TV, Mongera S, Carvalho B, Fijneman RJ, Meijer GA, Jiménez CR. Proteomics of differential extraction fractions enriched for chromatin-binding proteins from colon adenoma and carcinoma tissues. Biochim Biophys Acta. 2014 May;1844(5):1034-43.

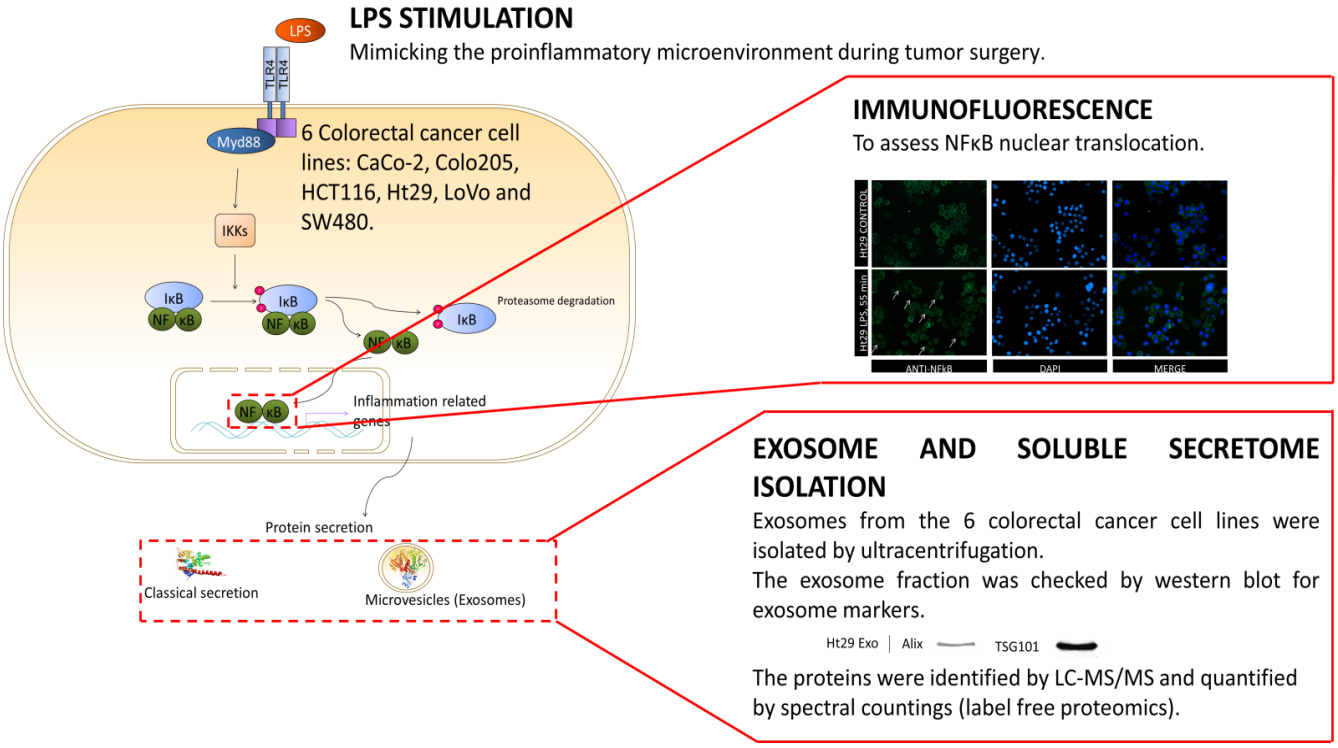
## Proteomic analysis of LPS-modified secretion in six colorectal cancer cell lines

García de Durango CR[1], De Wit M[2], Piersma SR[2], Pham TV[2], Pérez-Gordo M[1], Vidal-Vanaclocha F[1], Fijneman RJ[3], Jimenez CR[3].

[1]Institute of Molecular Applied Medicine (IMMA), CEU San Pablo School of Medicine, Madrid, Spain; [2] OncoProteomics Laboratory of the Department of Medical Oncology, VU University Medical Center; [3]Tumor Profiling Unit of the Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands

**Background** Colorectal cancer (CRC) continues to be among the four major cancers in developed countries (lung, colorectum, breast, and prostate) and has increasing numbers in some non-developed ones. It represents approximately 10% of deaths because of cancer. Surgery is the basis of therapy for colorectal cancer. Nevertheless, it has been seen that a high percentage of patients that did not have hepatic metastasis at the moment of the diagnosis will develop it within 5 years. There are evidences of the contribution of the primary tumor surgical removal to the metastatic process. This may be due to inflammatory mediator release in response to surgical trauma, which can promote the metastatic ability of circulating cancer cells.
During surgery, bacterial products such as lipopolysaccharide (LPS) from commensal bacteria translocate across the bowel wall and even reach systemic circulation. LPS is a potent inducer of inflammatory response through Toll like receptor-4 (TLR4) recognition, which implies Nuclear Factor-κB (NFkB) pathway: the recognition leads to a phosphorilation and subsequent degradation of IκB, a molecule that keeps the transcription factor NFkB cytoplasmic-inactive. Then, NFκB is free to translocate to the nucleus to initiate the transcription of inflammation-related genes.

**Aim** To identify proteins that are released by CRC cancer cells in response to LPS stimulation, via classical secretion or via extracellular vesicles.



**Approach** In order to mimic the microenvironment, we used an *in vitro* model we stimulated six colorectal cancer cell lines with LPS and studied the whole set of secreted proteins, defined as secretome (Fig. 1). This set spans several mechanisms of protein release: classical secretion, subset here termed Soluble Secretome (SS), cell death, ectodomain shedding of transmembrane proteins and release of endosome-derived extracellular vesicles termed exosomes (EXO). Exosomes are thought to have an important role in intercellular communication and immune response.
Here we first isolated EXO by ultracentrifugation and assessed exosome markers by western blot. We also applied high resolution MS/MS-based proteomics to analyze both the SS and EXO fraction from six CRC cell lines treated with LPS. We also assessed NFkB nuclear translocation by image analysis of immunofluorescenct staining of NFkB p65-subunit.

| **ALL GROUPS ANALYSIS** | | **SOLUBLE SECRETOME** | **EXOSOME** |
|---|---|---|---|
| Total Protein Ids | | 4987 | 4912 |
| Upregulated | p-value<0,05 | 33 | 35 |
| Downregulated | p-value<0,05 | 26 | 45 |

**Results** First we verified the whether LPS treatment affects NFkB sub-cellular localization. NFkB was found to translocate to the nucleus in waves, with different dynamics depending on the cell line.
Proteomics was used to study the repertoire of released proteins in the SS and EXO fractions of the secretome. The table below details the numbers of identified proteins in the different fractions. Regarding the regulated proteins between LPS-stimulated and control cell lines ($p < 0.05$), SS of treated cells was significantly enriched in ribosome structural proteins and chemokine and cytokine activity. SS of non-treated cells was enriched in protein binding and dimerization-related proteins.
As to the EXO fractions, all regulated proteins were vesicle-related. The upregulated proteins in EXO fraction of LPS-treated cells were related to cytoskeleton processes, cell motility and apoptosis regulation. The proteins enriched in the exosomes of non-treated cells were cytoskeletal as well as contractile proteins and carbohydrate metabolism and protein binding related.

**Conclusions and outlook** Despite differences due to cell line heterogeneity, a first overall analysis suggests an interesting subset of proteins that were consistently enriched in SS after LPS treatment related to inflammation and cytoskeletal binding, which confirms previous results seen by ELISA (Luminex® assay) as well as 2D-gel electrophoresis. EXO fractions of LPS-treated cell contained different sets of proteins associated with cell structure and motility functions among others.
Further research is needed to confirm these findings and in order to better understand the effect of the bacterial endotoxin on CRC cells.

# Cell surface proteomics identifies glucose transporter type 1 and prion protein as candidate biomarkers for colorectal adenoma-to-carcinoma progression

Meike de Wit[1,2], Connie R. Jimenez[2], Beatriz Carvalho[1], Jeroen A.M. Belien[1], Pien M. Delis-van Diemen[1], Sandra Mongera[1], Sander R. Piersma[2], Maindad Vikas[4], Sanjay Navani[4] , Fredrik Pontén[3], Gerrit A. Meijer[1], Remond J.A. Fijneman[1].

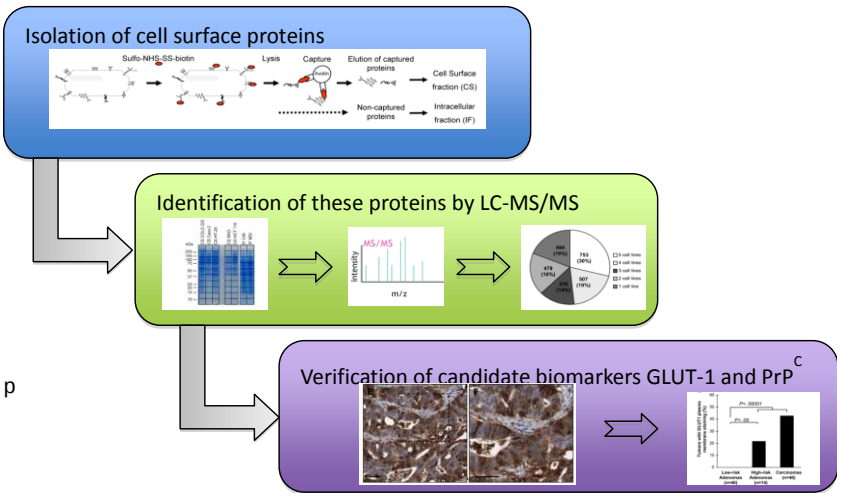
VU University Medical Center, Department of [1]Pathology (Tumor Profiling Unit) and [2]Medical Oncology (OncoProteomics Laboratory), De Boelelaan 1117, 1081HV Amsterdam, The Netherlands. [3]Uppsala University, The Rudbeck Laboratory, Department of Genetics and Pathology, SE-751 85 Uppsala, Sweden. [4]Lab Surg Path, Mumbai, India.

**Background and Objective:** Early detection of colon adenomas at high risk of progression and early stage colorectal cancer (CRC) is an effective approach to reduce CRC mortality rates. Current screening methods lack specificity as they detect many adenomas that will never progress to CRC. We aimed to identify cell surface protein biomarkers with extracellular domains that could be targeted for molecular imaging and discriminate low-risk adenomas and normal colon from high-risk adenomas and CRC.

**Approach:** Cell surface proteins of five CRC cell lines were biotinylated, isolated, and analysed by in-depth proteomics using gel -nanoLC-MS/MS. Differential expression in adenomas and CRCs was based on mRNA expression and verified by immunohistochemical staining of tissue microarrays.

**Results:** In total 2609 proteins were identified in the cell surface fractions. Of these, 44 proteins were selected as promising cell surface candidate biomarkers for adenoma-to-carcinoma progression based on the following criteria: protein identification in at least four out of five cell lines; a predicted (trans)membrane location; and increased mRNA expression in CRCs compared to adenomas. Increased protein expression in high-risk adenomas and CRCs compared to low-risk adenomas was confirmed by immunohistochemistry for the glucose transporter type 1 (GLUT1; gene symbol *SLC2A1*; $P < .00001$) and prion protein ($PrP^C$; gene symbol *PRNP*; $P < .005$).

**Conclusion:** This study revealed GLUT1, $PrP^C$, and 42 other cell surface candidate biomarkers for adenoma-to-carcinoma progression that could potentially serve as targets for emerging molecular imaging modalities like optical imaging, $^{19}$F-MRI and PET.

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*This research was supported by Phillips research and the VUmc Cancer Center Amsterdam*

**Reference** De Wit M, Jimenez CR, Carvalho B, Belien JA, Delis-van Diemen PM, Mongera S, Piersma SR, Vikas M, Navani S, Pontén F, Meijer GA, Fijneman RJ. Cell surface proteomics identifies glucose transporter type 1 and prion protein as candidate biomarkers for colorectal adenoma-to-carcinoma progression. Gut. 2012 Jun;61(6): 855-64.

# Colorectal cancer tissue spheroids: an in-depth proteomics analysis

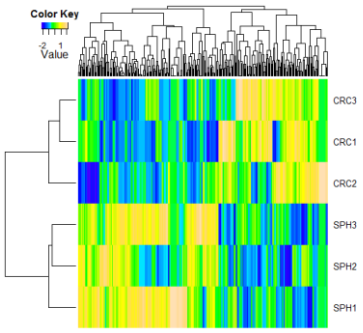
Uros Rajcevic[1], Jaco C. Knol[2], Sander Piersma[2], Sébastien Bougnaud[1], Thang V. Pham[2], Eirik Sundlisaeter[3], PerØyvind Enger[3], Rolf Bjerkvig[1,3], Simone P. Niclou[1] and Connie R. Jimenez[2]

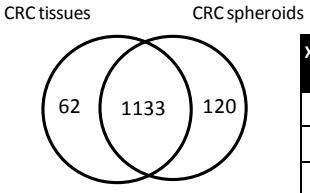
[1]NorLux Neuro-Oncology, CRP-Santé, Luxembourg, [2]OncoProteomics Laboratory, Dept. Medical Oncology, VU University Medical Center, Amsterdam; [3]Department of Biomedicine, University of Bergen, Bergen, Norway

**Background**. Organotypic tumor spheroids, a 3D in vitro model derived from patient tumor material, preserve tissue heterogeneity and retain structural tissue elements, thus replicating the in vivo tumor more closely than commonly used 2D and 3D cell line models. Such structures harbour tumorigenic cells, as revealed by xenograft implantation studies in animal models and maintain the genetic makeup of the original tumor material. The aim of our work was a morphological and proteomic characterization of organotypic spheroids derived from colorectal cancer tissue in order to get insight into their composition and associated biology.

**Aim and approach:** To characterize the spheroid-specific protein repertoire. To this end, we have performed an in-depth differential proteomics analysis of CRC tissue spheroids versus the original tissue resection material from which they were derived.

**Results**. Morphological analysis showed that spheroids were of about 250 µm in size and varied in structure, while the spheroid cells differed in shape and size and were tightly packed together by desmosomes and tight junctions. Our proteomic data revealed significant alterations in protein expression in organotypic tumor spheroids cultured as primary explants compared to primary colorectal cancer tissue. Components underlying cellular and tissue architecture were changed; nuclear DNA/ chromatin maintenance systems were up-regulated, whereas various mitochondrial components were down-regulated in spheroids. Most interestingly, the mesenchymal cells appear to be substantial component in such cellular assemblies. Thus the observed changes may partly occur in this cellular compartment. Finally, in the proteomics analysis stem cell-like characteristics were observed within the spheroid cellular assembly, reflected by accumulation of Alcam, Ctnnb1, Aldh1, Gpx2, and CD166. These findings were underlined by IHC analysis of Ctnnb1, CD24 and CD44, therefore warranting closer investigation of the tumorigenic compartment in this 3D culture model for tumor tissue.




| X/3 Pairs; X = | Fold Change | # Prots UP | # Prots DOWN |
|---|---|---|---|
| 3 | >2 | 70 | 35 |
| 3 | >1.5 | 109 | 65 |
| 2 | >2 | 296 | 193 |
| 2 | >1.5 | 385 | 284 |

**Conclusions** Our analysis of organotypic CRC tumor spheroids has identified biological processes associated with a mixture of cell types and states, including protein markers for mesenchymal and stem-like cells. This 3D tumor model in which tumor heterogeneity is preserved may represent an advantageous model system to investigate novel therapeutic approaches.

**Publication**

Rajcevic U, Knol JC, Piersma S, Bougnaud S, Fack F, Sundlisaeter E, Søndenaa K, Myklebust R, Pham TV, Niclou SP, Jiménez CR. Colorectal cancer derived organotypic spheroids maintain essential tissue characteristics but adapt their metabolism in culture. Proteome Sci. 2014 Jul 11;12:39.

*This research was supported by the Fonds National de la Recherche (FNR) of Luxembourg and the VUmc Cancer Center Amsterdam*

## The Secretome of Colon Cancer Stem Cells Contains Drug-metabolizing Enzymes

Emmink BL[1], Van Houdt WJ[1], Pham TV[2], Piersma SR[2], Verheem A[1], Fratantoni SA[2], Borel Rinkes IH[1], Jimenez CR[2], Kranenburg O[1].

[1] *University Medical Center Utrecht, Department of Surgery, Utrecht;* [2]OncoProteomics Laboratory*, VU University Medical Center, Amsterdam, The Netherlands*

**Background** Cancer stem cells (CSC's) are potential drug-resistant seeds of post-treatment tumor recurrence. However, the relationship between CSC's and drug resistance is incompletely understood.

**Aim and approach** To explore the relationship between CSC's and drug resistance by analyzing the secreted proteins from paired cultures of tumorigenic CSC-like cells and isogenic non-tumorigenic differentiated tumor cells isolated from three metastasized colon tumors.

**Results** Mass spectrometry-based analysis of conditioned media identified 156 proteins enriched in the CSC secretome. Ingenuity analysis revealed that out of all 'Molecular and Cellular Functions' categories, 'Cell Death' was most significantly associated with the CSC secretome. Interestingly, 37 of the 43 proteins in this category are anti-apoptotic, possibly reflecting increased CSC survival capacity. Interestingly, genome maintenance networks were largely absent from the CSC secretome, but proteome maintenance networks, including those governing protein synthesis, folding, modification and processing were highly enriched. The CSC secretome is also characterized by an Nrf2-like antioxidant signature. A striking observation was that CSC secretomes are enriched in drug-metabolizing enzymes, providing a potential direct link between the CSC phenotype and chemotherapy resistance.

**Conclusions and outlook** We conclude that CSC's are characterized by extensive survival, proteome maintenance and anti-oxidant networks. In addition specific enzymes secreted by CSC's can influence the activity of specific chemotherapeutic drugs by either detoxifying or activating them.

**Publication**

Emmink BL, Verheem A, van Houdt WJ, Steller EJ, Govaert KM, Pham TV, Piersma SR, Borel Rinkes IH, Jimenez CR*, Kranenburg O*. The secretome of colon cancer stem cells contains drug-metabolizing enzymes. J Proteomics. 2013 Jul 5;91C: 84-96. *Shared senior authorship.

# Tumor-specific protein biomarkers for early detection of colorectal cancer

Malgorzata A Komor[1], Thang V Pham[2], Annemieke C Hiemstra[1], Sander R Piersma[2], Meike de Wit[2], Beatriz Carvalho[1], Gerrit A Meijer[1], Connie R Jimenez[2], Remond JA Fijneman[1]

[1]Dept. Pathology and [2]Oncoproteomics Laboratory, Dept. Medical Oncology, VUmc

**Background:** Colorectal cancer (CRC) is a major health concern in the Western world. In the Netherlands the annual incidence exceeds 13,000 and mortality is over 5,000 patients. The 5-year survival rate depends on disease stage at diagnosis. Early detection of CRC and its precursor lesions, *i.e.* adenomas, offers a realistic opportunity to reduce CRC mortality rates. The currently used fecal immunochemical test (FIT) is a non-invasive population-wide CRC screening test that detects the blood-derived protein hemoglobin upon leakage from bleeding tumors. At the molecular level, the transition from benign lesions (adenomas at low risk of progression) to screen-relevant lesions (adenomas at high risk of progression and carcinomas) is accompanied by somatic mutations in DNA and alternative splicing of mRNA transcripts. Therefore, FIT sensitivity could be further improved using tumor-specific biomarkers such as mutated or alternatively spliced protein alterations that accompany adenoma-to-carcinoma progression. At present, the sequence databases against which mass spectra are searched lack such tumor-specific protein information, while ~50% of mass spectra are not identified due to the limitations of these databases.

**Aim:** We aim to identify and validate tumor-specific protein biomarkers for early detection of CRC to improve CRC screening test sensitivity without decreasing test specificity.

**Approach:** To identify tumor-specific splice variants, the splicing machinery was knocked down in CRC cell lines followed by RNA-seq massive parallel sequencing (Illumina HiSeq). Positive controls of differential splicing were established by qRT-PCR. Next, comparative RNA-seq analysis will be performed on normal human colon tissues and adenomas at low risk of progression *versus* adenomas at high risk of progression and CRCs. Proteins will be isolated from both cell lines and human tissues and analyzed by tandem mass spectrometry. Mass spectra will be searched against a database that is supplemented with predicted tumor-specific protein sequences translated from RNA splice variants and DNA mutations. The abnormal peptides detected with the use of this protocol will be validated in a large colonoscopy controlled stool sample collection by targeted proteomics, examined in terms of sensitivity and specificity and further analyzed for complementarity to the FIT test.

**Results:** Down-modulation of the splicing factors SF3B1, U2AF1, and SRSF1 in the SW480, Caco2 and HCT116 CRC cell lines resulted in quantitative changes on mRNA level of spliced isoforms of positive control genes, as detected by qRT-PCR. RNA-seq analysis of cell line SW480 before and after down-modulation of splicing factors confirmed splicing of these positive control genes and yielded hundreds of novel splice variant candidates. Further analysis of these candidates is ongoing.

**Conclusions and outlook:** Guided by RNA-seq analysis we will identify tumor-specific protein variants that will be evaluated for their performance as screening markers for CRC in both early and advanced stages. These markers will increase the sensitivity of the population wide CRC screening test without affecting its specificity.

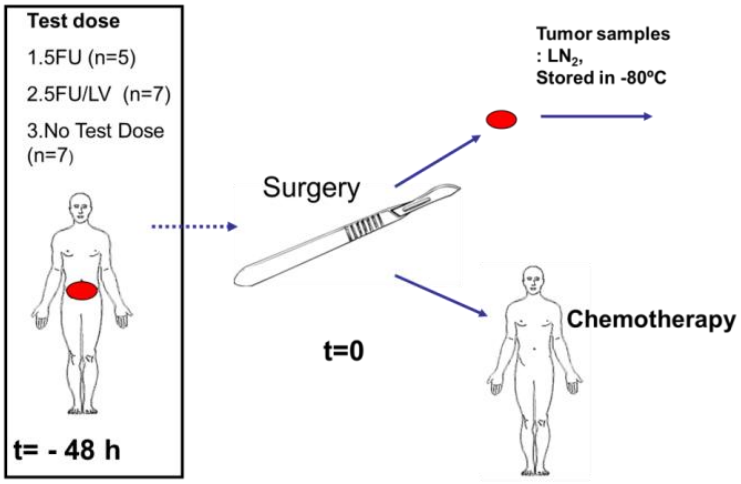
# Proteomics analysis of the effect of fluorouracil (5-FU) and 5-FU/leucovorin (LV) on colorectal cancer in patients.

E. Meijer, K. Smid , T.V. Pham, I. De Reus, S.R. Piersma, G.J. Peters, C.R. Jimenez
Department of Medical Oncology, VU University Medical Center, Amsterdam

**Background:**
5-Fluorouracil (5-FU) is a widely used chemotherapeutic drug and works mainly through the inhibition of thymidylate synthase (TS); leucovorin (LV) is often given in combination with 5-FU to enhance the TS inhibition. Although this main mechanism is known, the full mechanism remains elusive and there is a large variation in response between patients. In order to make better treatment decisions, several markers, including TS, have been investigated both in models systems and patients. Several studies on global molecular profiling of 5-FU have been performed on cell lines, but not in patients. Global protein profiling by mass spectrometry has emerged as an advantageous proteomics approach to identify and quantify thousands of proteins in small patient tissue samples.

**Aim:**
Identification of new potential mechanisms of action and potential biomarkers of 5-FU with or without LV treatment using in-depth proteomics analysis of patient colorectal cancer (CRC) tissues.

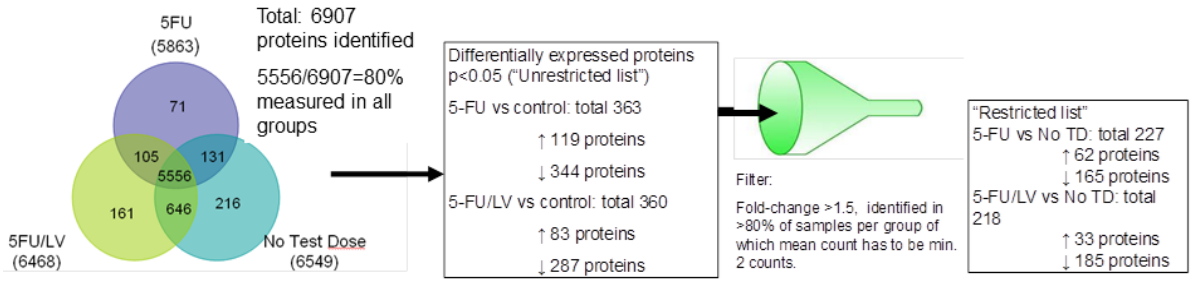
**Approach**:
Patients received approximately 48 hours before resection, a test dose of 5-FU (500 mg/m$^2$) (n=5), 5-FU with LV (500 mg/m$^2$) (n=7) or no test dose (n=7). For proteomics analysis, samples of CRC resection material were lysed in SDS sample buffer, fractionated by 1D gel electrophoresis, followed by in-gel digestion and nano-liquid chromatography coupled to tandem mass spectrometry (QExactive). The MaxQuant tool was used for protein identification and quantification by spectral counting.



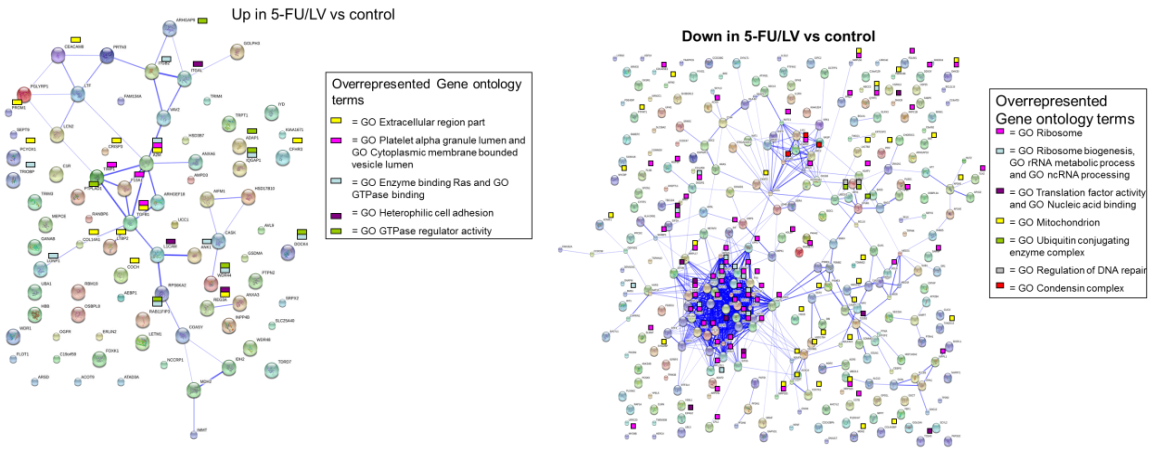

**Setup for tumor collection.** 48 Hours before resection of the tumors (red oval), patients were given a test dose of 5-FU, 5-FU/LV or no test dose. After resection, the tumor samples were put in liquid nitrogen, stored at -80ºC and patients received chemotherapy.

**Results:**
Our CRC proteome analysis identified a total of 6880 proteins and 874 proteins with altered abundance ($p< 0.05$) upon drug treatments. Our results indicate that 5-FU induces upregulation of proteins associated with the extracellular matrix, membrane vesicles, stress and immune responses and downregulates mitochondrial proteins, peroxisomes and (most profoundly) ribosomal proteins. Patients who received additional LV also displayed upregulation of extracellular matrix and vesicle/granule proteins in addition to cell adhesion proteins and showed an extensive downregulation of ribosomal proteins as well as downregulation of mitochondrial and ubiquitin proteins. In addition, there is an upregulation of the GO-term 'Extracellular region part' in both 5-FU and 5-FU/LV treated patients, when compared to the control group. Among the top upregulated proteins were isoform

NELF-D of negative elongation factor C (NELFCD), AMP deaminase 3 (AMPD) and myeloblastin (PRTN3), while neudesin (NENF), antigen KI-67 (MKI67) and HERC4 were in the top of the downregulated list.




**Protein selection and filtering.** A total of 6907 proteins were identified. After applying the beta-binominal test between the groups, there were 363 proteins differentially expressed between 5-FU and control and 360 in 5-FU/LV vs control. This list was called the 'Unrestricted list' and was used for gene ontology mining (DAVID) and for making protein-protein networks. This list was further filtered for fold change and minimum count into a 'restricted list' in order to get a list of more robust proteins.




**Protein-protein interaction networks of proteins up- or downregulated by 5-FU/LV.** The highly connected proteins seen in the downregulated proteins are mainly ribosomal proteins.

**Conclusions:**

Proteome analysis revealed strong changes in the CRC proteome upon 48 hrs of 5-FU +/- LV treatment, with regulated proteins involved in stress and immune responses, vesicular transport, protein synthesis and metabolism. A number of proteins were not yet associated to 5-FU actions. These proteins warrant further validation and may function as biomarkers for treatment decision making in the future

*Colon cancer proteomics subproject 11*

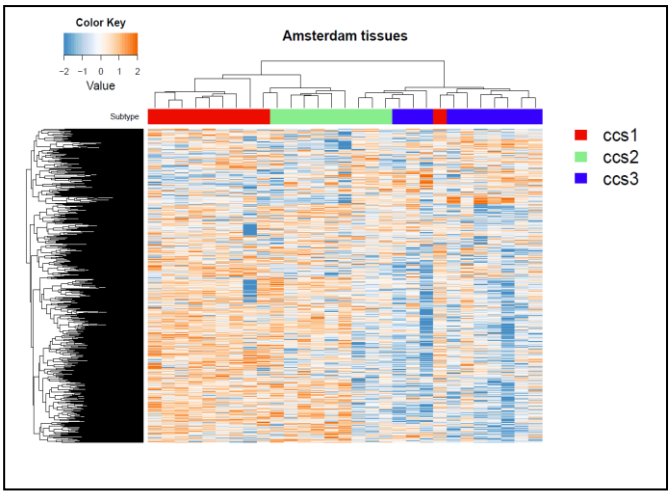
## Connection proteomics: Protein-based stratification of colon cancers

AMC, Lexor: Evelyn Fessler, Louis Vemeulen, Jan Paul Medema
VUmc, OPL: Thang V Pham, Sander R. Piersma, Connie R. Jimenez
Connection collaborators

**Background** Colorectal cancer (colon cancer) is, despite several novel therapeutic developments over the last decades, still a devastating disease with over 600.000 deaths per year worldwide. This is in part due to poor survival of patients with late stage disease where curative treatment options are limited, but even at stage II around 20% of the patients will develop a recurrence despite effective surgical resection of the primary tumor. Identification of these stage II patients at risk and optimization of the therapy provided is therefore an important goal to improve outcome in colon cancer. For patients with stage III disease the treatment routinely includes adjuvant chemotherapy, but here estimates are that around 30-40% of these patients are over-treated as they would have been cured by resection alone. Therefore also at this stage of disease identification of the patients at risk is important as it may spare patients the unwanted side effects of chemotherapy.
Recently, using gene expression profiling we and others have identified distinct biological subgroups in colon cancer. Common biological features are identified by the different teams and a consensus classification revealed four independent subgroups of which one is defined by mesenchymal features and has dismal prognosis. This subgroup analysis therefore allows us to separate patients into high and low risk for recurrences and may provide a new handle for the clinical management of colon cancer.

**Aim**: The overall aim of the connection project is to study is to use the biological differences that exist in subgroups of colon cancers to guide clinical decisions on the use and type of adjuvant therapy, thereby improving the outcome as well as the therapy burden for individual patients. The proteomics aim of the project is to develop and validate protein-based assays that depend on the biological differences in the distinct colon cancer subgroups, focusing specifically on the mesenchymal poor-prognosis subgroup. Such assays are currently not available and are absolutely vital to stratify colon cancer patients that are both in need to obtain adjuvant therapy as well as for the type of therapy.

**Results**: In total 29 CRC tumors of the AMC90 set have been analyzed by proteomics, yielding a dataset of ~8700 proteins that cluster largely according to CCS subtype (see figure unsupervised clustering). Several hundred subtype-enriched proteins have been identified using dedicated statistics. Poor prognosis subtype associated proteins are involved in extracellular matrix organization, regulation of response to wounding/ stress, cell adhesion, cell motility/ migration, developmental process and secretion. Integration with available proteome data derived from the CRC TCGA dataset has identified overlapping subtype-associated proteins.




**Outlook**: The resulting protein-based stratification will be validated in 200 tumor samples for which genetic data is assembled in WP2. Pathway hyperactivation will be distilled from the data and used to predict vulnerabilities that can subsequently be tested in vitro and in vivo (WP5) In addition, the most discriminatory proteins will be transformed to an immunohistochemistry-based assay which will be further analysed in WP6.

*This work is supported by Alpes d'HuZes (Connection project)*

# Proteomics of mouse BRCA1-deficient mammary tumors identifies DNA repair proteins with diagnostic and prognostic value in human breast cancer

Marc.O. Warmoes[1], Janneke Jaspers[2], Petra van der Groep[3,4], Thang V. Pham[1], Sander Piersma[1], Gideon Oudgenoeg[1], Maarten P.G. Massink[5], Hanne Meijers-Heijboer[5], Quinten Waisfisz[5], S. Rottenberg[2], Epie Boven[1], Elsken van der Wall[3], Paul van Diest[4], Jos Jonkers[2], Connie R. Jimenez[1]

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**Background:** Breast cancer is among the most common malignancies diagnosed in women worldwide and accounts for the majority of cancer-related deaths in women. In about 5% of breast cancer patients, the disease occurs as part of a hereditary cancer susceptibility syndrome. A substantial proportion of these hereditary breast cancers can be attributed to germline mutations in the *BRCA1* and *BRCA2* genes.
BRCA-associated breast cancer is hallmarked by genomic instability and high sensitivity to DNA double-strand break (DSB) inducing agents due to loss of error-free DSB repair via homologous recombination (HR). Therefore, predictive markers of HR deficiency are of great interest in order to individualize therapy in breast cancer and possibly also other tumor types where HR deficiency may play a role.

**Aim** To identify and validate proteins that are associated with BRCA1 deficient breast cancer. These proteins could have potential use as prognostic or predictive biomarkers.

**Approach** A proteomics approach was used to compare the protein profiles of breast tumor tissues of a mouse model deficient in BRCA1 with those of BRCA1 proficient genetic models. The deficient breast tumors share histopathological and molecular features with BRCA1-deficient basal-like breast cancers in women.

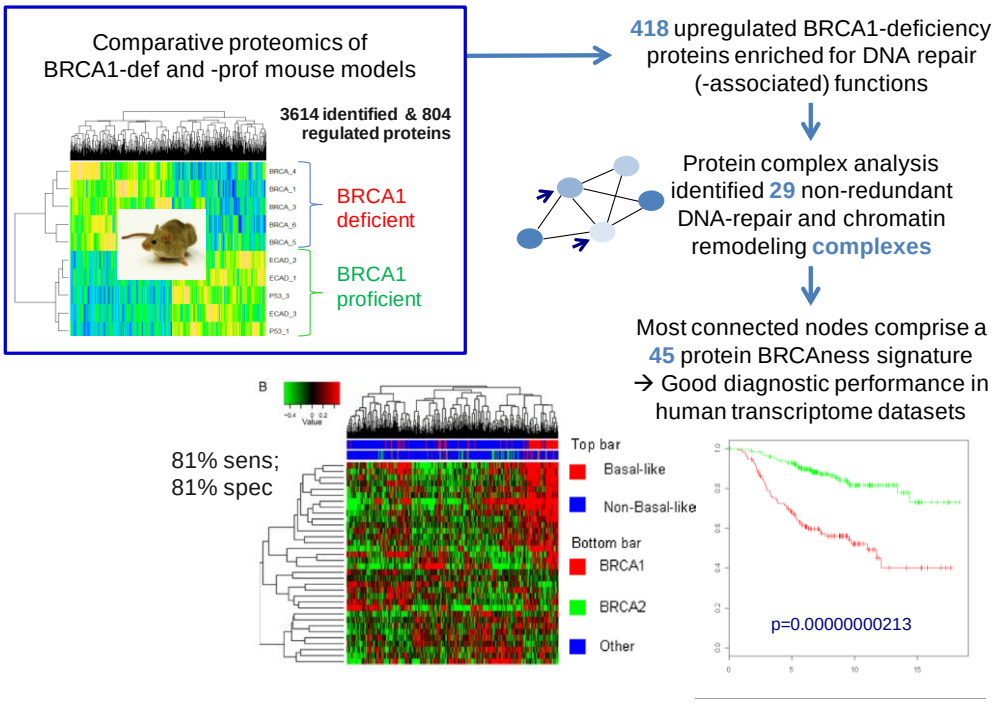


Figure 1. Graphical summary of comparative proteomics and data mining to identify and validate a protein signature for BRCA1-deficient breast tumors.

**Methods** Tumor proteins were fractionated using 1D gel electrophoresis followed by in-gel tryptic digestion and analysis using liquid chromatography coupled to tandem mass spectrometry, database searching and spectral counting. Statistical testing was applied to discover significantly differential proteins and pathway analysis tools were used to associate regulated proteins with biological functions and pathways. The diagnostic and prognostic power was assessed *in silico* by the use of publicly available gene expression human breast cancer data sets.
Selected markers were validated by immunohistochemical staining in a tissue microarray containing human breast tumor tissues. The study group comprised 160 cases of human invasive breast cancer with 38 *BRCA1* and 23 *BRCA2* germline mutation related cases and 99 cases with an unknown *BRCA* mutation status referred to as sporadic breast cancer cases. Scoring was performed by one observer (PJvD), who was blinded to the origin of the tumors. For all the markers the percentage of positive nuclei was scored. Associations between stainings were tested by Chi-square analysis.

**Results** Comparative analysis of BRCA1-deficient and proficient tumors identified a total of 3614 proteins, of which 804 were significantly regulated. Over 400 proteins in BRCA1-deficient tumors that were upregulated and linked to 29 non-redundant nuclear protein complexes, underscoring the importance of alterations in DNA repair pathways and chromatin remodeling. By selecting the highly connected nodes in each of the 29 protein complexes visualized as protein-protein interaction networks, we constructed a BRCA1-deficiency signature of 45 proteins (See Figure). In silico analysis of multiple public transcriptome datasets of human breast cancer (after orthologue mapping) revealed that this BRCA1 deficiency signature distinguishes human BRCA1- and BRCA2-mutated breast tumors from tumors lacking BRCA mutations (see Figure). This signature also exhibits prognostic power across 4 datasets (1114 tumors in total), with optimal performance in a dataset enriched for tumors deficient in homologous recombination (HR)-dependent DNA repair.

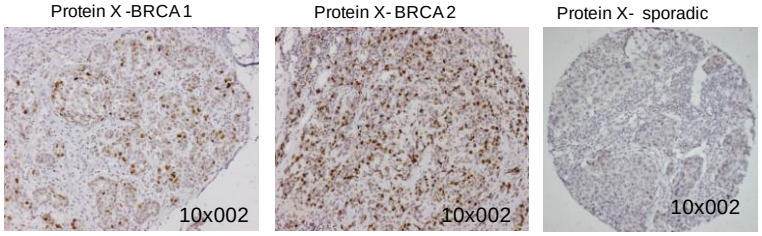


Figure 2 Representative immunohisto-chemical staining of marker X in 3 breast tumors.

Three candidates of the 45-protein signature were selected for validation by immunohistochemistry. The tumors on the tissue microarray were mostly of the ductal type and high grade. The mean age of the sporadic cases was 57, for the *BRCA1* mutation related cases 42 and for the *BRCA2* mutation related cases 47 years. Nuclear expression of marker X (figure 2) was present in 90% (28/31) and 85% (17/20) of the *BRCA1* and *BRCA2* related cases, respectively, which was significantly different compared to the expression of 30% (30/99) seen in the sporadic cases (p=0.000). Expression of marker Y was present in 61% (52/85) of the sporadic cases and seen in 43% (16/37) and 65% (13/20) of the *BRCA1* and *BRCA2* related cases, respectively (p=0.136). No significant differences in the expression patterns of marker Y between the three groups was observed (p=0.136). The expression of marker Z was observed in 79% (27/34) of the *BRCA1* and in 67% (12/18) of the *BRCA2* mutation related cases, significantly different compared to the expression of 20% (16/81) seen in the sporadic cases (p= 0.000).

**Conclusions and outlook** This study clearly illustrates how in-depth proteomics coupled to analysis of protein functions and networks can yield a signature covering the biology of BRCA1-deficiency with potential diagnostic and prognostic value in the human setting. The proteome changes that we identified in BRCA1-deficient breast tumors is indicative for a compensatory/rescue mechanism for the loss of HR repair. Tissue microarray analysis validated the increased expression of marker X and Z in *BRCA1 and BRCA2* deficient breast tumors in comparison to the expression of these markers in sporadic breast tumors. Future studies will explore the predictive potential of this signature and especially proteins X and Y for TNBC patient selection for DNA cross-linking therapy and PARP inhibitors.

*This research was supported by the CenE/Van Lanschot and the VUmc Cancer Center Amsterdam*

**Reference** Warmoes, MO. Jaspers, JE.,Pham TV., Piersma SR, Massink MPG, Waisfisz Q., Rottenberg S, Boven, E., Jonkers, J., Jimenez, CR. Proteomics of mouse BRCA1-deficient mammary tumors identifies DNA repair proteins with diagnostic and prognostic value in human breast cancer. Mol Cell Proteomics. 2012 Jul;11(7): M111.013334.

*Breast cancer proteomics, sub-project 2*

# Proteomics of mouse breast cancer models identifies fatty acid metabolism proteins as predictive markers for cisplatin resistance

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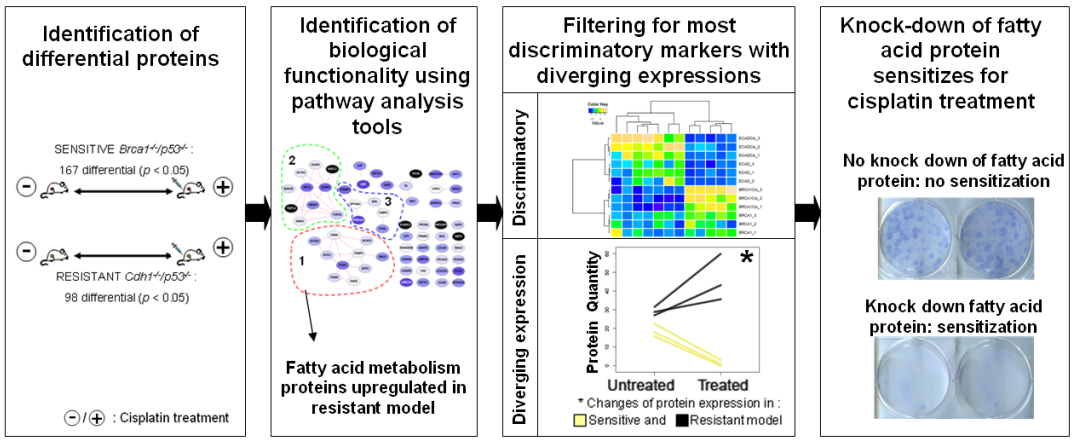
[2]Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

**Background**: Whereas a range of prognostic signatures are available for breast cancer, it is difficult to find signatures that predict the outcome of chemotherapy before treatment start. In fact, it may be easier to find a predictive profile shortly after the first treatment, when response-related genes are induced. We tested this approach using genetically engineered mouse models for breast cancer. Spontaneously developing *Brca1*$^{-/-}$*;p53*$^{-/-}$ mammary tumors of our mouse model for hereditary breast cancer (*K14cre;Brca1*$^{flox/flox}$*;p53*$^{flox/flox}$) are very sensitive to the maximum tolerable dose of cisplatin, and do not acquire resistance to this drug (Rottenberg et al., PNAS 2007). In contrast, several spontaneously developing *Ecad*$^{-/-}$*;p53*$^{-/-}$ mammary tumors of our mouse model for lobular breast cancer (*WAPcre;Ecad*$^{flox/flox}$*;p53*$^{flox/flox}$) do not shrink, but rather show a short growth delay or stable disease before becoming cisplatin resistant.

**Aim and approach:** To identify predictive biomarkers, we compared early changes in protein expression in cisplatin-sensitive BRCA1-deficient mammary tumors and cisplatin-resistant mammary tumors. The analyses were performed 24 hours after applying the maximum tolerable dose of cisplatin. At this time point drug-sensitive BRCA1-deficient tumors showed DNA damage, but cells were largely still viable.

**Results**: Comparative proteomics of treaded and untreated breast tumor samples of sensitive and resistant mice identified a total of 3486 proteins in 12 mammary tumor samples. By applying paired statistics and quantitative filtering, we identified highly discriminating markers for the sensitive and resistant models. Proteins upregulated in the sensitive model are involved in centrosome organization, chromosome condensation, (homology-directed) DNA repair and nucleotide metabolism. Major discriminating markers that were upregulated in the resistant model after treatment were predominantly involved in (fatty acid) metabolism. Specific inhibition of a fatty acid metabolism protein sensitized resistant lobular breast cells to cisplatin.




**Conclusions and outlook** Our data suggest that exploring the functional link between the DNA damage response and cancer metabolism shortly after the initial treatment may be a useful strategy to predict the efficacy of chemotherapy outcome.

*This research was supported by CenE/Van Lanschot and the VUmc Cancer Center Amsterdam*

**Reference** Warmoes M, Jaspers JE, Xu G, Sampadi BK, Pham TV, Knol JC, Piersma SR, Boven E, Jonkers J, Rottenberg S*, Jimenez CR*. Proteomics of genetically engineered mouse mammary tumors identifies fatty acid metabolism members as potential predictive markers for cisplatin resistance. Mol Cell Proteomics. 2013 May;12(5): 1319-34. *Shared senior authorship.

*Breast cancer proteomics, sub-project 3*

# Proteomics of mouse BRCA1-deficient and proficient mammary cancer cell secretomes reveals candidate biomarkers for non-invasive testing

Marc Warmoes[1,#], Siu W. Lam[1,#], Petra van der Groep[2,3], Janneke E. Jaspers[4,5], Yvonne H.C.M. Smolders[2], Leon de Boer[1], Thang V. Pham[1], Sander R. Piersma[1], Sven Rottenberg[5], Epie Boven[1], Jos Jonkers[4], Paul J. van Diest[2], Connie R. Jimenez[1]

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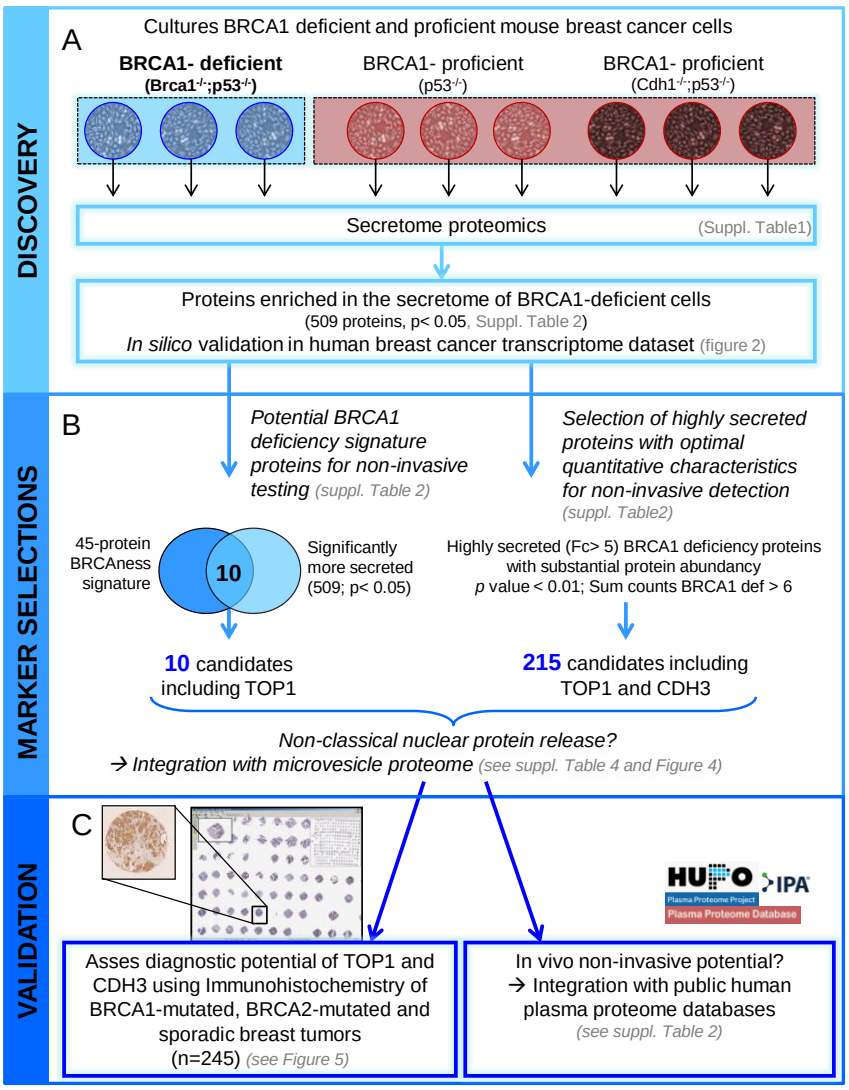
**Background:** Tumors arising in women carrying a BRCA1germline mutation are characterized by an aggressive behavior and early onset. Biomarkers that can be used to detect breast cancer in a non-invasive manner are scarce and may especially be helpful for early detection of breast cancer in BRCA1 mutation carriers.
Plasma proteomics is hampered by the protein complexity and large dynamic range of protein levels, obscuring tumor-derived proteins, Cancer cell secetome, comprising secreted proteins and extracellular vesicles has emerged as a valuable source for identification of candidate protein biomarkers potentially useful in non-invasive testing.



**Aim and approach**: The aim of this study was to identify extracellular proteins specifically related to BRCA1 status by differential protein profiling of secretomes of BRCA1-deficient and -proficient breast cancer cell lines. The whole strategy is outlined in the figure.

**Methods:** One BRCA1-deficient cell line and two BRCA1-proficient cells lines were cultured in triplicate from which secretomes were harvested. These cell lines were isolated from tumor-bearing genetic engineered mice.
The secretomes were first fractionated by one-dimensional gel electrophoresis, digested and subsequently analyzed by a nanoLC-MS/MS system. Differential expression of identified proteins between groups were tested using beta-binomial test. Pathway analyses were performed to describe differential biological process involved. Microvesicles were isolated and analyzed to explore non-conventional secretion of BRCA1-related proteins. Immunohistochemistry and gene expression analyses were performed to explore clinical relevance of secreted proteins.

**Results:**
The proteomic analysis identified a total of 2,107 secreted proteins in nine experiments. Reproducibility of triplicate experiments was high with an >80% overlap of protein identification. Overall, 59% of the identified proteins were predicted to be secreted via classical or non-classical secretion pathways. Interestingly, we found substantial amount of proteins of nuclear origin, because of which we explored the possibility of secretion of these proteins via microvesicular transport.

Proteomic analysis of microvesicles isolated from the same cell lines indeed confirmed the presence of relatively large amount of nuclear proteins, which were involved in DNA replication, RNA degradation and RNA splicing.

By using defined criteria ($p$-value $< 0.05$ and fold change $> 1.5$), we identified 509 upregulated proteins and 403 downregulated proteins in BRCA1-deficient secretomes relative to BRCA1-proficient counterparts. These upregulated proteins were involved in biological processes including translation, RNA spicing, angiogenesis, whereas the downregulated proteins were involved in proteolysis, actin cytoskeletal organization and nucleoside metabolism.

Mapping of 509 upregulated BRCA1-related proteins to mRNA expression dataset of human breast carcinomas with or without BRCA1/2 mutation as published by Johnson et al. revealed clustering according to BRCA1/2 mutation status.

Finally, by immunohistochemistry analyses on tissue-micro arrays containing 245 breast carcinomas (95 BRCA1-mutated, 44 BRCA2-mutated and 106 sporadic cases), we validated the overexpression of TOP1 and CDH3 in BRCA1-related breast cancer.

**Outlook**
Proteomic analyses of secreteome is a powerful strategy to identify candidate biomarkers potentially useful for non-invasive testing in the clinical practice. Here, we have identified proteins highly secreted by BRCA1-deficient breast cancer following in-depth comparative profiling of BRCA1-deficient and –proficient secretome, and with a substantial number being present in plasma proteome databases. Using in silico analyses and immunohistiochemistry, we have highlighted the potential clinical relevance of these BRCA1-related proteins. Although the clinical application of these proteins merits further exploration, we have provided preliminary evidence that several proteins may potentially be used for non-invasive detection of breast cancer in mutation carriers.

*This research was supported by CenE/Van Lanschot, the VUmc Cancer Center Amsterdam and CTMM Mammoth*

# Proteomic profiling of the murine mammary tumor secretome identifies candidate biomarkers for non-invasive breast cancer testing

Joey Lam[#1], Jeroen L.A. Pennings[#2], Thang V. Pham[1], Conny T.M. van Oostrom[2], Sander R. Piersma[1], Sandra Imholz[2], Epie Boven[1], Janneke Jaspers[3,4], Sven Rottenberg[4], Annemieke de Vries[2], Jos Jonkers[3], Connie R. Jimenez*[1], Wendy Rodenburg*[2]

[1]Dept. of Medical Oncology, VU University Medical Center, The Netherlands;[2]Laboratory for Health Protection Research (GBO), National Institute for Public Health and the Environment (RIVM), The Netherlands; [3]Division of Molecular Oncology, The Netherlands Cancer Institute, The Netherlands; [4]Division of Molecular Pathology, The Netherlands Cancer Institute, The Netherlands.
[#] contributed equally; [*] shared senior authorship

**Background**
Breast cancer is recognized as a heterogeneous disease consisting of multiple major subtypes as defined by gene expression microarray analysis or by immunohistochemistry. Early diagnosis of breast cancer is of paramount importance to improve outcome of patients. For this purpose of optimizing current breast cancer diagnosis, secreted proteins (secretomes) could provide complementary candidates for non-invasive testing.

**Aim and approach**
The aim of this study was to identify breast cancer subtype-specific, secreted proteins by differential protein profiling of secretomes of tumor-bearing genetically engineered mice, representing different breast cancer subtypes.

**Methods**
Breast tumor-bearing genetically engineered mice representing estrogen-receptor positive breast cancer, triple-negative breast cancer and healthy mice were used to harvest tissue secretome (N=3 per tissue type). Secretomes were fractionated by one-dimensional gel electrophoresis and digested, and the protein digests were subsequently analyzed by a nanoLC-MS/MS system. Differential expressions of identified proteins between groups were tested using beta-binomial test. Pathway analyses were performed to describe differential biological processes involved.

**Results**
A total of 3,796 secreted proteins were identified in 15 samples. Reproducibility of protein identification in biological replicates was generally high. Comparison between all tumor secretomes and normal breast tissue secretomes revealed 532 significantly upregulated proteins based on fold change ≥ 10 and a significance level of $p<0.001$, of which 109 upregulated proteins were present in all tumor secretomes. Gene ontology analysis of these 109 upregulated proteins pointed towards biological processes involving DNA replication, cell cycle, cell division, DNA helicase activity, transcription, regulation of transcription and transcriptional targets of E2F. In addition, we conducted comparison among tumor secretomes to identify subtype-specific proteins. This analysis revealed a total of 47 highly secreted proteins (fold change ≥ 5 and $p < 0.001$) among which were 23 proteins specific to estrogen-positive breast cancer and 24 proteins triple-negative breast cancer.

**Conclusion and outlook:**
In this study, we have identified breast cancer-specific proteins, which may capture the heterogeneous nature of breast cancer. Moreover, breast cancer subtype-specific proteins have been found. These proteins may allow personalized approach in the breast cancer management. Clinical relevance of selected proteins will be further investigated.

*This research was supported by the VUmc Cancer Center Amsterdam, CTMM Mammoth and RIVM funding*

## Proteomics of Brca1-deficient mouse tumors with acquired resistance for PARP inhibitors

Guotai Xu, Sven Rottenberg group NKI; Jos Jonkers group NKI; OPL

**Background**
We have previously shown that mammary tumors spontaneously arising in a genetically engineered mouse model for human BRCA1-associated breast cancer (K14Cre;*Brca1*$^{F/F}$;*p53*$^{F/F}$) are highly sensitive to the poly(ADP-ribose) polymerase (PARP) inhibitor olaparib due to their deficiency in homology-directed DNA repair (Rottenberg et al. PNAS 2008;105:17079-84). Despite the initial sensitivity, tumors are not eradicated and eventually develop drug resistance. Thus far, 3 mechanisms of PARP inhibitor resistance have been identified in BRCA1/2-deficient tumors: 1. Restoration of BRCA1/2 functions by genetic reversion; 2. P-glycoprotein-mediated efflux of olaparib; 3. loss of 53BP1. In this project we aim to identify additional mechanisms that explain resistance in our spontaneous BRCA1-deficient mouse mammary tumors which acquired PARPi resistance *in vivo*, in which we eliminated the first two mechanisms. In particular we are interested in alterations that result in restoration of DNA repair by homologous recombination (HR).
To explore the remain mechanisms of PARPi resistance, in collaboration with the group of Prof. Jos Jonkers we are combining the analysis of shRNA screens in cell lines with the study of tumors that acquired resistance *in vivo*. In our screen, we included 1976 hairpins targeting 391 DNA damage response related genes using the *Brca1*$^{-/-}$; *p53*$^{-/-}$ mammary tumor cell lines B11 and G3 derived from our mouse model. We found a reproducible enrichment of hairpins targeting *Rev7* (also known as *Mad2l2*) in those olaparib-surviving colonies from the screen and the results were validated both *in vitro* and *in vivo*.

**Aim and Approach** To investigate whether loss of REV7 may explain PARPi resistance in some of our spontaneous BRCA1-deficient mouse mammary tumors which acquired PARPi resistance in vivo, we performed RNAseq analysis of our collection of PARPi resistant tumors and their matched controls. In total, there is a more than 50% inhibition of the transcript levels in 10 out of 76 PARPi resistant tumors based on the RNASeq analysis, and this result was confirmed by RT-qPCR. However, we didn't have a good antibody to show the protein levels of REV7 in those tumors at that moment. Therefore, in collaboration with Prof. Jimenez, it was our main goal to determine expression of REV7 levels and the associated DNA damage network of matched PARPi-sensitive and resistant tumors using unbiased proteomics.

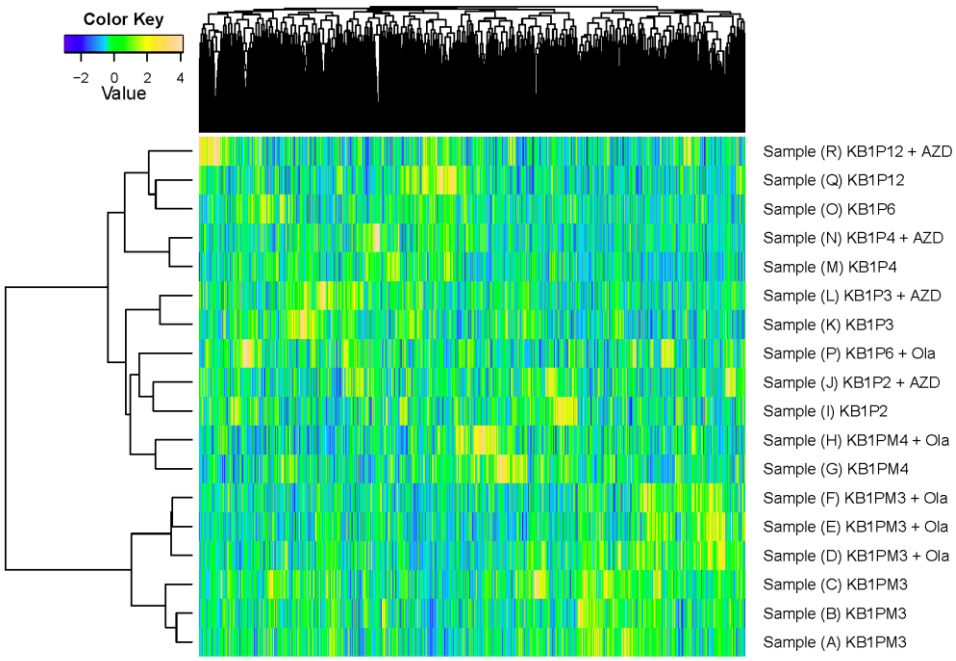



Figure. Usupervised clustering.

**Results**

The measurements and statistical analysis of all the samples have been finished successfully. Overall we detected more than 6600 proteins with about 5000 proteins per sample. Using unbiased clustering analysis, we observed that tumors derived from the same tumor cluster together and that the individual fingerprint is stronger that the difference between sensitive and resistant tumors. This is consistent with RNASeq analysis.

Unfortunately, REV7 was not detected due its low abundance in expression. However, we identified other interesting hits in the DNA damage network by combining the protein data with results from functional shRNA screens. We are now validating these hits by targeted genetic inhibition and subsequent clonogenic assays.

In some tumors we observed a slight decrease of PARP1 in PARPi-resistant tumors using proteomics. Since PARP inhibitors act as poisons that trap PARP1 at damaged DNA, reduced PARP levels may contribute to resistance. However, this finding could not be validated by Western Blotting using re-transplanted stably PARPi-resistant tumors.

**Conclusions and outlook**

The proteomic analysis of matched PARPi-sensitive and –resistant tumors did not succeed in confirming a decrease of REV7 proteins in the resistant tumors because of the low abundance of REV7. Nevertheless, proteomics appear to be useful to identify other players in the DNA damage response network that may also impact PARPi sensitivity and that may be linked to REV7. As future experiments we think that it may be very interesting to challenge the tumors by inducing DNA damage (e.g. using ionizing irradiation) and study differential expression of proteins of resistant versus sensitive tumors under these stress conditions. Moreover, the analysis of phosphoproteomics may be a useful complementary approach.

# Novel candidate biomarkers for cisplatin response prediction in NSCLC

Tieneke B.M. Schaaij-Visser[1], Inge de Reus[1], Natalie Proost[2], Remco Nagel[3], Sander R. Piersma[1], Thang V. Pham[1], Ruud H. Brakenhoff[3], Erik FBJM Thunissen[4], Egbert F. Smit[5], Anton Berns[2], Connie R. Jimenez[1]

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**Background:** Lung cancer is currently the number one cause of cancer-related deaths worldwide. Five-year survival rates for both non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) are still less than twenty percent due to late stage of presentation, treatment failure and lack of biomarkers for personalized therapy. The majority of patients with metastatic non-small cell lung cancer (NSCLC, stage IIIB/IV) are treated with cisplatin-based chemotherapies in a one-size-fits-all approach. However, a significant number of patients do not benefit in terms of survival and, moreover, quality of life detoriates due to side effects of this treatment. Pinpointing NSCLC cancer patients who are more likely to derive clinical benefit from cisplatin-based chemotherapies using molecular markers, will improve clinical outcome and reduce both toxicity and health care costs.

**Aim and approach** To identify potential predictive biomarkers for cisplatin sensitivity and resistance, we performed proteomics of 6 NSCLC cell lines with various IC50 values (range 1.5-14.6 µM) for cisplatin.

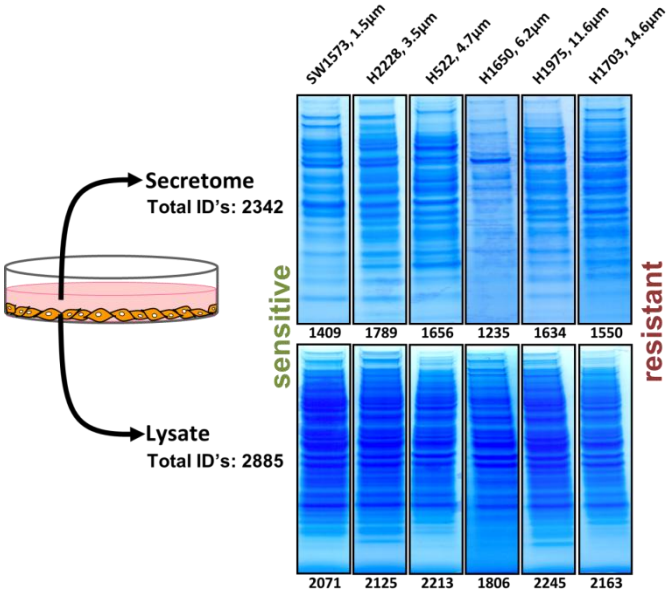


Figure 1. 1D-gel separation of secretomes and cellular lysates of 6 NSCLC cell lines. For analysis by nanoLC-MS/MS, gel lanes were cut into 10 pieces , followed by in-gel digestion and peptide extraction.

**Results:** We profiled intracellular proteins (cell lysates) and extracellular proteins (secretomes) and generated a datasets of 2885 lysate and ~2342 secretome proteins. Supervised clustering based on differential proteins revealed clear clustering of resistant and sensitive cell lines.
Based on the level of significant regulation between sensitive and resistant cells and the correlation to the cisplatin IC50 value (Fig. 2) a total of 38 predictive cell lysate candidates (19 sensitivity and 19 resistance markers) and 47 secretome candidates (34 sensitivity and 13 resistance markers) were identified that may be coupled to immunohistochemistry and enzyme-linked sorbent assays, respectively.

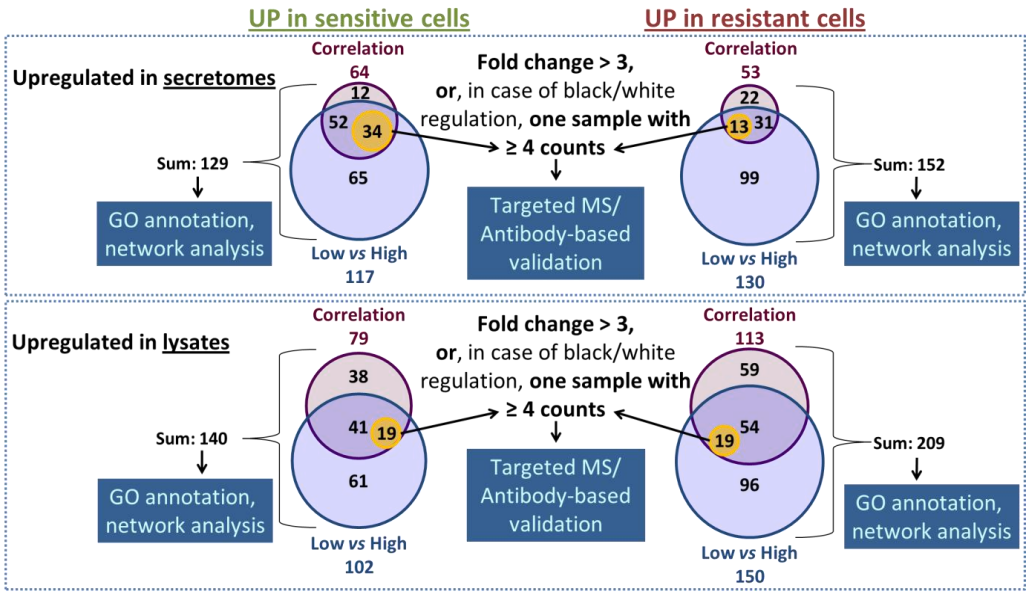


Figure 2. Numbers of differentially expressed proteins in secretomes and lysates of sensitive and resistant lung cancer cell lines. The numbers of differential proteins are displayed for the correlation analysis and for the group comparison between the two cell lines with low and the two cell lines with high $IC_{50}$-values.

For functional data mining, the combination of all differential proteins was divided into those higher expressed in the cell lines with a low $IC_{50}$-value for cisplatin (designated: UP in sensitive cells) and those higher expressed in the cell lines with a high $IC_{50}$-value for cisplatin (designated: UP in resistant cells). These separate sets of proteins were used as input for network, gene ontology, and pathway analyses. Notably, the secreted/released proteins are linked to different biological processes. Functions and pathways known to be involved in chemotherapy sensitivity and resistance were identified. For example, DNA packaging, as part of the DNA damage response, was overrepresented in the functions linked to proteins exhibiting higher levels in sensitive cells than in resistant cells, as described before by others. In turn, protein trafficking and vesicle transport were overrepresented functions associated with proteins found at higher levels in the secretome of resistant cells, in agreement with other reports that indicated a role for extracellular vesicle release by cancer cells and in tumor biology. Figure 3 highlights the identified proteins and processes in relation to known cisplatin sensitivity and resistance mechanisms.

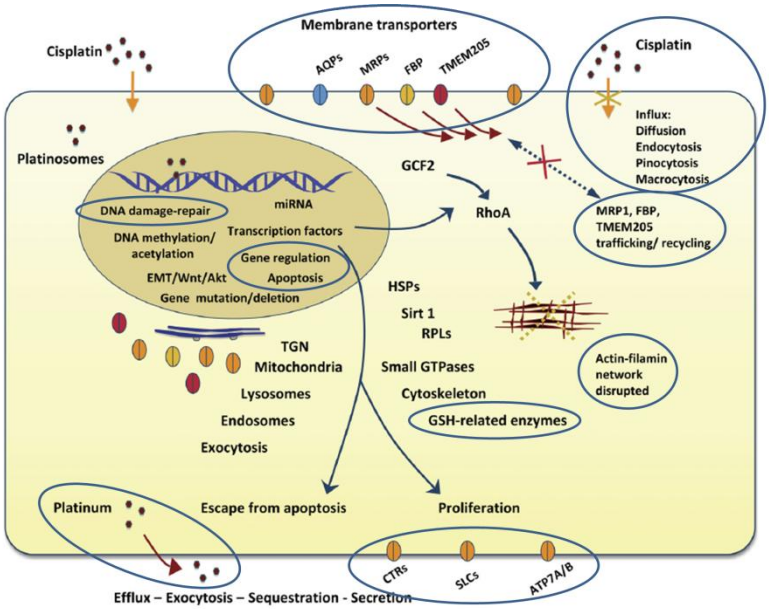


Figure 3. Cisplatin resistance mechanisms as described by Shen et al. (Pharmacol. Rev. 2012), with highlighting (encircling) of processes to which differential proteins identified in our study are linked.

Because of the availability of NSCLC tumor tissue collections of patients treated with adjuvant cisplatin-based chemotherapy with clinical follow-up, we focused on exploring the link with survival

using immunohistochemistry (IHC) of 5 selected predictive DNA repair related cell lysate candidates with available antibodies. For 3 antibodies we were able to develop suitable staining protocols and performed IHC analysis of a series of ~30 tumor tissues of patients samples. In this small cohort, the Kaplan Meijer analysis of IHC data for one DNA repair protein revealed some potential in squamous NSCLC carcinoma, where patients with a relatively high percentage of positive tumor cell nuclei in the basal layer exhibited a better survival ($p < 0.01$). The results for the second protein were sub-significant ($p=0.24$) in the squamous cell carcinoma group, though there may be a correlation with survival with a higher staining intensity being indicative of a better survival. A larger sample size is needed to reach significant findings. Staining ofr candidate 3 was not significantly correlated to survival. We are currently collecting a larger patient cohort for further clinical validation by IHC.

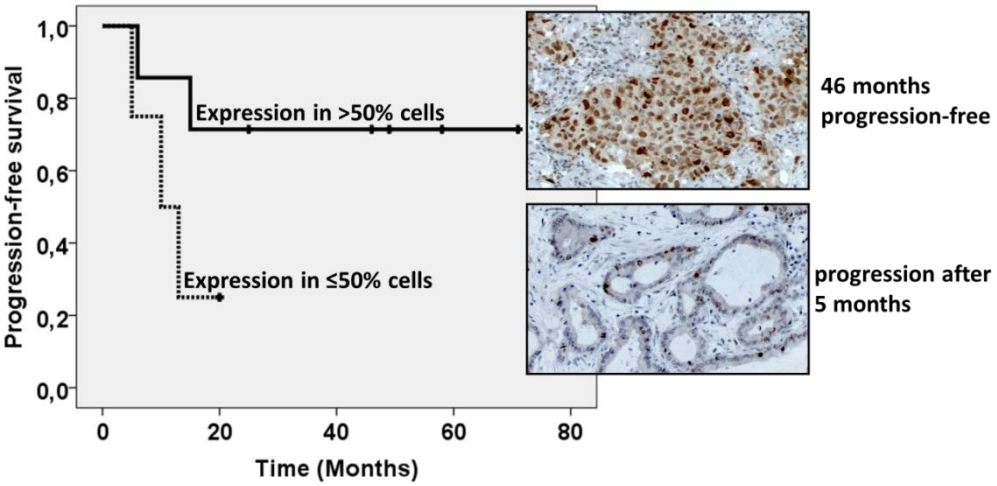


Figure 4. Progression-free survival associated with expression of a candidate protein biomarker for cisplatin response prediction.
Eleven patients were diagnosed with adenocarcinoma stage 1 or 2 and treated by surgery and adjuvant platinum-based chemotherapy. Protein expression was examined by immunohistochemistry on the resected tumor tissue. Low expression was defined as ≤50% positive tumor cell nuclei; high expression was defined as >50% positive tumor cell nuclei.

**Conclusions and outlook** In conclusion, our proteomics analysis of a panel of NSCLC cell lines identified proteins involved in numerous mechanisms previously implicated in treatment response or cisplatin resistance, and yielded promising new potential markers for platinum response prediction. Preliminary validation of one candidate protein biomarker demonstrated the potential value of this marker for prediction of response to adjuvant platinum-based therapy after surgery. Targeted mass spectrometry and antibody-based methods will be used in the proposed project to further validate the panel of most promising markers in patient tissue and body fluid material. Together, these data show the potential of proteomics to identify novel protein biomarkers for cisplatin response prediction and their potential clinical value in treatment selection for human NSCLC.

*This research was supported by the CTMM Airforce project*

# Proteomics of FFPE tumors of patients with lung cancer to identify prognostic/ predictive biomarkers for cisplatin response

Tieneke BM Schaaij-Visser[1], Inge de Reus[1], Katrien Grunberg[3], Egbert F. Smit[3], Connie R. Jimenez[1]

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**Background:** Lung cancer is the deadliest malignancy worldwide accounting for ~twenty percent of all cancer deaths. The overall five-year survival rate is less than 15% and has barely increased in the last decades. This poor overall survival is mainly due to late detection of the tumor, which causes the majority of patients to present with (inoperable or) metastasized disease. Most NSCLC patients are treated with palliative or adjuvant chemotherapy, and cisplatin is the major component of most chemotherapy regimens. However, approximately 75% of all treated lung cancer patients are resistant to cisplatin-based regimens, and virtually all develop resistance during therapy. The availability of biomarkers (detected in tissue or body fluids) that can predict cisplatin treatment response could avoid over-treatment and its associated toxicities, and may guide treatment decisions towards non-platinum-based regimens.

**Aim** To identify novel biomarkers for cisplatin response prediction and correlate our set of recently identified novel predictive cisplatin response markers to clinical outcome in NSCLC patients treated with adjuvant chemotherapy.

**Approach** In total 23 FFPE tumor tissue blocks were selected based on clinical outcome, ie., shorter or longer median recurrence-free survival interval after a cisplatin-based treatment and adjusted for clinical variables (age, gender, performance status, smoking history, stage. The median recurrence-free survival interval was 16 months for the VUmc cohort. NSCLC patient samples included in the analysis have at least 3 years of follow-up and are well-annotated (eg for age, gender, performance status, smoking history, histology, stage).
In total 14 squamous NSCLC patients (8 with short and 6 with long progression-free survival (PFS)) and 9 adenocarcinoma patients (5 with short patients and 4 with long PFS) were included for the analysis. Areas with tumor tissue were manually macrodissected from slides demarcated by a pathologist.

**Methods** Comparative proteome profiling of lysed FFPE samples was performed as described before (Piersma et al., 20103) with modifications: proteome analysis was by single fraction GeLC-MS/MS.

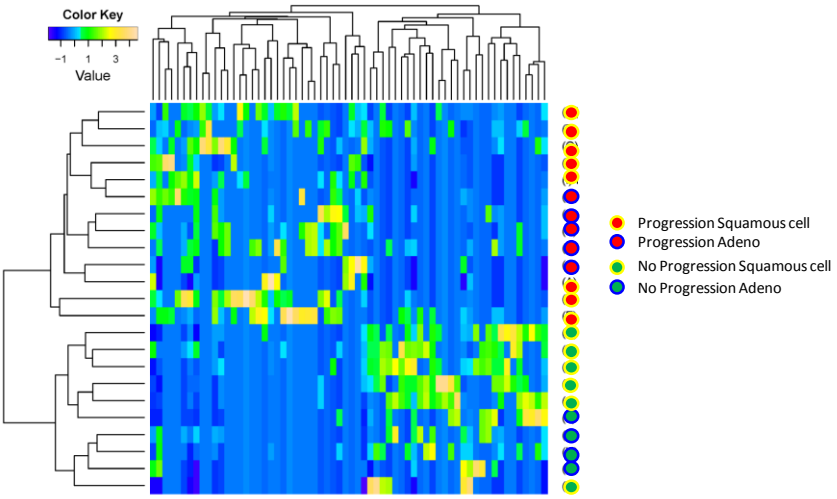
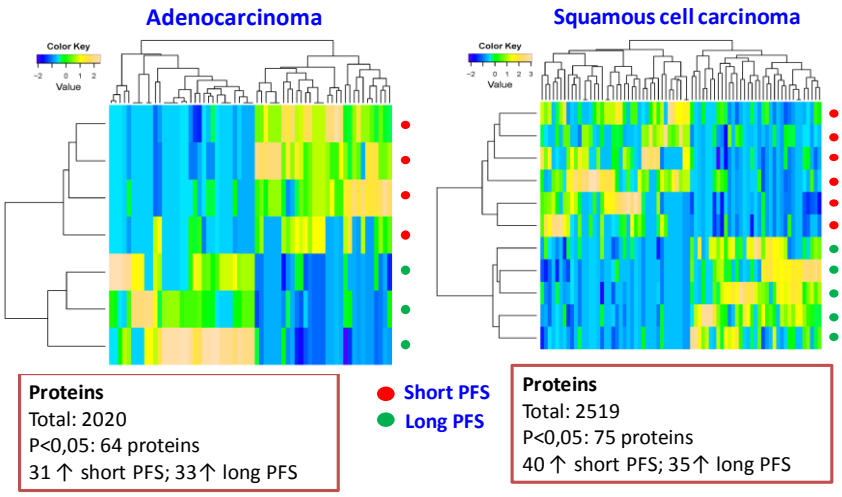



Fig. 1. Heat map of supervised cluster analysis using differential proteins ($p < 0.05$) between all lung NSCLC cancer patients with short and long PFS.

**Results** In total 2519 proteins were identified in all 23 FFPE NSCLC tumors of which 64 proteins were significantly regulated (p-value <0,05) between patients with long and short FPS. Of these 64 regulated proteins, 35 proteins were upregulated in patients with short FPS and 29 proteins were upregulated in patients with long PFS. These proteins could separate the patient groups according to PFS (Fig 1) and sub-clusters were observed largely according to histology.

Poteins associated with short PFS were associated with the protein extracellular matrix, ECM binding, Cell matrix adhesion, integrin complex, and the cell surface while proteins associated with long PFS were involved in protein transport, protein localization and the ribosome. The table indicates the sub-analysis of PFS-related processes in the lung cancer histology types.




**Adenocarcinoma**

- RNA-binding, transcription cofactor activity, TF binding
- **Extracellular matrix**
- Glycosphingomyelin metabolic process
- Lysosome, lytic vacuole
- Endopeptidase inhibitor activity
- Acute inflammatory response
- Melanosome
- Ribonucleoprotein complex

**Squamous carcinoma**

- **Extracellular matrix**, cell adhesion
- Cell migration
- Response to organic substance/ hormone
- Polysaccharide binding
- Protein maturation
- Protein transport/ localization
- Regulation of protein ubiquitin ligase activity during cell cycle
- Lamellipodium

**Short PFS**

**Long PFS**

**Conclusions and outlook**

Together, the NSCLC FFPE tumor proteome data show the potential of proteomics to identify novel protein biomarkers for cisplatin response prediction and their potential correlation to clinical outcome in NSCLC. Some of the identified candidates in this analysis of clinical tissues overlap with our previous cell line based candidates that correlated with cisplatin IC50 values and provide relevant markers for follow-up analysis by immunohistochemistry in a larger cohort.

# Exploration of sputum to develop protein-based assays for early detection, prognosis and drug response of lung cancer

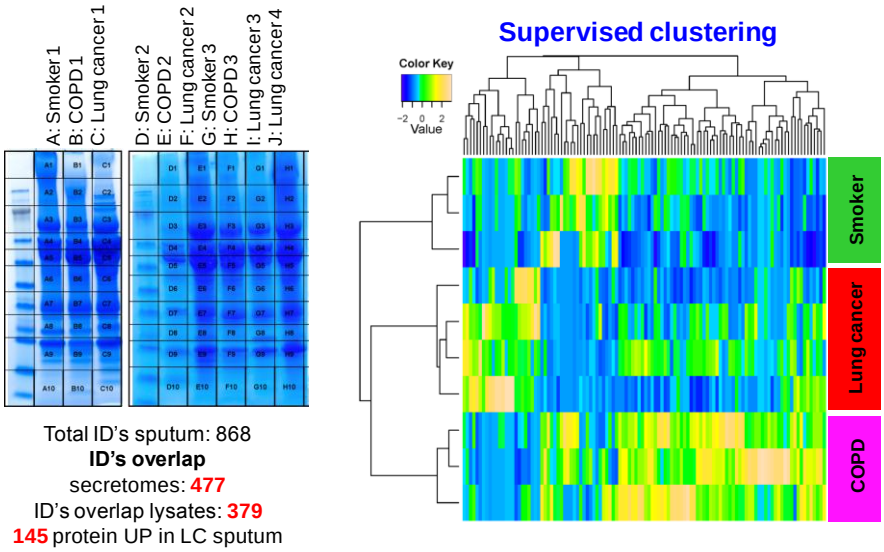
Tieneke BM Schaaij-Visser[1], Inge de Reus[1], Burçin Colak[1], Jasmijn Hubers[2], Egbert F. Smit[3], Erik FBJM Thunnissen[2], Connie R. Jimenez[1]

[1] OncoProteomics Laboratory, Department of Medical Oncology; [2] Department of Pathology, [3]Dept. Pulmonology, VU University Medical Center, Amsterdam, The Netherlands

**Background:** The five-year survival rate of patients with late stage lung cancer (80% of the cases) remains very disappointing. Early detection of lung cancer can dramatically increase survival. However, early detection is still difficult at the moment, since current molecular tests have relatively low sensitivity and specificity. This stresses the need to identify ideal new biomarkers that can be collected non-invasively from proximal biological fluids. Sputum is mucus and other matter from the lungs that can be coughed up, for example in lung disease patients and smokers. It is an accessible non-invasively collected biofluid and contains cells as well as soluble molecules such as secreted and externalized proteins. Currently, DNA, RNA and methylation biomarkers are described that can be found in sputum of lung cancer patients. The concentrations of biomarkers in sputum are considerably higher as compared to blood. Therefore it provides a suitable starting point for biomarker discovery at the proteome level.

**Aim** of this project is: 1. To set up a robust and reproducible protocol for proteomics of sputum samples; 2. To explore the potential of sputum for protein biomarker discovery in a pilot analysis of NSCLC patients, COPD patients and healthy smokers

**Approach:** 1. Protein extraction will be optimized by assessment of different conditions and protein yield from sputum will be monitored by visualization of proteins in coomassie-stained 1D gels. 2. Comparative proteomics of sputum of cancer patients, COPD (control) patients and healthy smokers (controls).




**Results and outlook:** A protocol for protein isolation from the soluble fraction of freshly collected sputum has been set up and optimized. The soluble fraction of freshly collected sputum is rich in proteins: the 1D gel protein patterns show good yield over the whole mass range. The sputum dataset of 4 NSCLC patients, 3 COPD patients and 3 smokers comprised ~800 human proteins, including known and novel potential biomarkers whose abundance was significantly increased in the sputum of lung cancer patients. Previously reported tumor markers include Carcinoembryogenic Antigen, heterogeneous nuclear ribonucleoproteins A2/B1 and isoform 1 of Kallikrein-11, underscoring the sensitivity of our proteome analysis. Validation of the candidates in a larger cohort is required.

*This research was supported by the VUmc Cancer Center Amsterdam*

# An accurate paired sample test for count data

Thang V. Pham, Connie R. Jimenez
OncoProteomics Laboratory, Department of Medical Oncology, VU University Medical Center, the Netherlands

**Background**
Recent technology platforms in proteomics and genomics produce count data for quantitative analysis. Previous works on statistical significance analysis for count data have mainly focused on the independent sample setting, which does not cover the case where pairs of measurements are taken from individual patients before and after treatment. This experimental setting requires paired sample testing such as the paired *t*-test often used for continuous measurements.

**Aim** To derive a method for significance analysis of count data with a paired sample design.

**Approach**
We formulate the problem of paired sample test for count data in a framework of statistical combination of multiple contingency tables. In particular, we specify explicitly a random distribution for the effect with an inverted beta model. The technical variation can be modeled by either a standard Poisson distribution or an exponentiated Poisson distribution, depending on the reproducibility of the acquisition workflow.

**Results**
We compare the performance of the new method to a state-of-the-art method, edgeR on two real-life datasets. The first dataset (van Houdt dataset) is a comparative proteomics analysis of an in vitro model of colon cancer stem cells and differentiated tumor cells (van Houdt et al., 2011). Each sample pair was derived from freshly resected liver metastases. The second dataset (Tuch dataset) is an RNA-Seq dataset in a study of the development of oral squamous cell carcinoma (OSCC) (Tuch et al., 2010). Three OSCC tumors and three matched normal tissues were analyzed to obtain transcriptome read counts.
The new statistical test shows a comparable performance to the state-of-the-art methods in general (Fig. 1), and in several cases where the methods differ, the proposed test returns more reasonable *p*-values (Fig. 2).

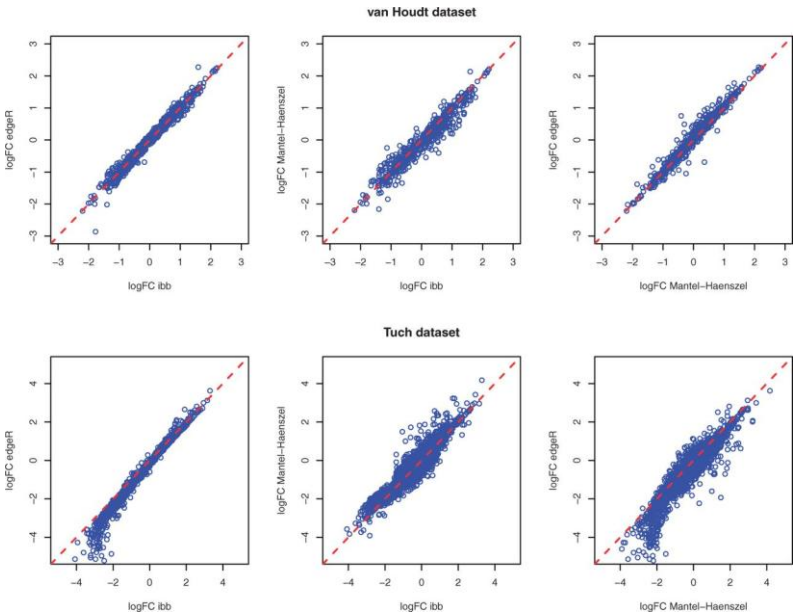
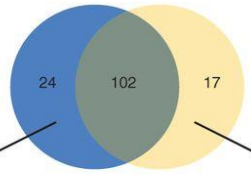



Fig. 1. Fold changes estimated by the new method (ibb - inverted beta binomial), edgeR and Mantel-Haenszel test on two datasets show consistent results.

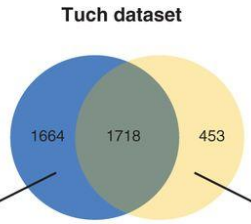



(a)

| Diff | | | Sph | | | ibb | | edgeR | |
|---|---|---|---|---|---|---|---|---|---|
| L145 | L146 | L167 | L145 | L146 | L167 | *fc* | *p-value* | *fc* | *p-value* |
| 28 | 16 | 13 | 17 | 11 | 8 | -1.5390 | 0.0472 | -1.4898 | 0.1513 |
| 0 | 31 | 44 | 49 | 39 | 49 | 3.4249 | 0.0226 | 2.9674 | 0.1137 |
| 10 | 21 | 12 | 7 | 11 | 8 | -1.7132 | 0.0426 | -1.6563 | 0.1066 |
| 18 | 14 | 7 | 31 | 16 | 13 | 1.5612 | 0.0497 | 1.6014 | 0.0976 |
| 27 | 28 | 23 | 16 | 18 | 14 | -1.5790 | 0.0217 | -1.5419 | 0.0788 |

(b)

| Diff | | | Sph | | | ibb | | edgeR | |
|---|---|---|---|---|---|---|---|---|---|
| L145 | L146 | L167 | L145 | L146 | L167 | *fc* | *p-value* | *fc* | *p-value* |
| 23 | 3 | 23 | 42 | 19 | 25 | 1.9648 | 0.1016 | 2.0592 | 0.0460 |
| 34 | 38 | 3 | 6 | 17 | 4 | -2.5449 | 0.0949 | -2.4981 | 0.0144 |
| 5 | 3 | 2 | 7 | 11 | 12 | 2.6302 | 0.0779 | 2.6998 | 0.0231 |
| 7 | 18 | 11 | 7 | 3 | 4 | -2.5358 | 0.0739 | -2.5295 | 0.0265 |
| 2 | 7 | 9 | 12 | 10 | 15 | 1.9900 | 0.0519 | 2.0954 | 0.0475 |




(c)

| Normal | | | Tumor | | | ibb | | edgeR | |
|---|---|---|---|---|---|---|---|---|---|
| N8 | N33 | N51 | T8 | T33 | T51 | *fc* | *p-value* | *fc* | *p-value* |
| 126 | 111 | 145 | 142 | 188 | 230 | 1.5362 | 0.0126 | 1.2412 | 0.4998 |
| 193 | 129 | 172 | 221 | 215 | 285 | 1.5060 | 0.0173 | 1.2583 | 0.4704 |
| 234 | 165 | 231 | 335 | 238 | 363 | 1.5049 | 0.0009 | 1.2684 | 0.4564 |
| 157 | 111 | 124 | 196 | 157 | 230 | 1.5247 | 0.0171 | 1.2770 | 0.4496 |
| 257 | 220 | 238 | 294 | 349 | 427 | 1.5099 | 0.0223 | 1.2720 | 0.4490 |

(d)

| Normal | | | Tumor | | | ibb | | edgeR | |
|---|---|---|---|---|---|---|---|---|---|
| N8 | N33 | N51 | T8 | T33 | T51 | *fc* | *p-value* | *fc* | *p-value* |
| 219 | 86 | 60 | 43 | 157 | 26 | -1.6647 | 0.2875 | -2.1407 | 0.0466 |
| 119 | 79 | 63 | 34 | 158 | 15 | -1.6889 | 0.2746 | -2.2045 | 0.0464 |
| 188 | 184 | 643 | 56 | 331 | 187 | -1.6793 | 0.2713 | -2.1808 | 0.0371 |
| 711 | 188 | 365 | 137 | 242 | 272 | -1.6338 | 0.2670 | -2.0168 | 0.0497 |
| 473 | 210 | 944 | 168 | 366 | 246 | -1.6817 | 0.2648 | -2.1703 | 0.0360 |

Fig. 2. Overlap analysis of the proteins and genes with p-values < 0.05 by either ibb or edgeR.

## Conclusions and outlook

A software tool is available for download at http://www.oncoproteomics.nl/

## References

Pham TV and Jimenez CR *Bioinformatics* 2012;28:i596-i602.

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*Data mining, sub-project 2*

# Computational analysis of phosphoproteomics data

Thang V. Pham, Sander R. Piersma, Jaco Knol and Connie R. Jimenez

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**Background** Phosphoproteomics profiling is a strategy to identify aberrant signaling pathways in cancer cells and tissues. Phosphoproteomics can be used to identify candidate drivers of cancer progression and drug targets. Hyperphosphorylation of protein kinases is an indication of increased kinase activity and restoring this activity to normal levels by therapeutic intervention is the aim of many novel targeted therapies based on kinase inhibitors.
Analyzing phosphoproteomics remains a challenge because of [1)] the low intensity of phosphorylated peptides detected by LC-MS/MS as compared to unmodified peptides and the lability of phosphopeptides, because [2)] phosphorylation site localization algorithms are not perfect and because of [3)] the complicated dynamic biochemical process of phosphorylation and dephosphorylation and its feed-back loops.

**Aim** This project aims to establish robust data analysis workflows for phosphoproteomics data with sound bioinformatics and statistical methods to enable novel molecular insights into signaling network state and to identify candidate driver kinases in model systems and clinical material.

**Approach**
The main steps in phosphoproteomics data analysis are: phosphopeptide identification, phosphosite localization, phosphosite quantification (intensity-based and spectral-counting based), normalization of phosphosite intensities, kinase-centered analysis including ID mapping (activation loop phosphorylation and kinase motif analysis of phosphoproteins) and ultimately, downstream data mining including GO analysis, STRING analysis, and mapping to known signaling pathways and assembly of novel signaling pathways.

**Methods**
*ID-Propagation*
With respect to the first step, we have adopted MaxQuant as the main tool for mass spectrometry data processing. The software package implements a robust signal processing algorithm to extract MS1 intensity information. MS/MS data is used for peptide identification, however the stochastic nature of data-dependent MS/MS acquisition results in incomplete detection of phosphopeptides across samples. This results in zero intensities, which hampers downstream analysis. A crucial step here is to propagate identified (phospho)peptides in one or more samples to other samples that satisfy constraints in MS1 and LC retention time. We have evaluated MaxQuant extensively in this aspect and provided appropriate adaptation of the software. Starting from the evidence.txt file an R-script was developed to propagate ID's and phosphosite intensities across samples.

*Phosphopeptide quantitation by MS/MS spectral counting*
Guo et al in PNAS 2007 and Rikova in Cell 2007 have shown that counting the number of phospphopeptide MS/MS spectra is a straight-forward method to quantify protein phosphorylation aimed at finding hyperphosphorylated driver protein kinases. The basic assumption in spectral counting-based protein quantitation (Liu, Analytical Chemistry 2004) is that the number of MS/MS spectra acquired for a protein scales linearly with protein abundance in a complex background. Here this approach is extended to phosphopeptides. Again, the assumption is that increased phosphorylation points at (over-)active signaling. We have applied the approach to identified phosphopeptides, focusing on protein kinases as being the target of many novel small-molecule inhibitors. Based on MaxQuant identification and quantification output we have developed an excel-based workflow for calculation of 'phosphopeptide MS/MS spectral counts' per kinase.

*Kinase activation loop phosphorylation analysis*
In the human genome 518 protein kinases have been identified, of which 90 are tyrosine kinases. Many kinases have been associated with tumor cell proliferation, migration and survival. Kinases have a conserved activation loop whose degree of phosphorylation is highly correlated with catalytic activity.

The intensity of kinase activation loop phosphopeptides detected by MS/MS can be used as surrogate read-out for kinase activity. The kinase activation loop region is characterized by two conserved tripeptides: DFG at the start and APE at the end of the loop. We have developed an excel-based workflow to quantify activation-loop phosphorylation based on peptide intensities from MaxQuant.

*Signaling network analysis*
Phosphoproteomics data (incl preclinical +/- drug), protein-protein interaction data with driver mutations from cancer genomics (eg., TCGA project) and key targets from functional genomics are integrated to identify causally implicated drug targets. We rank on quantitative data at the level of phosphosites/ phosphoproteins/ phosphorylated kinases. If no hyperphosphorylated driver kinase stands out, network-based analysis may provide a solution based on the rationale that drug targets are often highly connected nodes; drug targets show regulation upon drug exposure (integrate with preclinical +/- drug phospoproteomics); drug targets are more often connected to cancer genes or are mutated themselves.

**Results**

*ID-Propagation to improve reproducibility of phosphopeptide detection*
In Fig 1. the effect of proper ID propagation is shown for low-level P-Tyr phophopeptide IP samples. ID propagation improves the phosphopeptide quantitation based on MS1 intensities by solving the zero's problem and works best with multiple replicate samples and samples of different protein amounts.

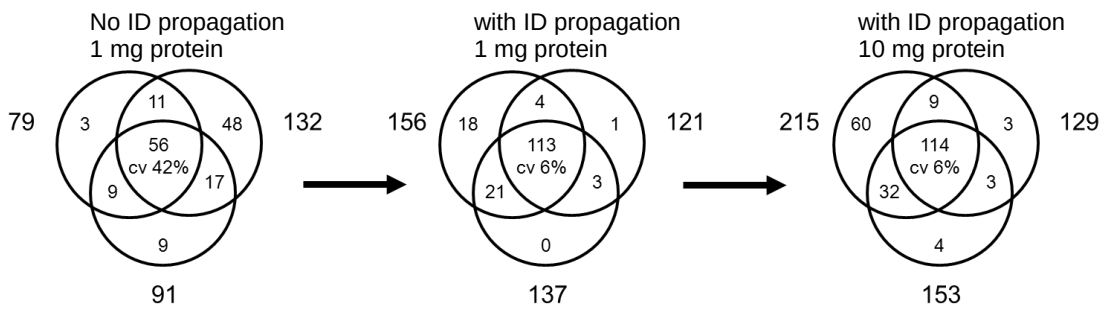



Fig. 1. Effect of ID-propagation on low level pTyr phosphopeptide identification and quantification. Triplicate analysis of phosphopeptide IDs from 1 mg protein of a cancer cell line lysate shows that the overlap of identified peptides increases when the peptide identities are propagated from runs with MS/MS identification to runs with only MS signals.

*Phosphopeptide MS/MS spectral counting to identify candidate driver kinases*
In Figure 2 the workflow for extraction of phosphopeptide MS/MS counts from MaxQuant Evidence.txt output is shown. Several SORT, COPY and SUM steps are required to extract the relevant intensity values form the Evidence.txt file as well as an ID mapping step to extract protein kinases from the file. Combination of these two steps results in summed spectral counts per protein kinase. In a differential experiment this value is used to find increased kinase phosphorylation, which could have predictive value for therapy selection.

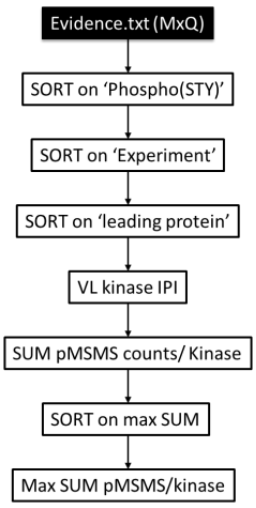
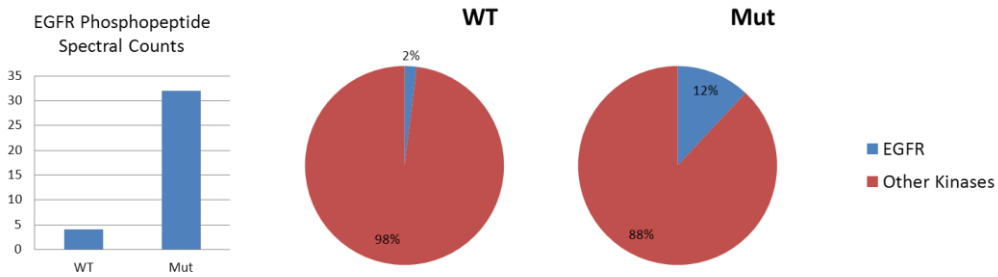


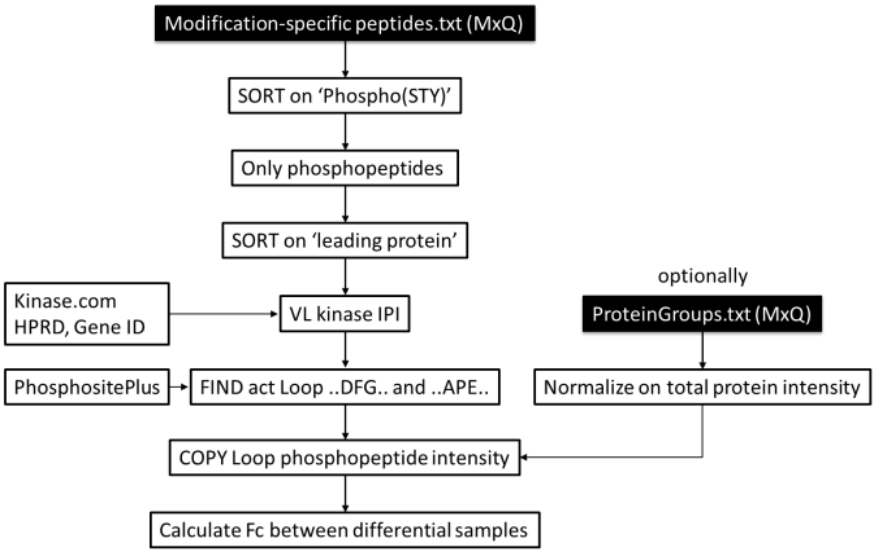

Fig 2. EGFR phosphopeptide Spectral Counts U87 lysate pTyr IP



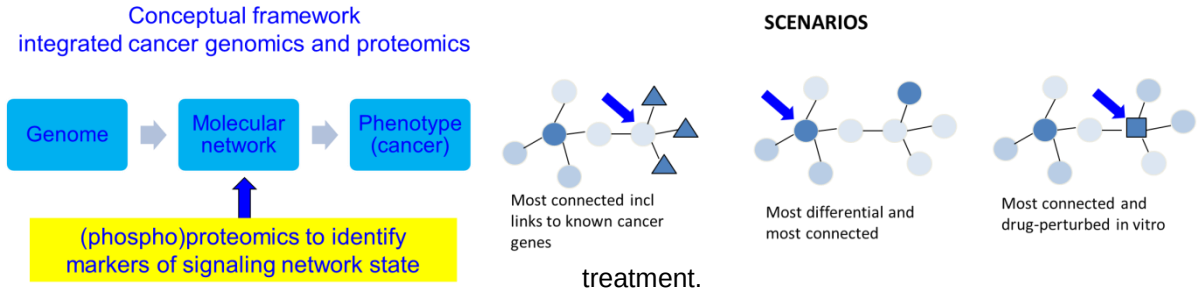
A pie chart and bar graph representation is convenient for identification of hyperphosphorylated protein kinases: see example phosphopeptide spectral counts for EGFR WT vs ΔVIII Mutant (Figure 2; see abstract on page 44).

*Kinase activation loop phosphorylation analysis to identify activated protein kinases*
In Figure 3 the workflow for extraction of activation loop phosphopeptide intensities from the MaxQuant phosphoSTY.text export is shown. Kinase ID mapping like shown for phosphopeptide spectral counting is performed using kinase sequences from kinase.com/Kinbase and ID mapping from Gene ID to IPI using the human protein reference database (HPRD). As quantitative option, loop phosphopeptide intensity can be normalized for protein expression changes in a differential experiment. Additional input is then required for normalization on (kinase)protein intensity (from MaxQuant ProteinGroups.txt). Currently we are working on construction of a targeted comprehensive assay for all kinase activation loop phosphopeptides. The bioinformatics analysis of all kinase amino acid sequences and extraction of activation loop sequences is performed in Clustal Omega, R and excel. Targeted assays for SRM and accurate inclusion mass screening on Q Exactive are being developed based on the bioinformatics sequence analysis.

Fig 3. Data analysis workflow for Protein kinase activation loop phosphorylation analysis




*Signaling network analysis to prioritize candidate driver/acitvated kinases*
In Figure 4 network-based analysis integrating OMICS data is shown. Various datasets from a variety of sources including (phospho)proteomics, public repositories with cancer genome sequencing data, expression arrays and data from functional screens may be integrated into a signaling network view. Visualisation is a key ingredient; our preferred network visualization tool is Cytoscape, combined with network input from STRING. The central assumption is that the most differential, hyperactive, and connected nodes are target for




treatment.

**Outlook**
The maturity of phosphopeptide data analysis modules in the MaxQuant proteomics data analysis environment is not at the same level as those available global protein expression profiling (protein Identification and quantification). Custom bioinformatics tools for phosphoproteomics data analysis and downstream network and GO analysis have been and are being developed at the OPL to answer

questions relevant for cell signaling state and therapy selection.

*Data mining, sub-project 3*

## Constructing spectral library from multiple proteomics experiments

Thang V. Pham, Connie R. Jimenez
OncoProteomics Laboratory, Department of Medical Oncology, VU University Medical Center, the Netherlands

**Background** Tandem mass spectrometry-based proteomics aims to translate sets of mass spectra (MS & MS/MS) to peptide and protein identification and quantification, and subsequently to link proteome data to clinical information. Currently, more than half of the MS/MS spectra are not identified in a typical experiment due to protein forms such as rare post-translational modifications, mutations, and splicing variants that cannot be handled by standard techniques. The set of unidentified spectra offers a rich source of biomarkers, especially for proteins at a low abundance level. The set of identified MS/MS spectra is a valuable resource to create spectral library for targeted proteomics such as SRM and SWATH.

**Aim** To exploit new computing infrastructures to characterize the large amount of unidentified MS/MS spectra for disease biomarker discovery and to construct high-quality spectral library.

**Approach** Our approach is to organize all MS/MS data into clusters of similar spectra (Frank AM, Nature method 2011). Clusters of high-quality, unidentified peptides will be identified by specialized peptide identification methods.

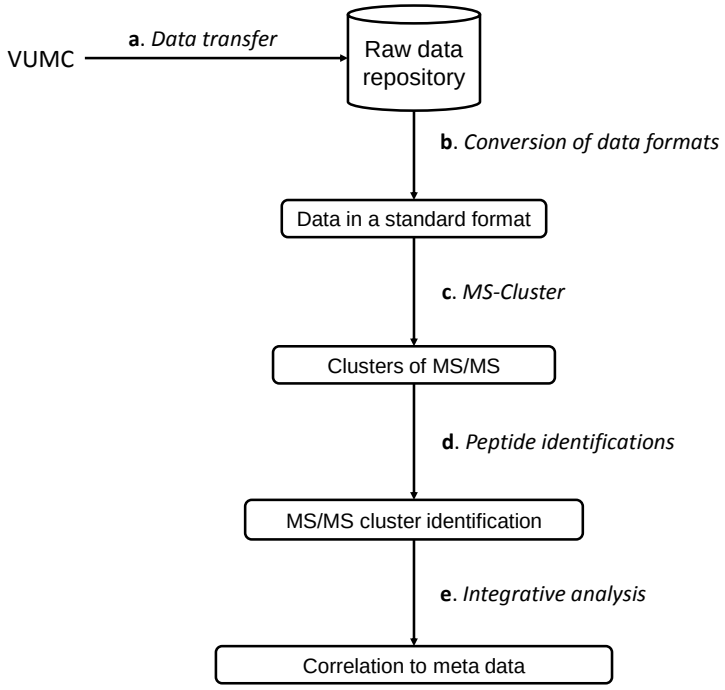



Fig. 1. Five steps in integrative analysis of large-scale MS/MS datasets.

**Results** We have gathered MS/MS data in the VUmc store4ever system. In total we have generated 16TB of raw data, containing 235 million MS/MS spectra. We are in the process of implementing a clustering algorithm capable of dealing with this large amount of spectra.

**Conclusions and outlook** We expect that a global analysis of a large number of proteomics datasets will lead to novel peptides as well as biological insights obtained from multiple experiments.

*Data mining, sub-project 4*

# Accelerating the analysis of large-scale targeted proteomics data

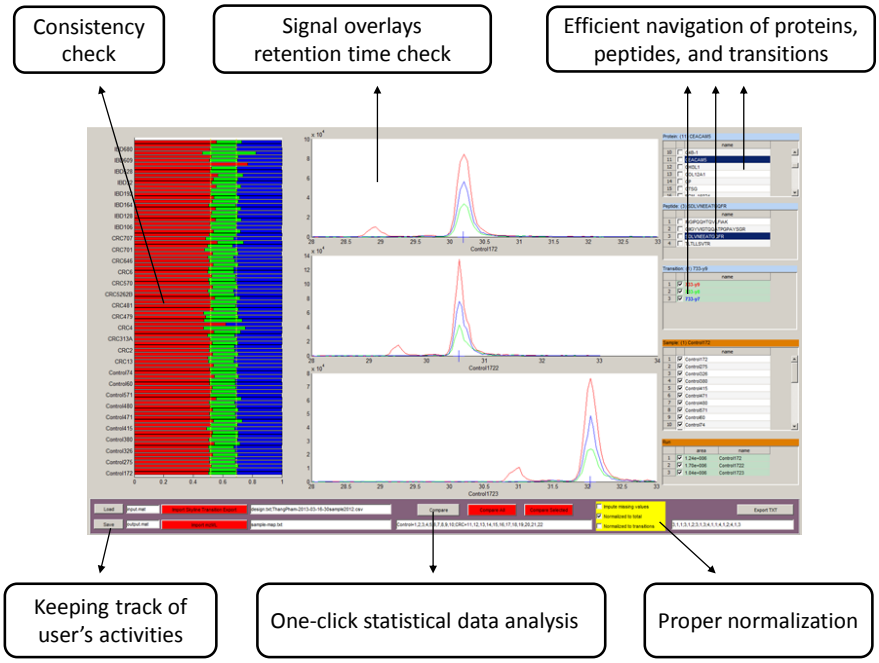
Thang V. Pham, Connie R. Jimenez
OncoProteomics Laboratory, Department of Medical Oncology, VU University Medical Center, the Netherlands

**Background** Mass spectrometry-based targeted proteomics was selected as the “Method of the Year” by the prestigious journal Nature Method in 2012. This technology offers biomedical researchers the opportunity to quantitatively monitor proteins of interest in biological samples with high sensitivity and reproducibility. In cancer research, this opens up an array of applications for early diagnosis and personalized therapy. In particular, it bridges the gap between candidate biomarker discovery and biomarker validation, departing from experiments involved a few samples to ones that require a multiplexed analysis of hundreds of samples.
The current practice in analyzing the new type of data using Skyline software tool involves a large number of manual intervention steps. It has been estimated that a typical experiment will require a 10-month effort of a full time data analyst (Whiteaker et al., Nature Biotechnology 2011).

**Aim** To develop a software tool that provides more efficient processing of mass spectrometry data, and consequently, more reproducible results.

**Approach** We use a novel mathematical modeling of user interaction and expert knowledge whereby a machine learning algorithm replaces rule-based heuristics.

**Results** We have implemented a prototype of the software, allowing a one-click statistical analysis of Skyline exports as well as spectral overlay of raw data for visual validation.




**Conclusions and outlook** This software tool will offer users of targeted proteomics a substantial added value to their analysis workflows.

# Simulated linear tests applied to quantitative proteomics

Thang V. Pham, Connie R. Jimenez
OncoProteomics Laboratory, Department of Medical Oncology, VU University Medical Center, the Netherlands

**Background** Experimental proteomics data exhibits inherent technical variation (see Fig. 1). 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 approaches have placed both technical and biological variations in tightly integrated mathematical models, which are difficult to adapt for different sources of technical variation.

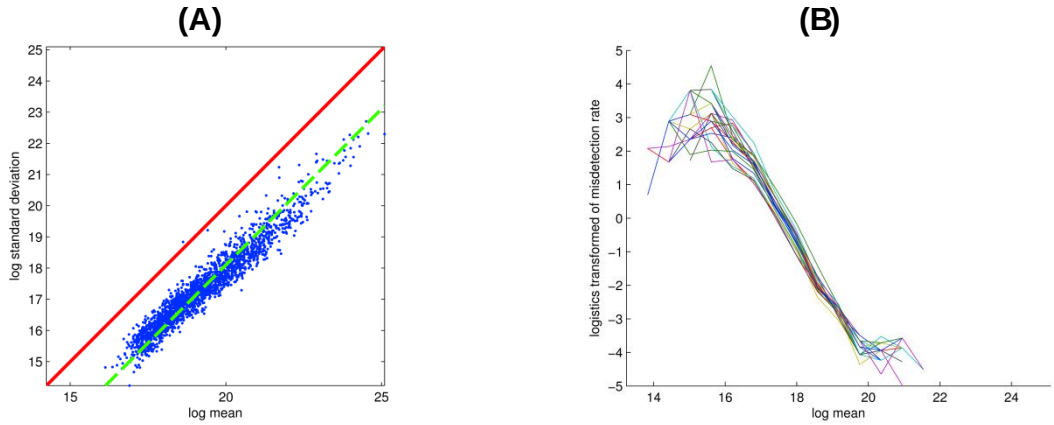



Fig. 1. (A) Variation in protein quantification in 23 LCMS runs of Jurkat cell lines shows a linear trend with respect to protein abundance. (B) The rate of missed identification reduces for higher abundance proteins. These types of knowledge can be included in a statistical model for accurate significance analysis.

**Aim** To derive a statistical framework that allows the inclusion of a wide range of technical variability.

**Approach** We propose a generic statistical model that allows for the inclusion of a variety of technical models. Our approach is based on linear modeling which supports a wide range of statistical designs including independent sample test, paired sample test and time course experiments. The central idea is to simulate likely data points from the technical distribution. The resulting test is therefore called the simulated linear test. We provide a generic and efficient method for model fitting. Devising a new test is then considered an engineering task, where most of the effort can be spent on obtaining an appropriate model for technical variation.

**Results** We demonstrate the applicability of the proposed approach by deriving a new significance tests for quantitative discovery proteomics for which missing values have been a major issue for traditional methods such as the *t*-test. We evaluate the results on three label-free (phospho) proteomics datasets with both spectral counting and ion-intensity based quantitation.

**Conclusions and outlook** Experimental results suggest that the simulated linear test is an attractive method for significance analysis of proteomics data.

# Other core/ collaborator abstracts

**Miscellaneous cancer proteomics**
**Neuroproteomics**
**Proteomics of signaling protein complexes**
**Proteomics of perturbation –induced cell states**
**Proteomics of mycobacteria**

# Maspin is a marker for early recurrence in primary stage III and IV colorectal cancer

Snoeren N[1*], Emmink BL[1*], Goos JA[3], Piersma SR[2], de Wit M[3], Pham TV[2], Belt EJ[4], Bril H[5], Stockmann HB[6], Fijneman RJ[3], Meijer GA[3], Jimenez CR[2], Kranenburg O[1], Borel Rinkes IHM[1].

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[4] *Department of Surgery, VU University Medical Center, De Boelelaan 1117, 1081HV Amsterdam, The Netherlands*
[5] *Department of Pathology, Kennemer Gasthuis, Boerhaavelaan 22, 2035 RC Haarlem, The Netherlands*
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** equal contribution to study*

**Background**: Little is known about the factors that drive metastasis formation in colorectal cancer (CRC). Here, we set out to identify genes and proteins in patients with colorectal liver metastases that correlate with early disease recurrence. Such factors may predict a propensity for metastasis in earlier stages of CRC.

**Methods**: Gene expression profiling and proteomics (n=5 per group) were used to identify differentially expressed genes/proteins in resected liver metastases that recurred within 6 months following liver surgery vs those that did not recur for >24 months. Expression of the identified genes/proteins in stage II (n=243) and III (n=176) tumours was analysed by immunohistochemistry on tissue microarrays. Correlation of protein levels with stage-specific outcome was assessed by uni- and multivariable analyses.

**Results**: Both gene expression profiling and proteomics identified Maspin to be differentially expressed in colorectal liver metastases with early (<6 months) and prolonged (>24 months) time to recurrence. Immunohistochemical analysis of Maspin expression on tumour sections revealed that it was an independent predictor of time to recurrence (log-rank P=0.004) and CRC-specific survival (P=0.000) in stage III CRC. High Maspin expression was also correlated with mucinous differentiation. In stage II CRC patients, high Maspin expression did not correlate with survival but was correlated with a right-sided tumour location.

**Conclusion**s: High Maspin expression correlates with poor outcome in CRC after spread to the local lymph nodes. Therefore, Maspin may have a stage-specific function possibly related to tumour cell dissemination and/or metastatic outgrowth.

**Publication**

Snoeren N, Emmink BL, Koerkamp MJ, van Hooff SR, Goos JA, van Houdt WJ, de Wit M, Prins AM, Piersma SR, Pham TV, Belt EJ, Bril H, Stockmann HB, Meijer GA, van Hillegersberg R, Holstege FC, Jimenez CR, Fijneman RJ, Kranenburg OW, Rinkes IH. Maspin is a marker for early recurrence in primary stage III and IV colorectal cancer. PLoS One. 2013 Sep 17;109(6): 1636-47.

# miR-200a-mediated suppression of non-muscle heavy chain IIb inhibits meningioma cell migration and tumor growth in vivo

Senol O[1], Schaaij-Visser TB[2], Erkan EP[3], Dorfer C[4], Lewandrowski G[5], Pham TV[2], Piersma SR[2], Peerdeman SM[6], Ströbel T[7], Tannous B[5], Saydam N[3], Slavc I[3], Knosp E[4], Jimenez CR[2], Saydam O[3].

1Department of Neurology and Radiology, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA. 2OncoProteomics Laboratory, Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands. 3Molecular Neuro-Oncology Research Unit, Department of Pediatrics, Medical University of Vienna, Vienna, Austria. 4Department of Neurosurgery, Medical University of Vienna, Vienna, Austria. 5Experimental Therapeutics and Molecular Imaging Laboratory, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA. 6Department of Neurosurgery, VU University Medical Center, Amsterdam, The Netherlands. 7Institute of Neurology, Medical University of Vienna, Vienna, Austria.

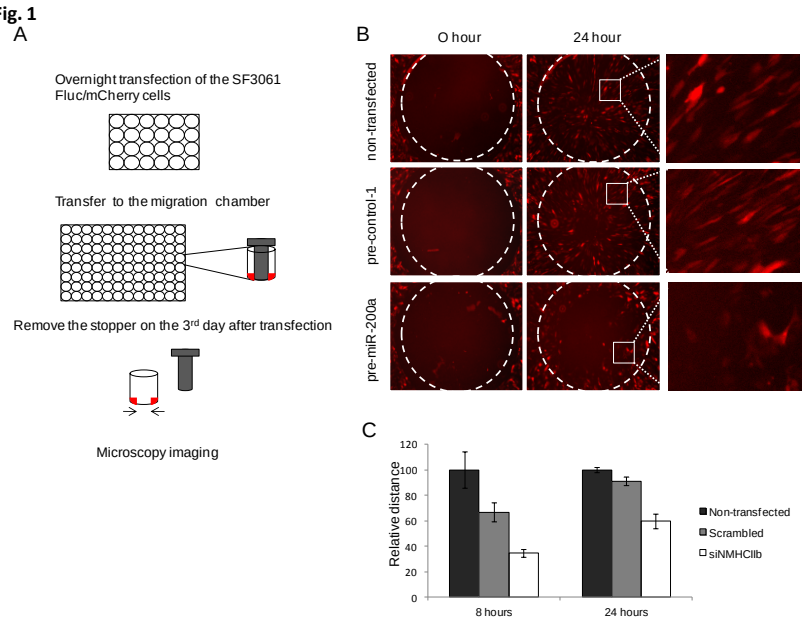
**Background** miR-200a has been shown to be implicated in the pathogenesis of meningiomas, one of the most common central nervous system tumors in humans.

**Aim and Approach** Identify novel target genes of miR-200a in primary meningioma cell lines by comparative protein profiling of meningioma cell lines +/- miR-200a

**Results** Out of a dataset pver 2000 proteins, 130 proteins were dysregulated in response to miRNA manipulation. Following the bioinformatic analysis to identify potential genes targeted by miR-200a, we focused on the non-muscle heavy chain IIb (NMHCIIb), and showed that miR-200a directly targeted NMHCIIb. Considering the key roles of NMHCIIb in cell division and cell migration, we aimed to identify whether miR-200a regulated these processes through NMHCIIb. We found that NMHCIIb overexpression partially rescued miR-200a-mediated inhibition of cell migration (Fig 1), as well as cell growth in vitro and in vivo. Moreover, siRNA-mediated silencing of NMHCIIb expression resulted in a similar migration phenotype in these cells and inhibited meningioma tumor growth in mice.

**Conclusions** Taken together, these results suggest that NMHCIIb might serve as a novel therapeutic target in meningiomas.

**Fig. 1**



# HP1-gamma's expression correlates with glioma grade and survival and is a putative marker of glioma stem like cells

Uros Rajcevic[1, 5], Sabrina Fritah[2], Petr V. Nazarov[3], Jaco Knol[4], Thang Pham[4], Sander Piersma[4], Mara Popovic[5], Rajko Kavalar[6], Tadej Strojnik[7], Neza Podergajs[8], Connie R. Jimenez[4] and Simone P. Niclou[2]

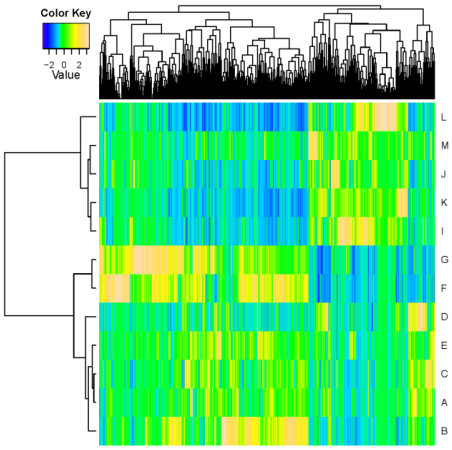
1 Blood Transfusion Centre of Slovenia, R&D, Šlajmerjeva 6, SI-1000 Ljubljana, Slovenia; 2 NorLux Neuro-Oncology Laboratory, Department of Oncology, CRP-Santé, Luxembourg, Luxembourg; 3 Genomics Research Unit, CRP-Santé, Luxembourg, Luxembourg; 4 Oncoproteomics Laboratory, VU University Medical Center, Amsterdam, The Netherlands; 5 Institute of Pathology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia; 6 Department of Pathology and; 7 Neurosurgery, Maribor University Medical Center, Maribor, Slovenia; 8 Department of Genetic Toxicology and Cancer Biology, National Institute of Biology, Ljubljana, Slovenia

**Background**: Genetic and signaling pathways involved in the development of glioblastoma have been relatively well characterized. Glioblastoma appears to be composed of heterogeneous cell populations, but the detailed cellular origin of these tumors is unknown. Gene/protein expression profiles of glioblastoma have been described that reflect stem-like characteristics of the tumor. However, no reliable markers exist today that differentiate glioblastoma stem-like cells from normal neural stem cells.

**Aim**: In this project we aimed at identifying novel markers linked to the phenotype of cancer cell stemness and analyzing their expression on a large number of clinical samples of patients with astrocytoma grades I-IV to establish their clinical relevance in glioma.

**Approach**: Using a label-free proteomic approach (geLC MSMS and spectral counting), we analyzed two different glioblastoma models with proven *de novo* tumorigenic properties (being enriched in putative glioma stem like cells), and compared them with primary neurospheres. To validate expression of novel markers in glioma patients, we built a large tissue microarray, composed of 330 patients with astrocytoma grades I-IV and screened the putative markers. Our data were also linked to publicly available glioma patient data.

**Results**: Of almost 2000 identified proteins, we found 482 to be significantly differentially expressed among the three groups. Among the identified substrates, the clusters of proteins with highest density of protein-protein interactions were functionally enriched in RNA processing related to development and metabolic processes.




We then focused on two putative quantitative markers of glioma stem-like cells, HP1-gamma (Heterochromatin Protein 1- gamma) (also known as CBX3 (chrome box 3)), an epigenetic regulator involved in RNA processing, and LDHA (Lactate Dehydrogenase A), a key metabolic enzyme of anaerobic glycolysis. We quantified their expression in a large set of clinical samples of gliomas of different grades by IHC on a tissue microarray and were able to show that the expression of HP1-gamma, highly upregulated in glioblastoma stem-like cells, correlates to glioma grade and overall patient survival. Independent analysis of publicly available glioma patient data showed that LDHA expression is linked to IDH1 mutation status both in high grade and low grade gliomas. The same analysis reinforced our findings towards an increased involvement of HP1-gamma in high grade gliomas.

**Conclusions and outlook**: Altogether, HP1-gamma and LDHA may reflect novel biomarkers for a cancer stem cell phenotype with different clinical importance in gliomas.
As adapted cancer metabolism and epigenetic regulation appear to be landmarks of cancer cell stemness, screening other markers related to these phenomena will strengthen our understanding of glioma stem-like cells.

*'This project is supported by the Fonds National de la Recherche (FNR) of Luxembourg.'*

# A factor that rebuilds immunity in mice and humans

Loredana Ruggeri, Elena Urbani, Federica Susta, Pier Luigi Orvietani, Davide Chiasserini, Emanuela Burchielli, Antonella Tosti, Antonella Mancusi, Fabiana Topini, Dunia Ramarli[1], Stefano Bruscoli, Connie, Jimenez[2], Georg Hollander[3], Luciano Binaglia and Andrea Velardi

University of Perugia, University of Verona[1], VU University Amsterdam[2], University of Basel[3].

**Background** Long-lasting immune incompetence underlies up to 40% infection-related deaths after allogeneic hematopoietic cell transplantation for leukemia. To date, attempts to improve immune reconstitution have been disappointing. We previously found that, in HLA haplotype-mismatched transplants, donor-versus-recipient natural killer (NK) cell alloreactivity eradicated leukemia without causing GvHD (Ruggeri et al., Science 2002; Blood 2007; Mancusi et al., 2015 submitted).

We next discovered alloreactive natural killer (NK) cells accelerated post-transplant immune cell recovery, i.e., dendritic cells (DCs), B- and T-cell precursors, and their transition to immune effector cells. Murine transplant in vivo models and human in vitro systems concurred to show that donor-versus-recipient alloreactive NK cells promote an extraordinarily accelerated maturation of donor-type B- and T-cell precursors. Donor-versus-recipient alloreactive NK cells triggered recipient DCs to synthesize and release protein factor in a DNA translation-depended fashion (i.e., blocking DNA transcription in DCs abrogated the DC ability to produce the factor). This factor, in turn, induced bone marrow and thymic stromal cells to produce IL-7 and c-Kit ligand and, thereby, the extraordinarily accelerated maturation of donor DCs, B- and T-cell precursors.

**Aim and approach** To isolate de-novo synthesized functional protein(s), NK:DC culture supernatants were fractionated under non-denaturating conditions by hydrophobic interaction or anion-exchange chromatography. Murine fractions were infused into conditioned mice to test their “immune rebuilding” effect by thymocyte counts. Human fractions were were tested for their ability to support human thymocyte proliferation and differentiation in vitro. In both instances, only one out of several fractions contained all of the biological activity. In humans as in mice, the protein had a specific, identical molecular weight.

In view of the striking biological and biochemical similarities displayed by the murine and the human “immune rebuilding” factors, we hypothesized they might indeed be the same molecule. Mass spectrometry analysis by stable isotope labelling with amino acids in cell culture (SILAC) was used to identify newly synthesized protein(s) in murine and human alloreactive NK cell+DC samples.

**Results** In human samples we identified 8 proteins showing heavy label in NK+DC samples and not in controls. The total number “heavy label” peptides in the mouse experiments was 144. The overlap analyses between the human and mouse samples showed two specific proteins as the only ones with at least one heavy peptide identified in both human and mouse NK+DC samples and not present in the control.

Direct evidence that one of the two proteins was indeed the “immune rebuilding factor” was obtained by: 1) Loss of “immune rebuilding” function when such protein was removed from murine and human alloreactive NK-DC cell culture supernatants by antibodies. 2) Loss of “immune rebuilding” function in mice that were knock-out for the specific gene. 3) Repair of function infusing the KO mice with alloreactive NK-DC co-culture supernatants from healthy mice. 4) Finally, RNA interference silenced the specific gene in human dendritic cells, as their co-culture with alloreactive NK cells and functional assessment of supernatant demonstrated loss of biological activity.

# Target identification for microRNAs that play a role in the persistence of acute myeloid leukemia stem cells

Dave de Leeuw[1], Han Verhagen[1], Fedor Denkers[1], Inge de Reus[2], Jaco Knol[2], Arjo Rutten[1], Gerrit Jan Schuurhuis[1], Gert Ossenkoppel[1], Connie Jimenez[2] and Linda Smit[1]

[1]**Department of Hematology and [2] Oncoproteomics laboratory, Cancer Center Amsterdam, VU University Medical Center, Amsterdam**

**Background:** Despite high remission rates after chemotherapy, only 30-40% of acute myeloid leukemia (AML) patients survive five years after diagnosis. This poor outcome is mainly due to relapse, caused by a subpopulation of chemotherapy resistant leukemic cells with stem cell-like properties, often referred to as “leukemic stem cells” (LSC). LSC co-exist with normal hematopoietic stem cells (HSC) in the AML bone marrow. To improve AML outcome, it will be crucial to develop alternative therapies that specifically target LSC while sparing HSC.
The major challenge in targeting these LSC includes their heterogeneity and stem cell-like properties. As microRNAs (miRNAs) target multiple genes simultaneously and as manipulation of their expression can therefore affect multiple pathways, their modulation may hold the key to successful elimination of therapy resistant leukemic (stem) cells.
For development of specific anti-LSC therapies the identification of differences between LSC, HSC and the AML bulk will be critical. Identification of these differences is most relevant in cell fractions obtained from the same AML bone marrow, taking into account effects of the leukemic microenvironment.

**Preliminary Results:** To identify and purify LSC and discriminate them from HSC, we described leukemia-associated markers and showed that LSC have lower aldehyde dehydrogenase (ALDH) activity than HSC co-existing in the AML bone marrow. Using these markers we purified LSC, HSC and leukemic progenitors (LP) from AML bone marrows and reported for the first time the comparison between expression of miRNAs in LSC and LP and between LSC and residual HSC [De Leeuw et al. Cancer Research 2014]. In this manner we identified several LSC- and HSC-specific miRNAs (Figure 1A and 1B).
For a number of these miRNAs we correlated expression with outcome and performed functional studies. So far, we showed that the expression of miR-126 is associated with a poor AML outcome and that miR-126 knockdown results in reduced LSC survival while HSC are not affected [De Leeuw et al. Cancer Research 2014]. Moreover, we showed that miR-551b is highly expressed in HSC and that its expression in AML is an independent prognostic marker for relapse (submitted). In addition, we showed that miR-22, miR-151, miR-221 and miR-335 are differentially expressed between LSC, HSC and LP that reside within AML bone marrows.

**Aim:** In this project we aim to identify targets for miR-22, miR-151, miR-221, miR-335 and miR-551b and study their functional role in AML stem cell persistence.

**Approach:** We will identify targets for these miRNAs by searching for miRNA target predictions in available online resources, such as miRanda, MirBase, Pictar and Targetscan, and also in databases that contain predictive miRNA targets including experimental support, such as Tarbase, Argonaute and miRNAMAP. We will also experimentally search for miRNA targets by studying changes in mRNA (RNA sequencing in collaboration with Dr. R. Kerkhoven, NKI, Netherlands) and protein expression after overexpression of the particular miRNA in AML cell lines. After identification of targets we will study their expression in the bulk, and LSC, HSC and LP fractions of AML bone marrows. Depending on the identity of the target gene and available inhibitors we will study the function of the target gene in leukemic cell maintenance and its potential as a therapeutic target in elimination of leukemic cells.

**Results:** We started by generating AML cell lines that ectopically express miR-551b (Figure 1C). Next, we compared the transcriptome and proteome of AML cells that overexpress miR-511b with control cells and showed that 999 genes were significantly upregulated and 945 significantly downregulated in two or more cell lines after overexpression of miR-551b (Figure 1D). From the 990 genes that were upregulated in at least two cell lines, 70 genes were positively co-expressed with miR-551b in leukemic cells of AML patients. From the 945 genes that were downregulated in two or more cell lines, 22 genes were negatively correlated with miR-551b in AML and might be direct targets. Comparing the expression of proteins before and after the ectopic expression of miR-551b in the AML cell lines showed many up- or down-regulated proteins induced by miR-551b (Figure 1E). Nine of all these proteins are significantly up- and 38 are significantly down-regulated after miR-551b overexpression. Comparing protein expression in KG1, THP1, OCE-AML3, Kasumi-1 and Hel overexpressing miR-551b with their control counterparts resulted in respectively 28, 52, 15, 24 and 63 proteins that are significantly up- or down-regulated.

**Figure 1**

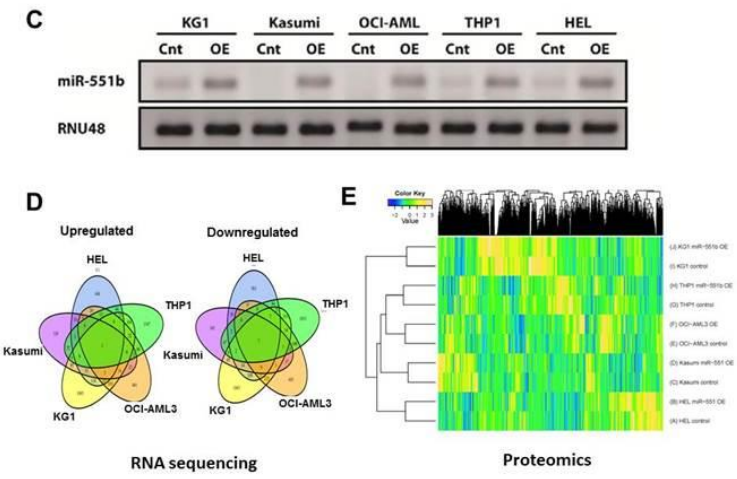
**A**

| Ratio LSC/Progenitor | Fold change | | | | | | | Number of patients |
|---|---|---|---|---|---|---|---|---|
| | AML1 | AML2 | AML3 | AML4 | AML5 | AML6 | Average | |
| hsa-miR-1274a | 0.801 | 0.887 | 0.941 | 0.850 | 0.862 | 0.983 | 0.887 | 6/6 |
| hsa-miR-886-3p | 0.880 | 0.922 | 0.995 | 0.849 | 0.855 | 0.978 | 0.913 | 5/6 |
| hsa-miR-1305 | 0.944 | 0.853 | 0.995 | 0.917 | 0.910 | 0.968 | 0.931 | 5/6 |
| hsa-miR-18a | 0.964 | 0.799 | 0.949 | 0.945 | 1.003 | 0.979 | 0.940 | 5/6 |
| hsa-miR-1260 | 0.929 | 0.915 | 0.949 | 0.957 | 0.902 | 1.000 | 0.942 | 5/6 |
| hsa-miR-1914* | 0.971 | 0.909 | 0.981 | 0.940 | 0.942 | 0.975 | 0.953 | 6/6 |
| hsa-miR-93 | 0.978 | 0.861 | 0.969 | 0.971 | 0.944 | 1.000 | 0.954 | 5/6 |
| hsa-miR-150 | 1.039 | 0.993 | 1.029 | 1.025 | 1.060 | 1.022 | 1.028 | 5/6 |
| hsa-miR-335 | 1.070 | 0.954 | 1.063 | 1.032 | 1.052 | 1.052 | 1.037 | 5/6 |
| hsa-miR-126* | 1.032 | 1.079 | 1.028 | 1.083 | 1.048 | 1.083 | 1.059 | 6/6 |
| hsa-miR-22 | 1.022 | 1.154 | 1.037 | 1.084 | 1.062 | 1.024 | 1.064 | 6/6 |
| hsa-miR-126 | 1.070 | 1.386 | 1.048 | 1.078 | 1.048 | 1.058 | 1.115 | 6/6 |

**B**

| Ratio LSC/HSC | Fold change | | | |
|---|---|---|---|---|
| | AML2 | AML3 | AML6 | Average |
| hsa-miR-551b | 0.691 | 0.881 | 0.717 | 0.693 |
| hsa-miR-10a | 0.929 | 0.664 | 0.654 | 0.749 |
| hsa-miR-151-5p | 0.805 | 0.799 | 0.820 | 0.808 |
| hsa-miR-29b | 0.765 | 0.869 | 0.886 | 0.841 |
| hsa-miR-125b | 0.741 | 0.942 | 0.909 | 0.864 |
| hsa-miR-23b | 0.836 | 0.859 | 0.914 | 0.870 |
| hsa-miR-196b | 0.918 | 0.868 | 0.846 | 0.877 |
| hsa-let-7c | 0.934 | 0.946 | 0.915 | 0.931 |
| hsa-miR-130a | 1.069 | 1.124 | 1.121 | 1.105 |
| hsa-miR-22 | 1.262 | 1.120 | 1.090 | 1.157 |
| hsa-miR-21 | 1.237 | 1.061 | 1.242 | 1.187 |
| hsa-miR-221 | 1.073 | 1.191 | 1.496 | 1.240 |
| hsa-miR-181b | 1.217 | 1.123 | 1.488 | 1.276 |

**MiR-22,**
**MiR-151**
**MiR-221**
**MiR-335**
**MiR-551b**




(A) MiRNA expression ratios between LSC and LP. Italic printed ratios are non-significant differences for that individual patient. The average ratio is calculated for all 6 AML patients. (B) MiRNA expression ratios between LSC and HSC. The average ratio is calculated for 3 patients. In both A and B, colors represent strength of the ratio in an individual patient. More intense red; higher expressed in LSC than in LP (A) or higher in LSC than in HSC (B), more intense blue; higher expression in LP compared to LSC (A) or in HSC compared to LSC (B). Several miRNAs were selected for target identification.
(C) MiR-551b was overexpressed in AML cell lines and expression was measured by QRT-PCR. (D) RNA was isolated and RNA-sequencing was performed. Genes down- or up-regulated in cells with miR-551b overexpression were compared with expression of genes in control cells and overlap of gene expression differences was determined. (E) Proteins were isolated from the cell lines and identified by proteomics. Unsupervised clustering showed that AML cell lines with the control vector and overexpression of miR-551b cluster together.

**Conclusions and outlook**

We have completed the target identification of miR-551b and will validate and further explore the function of these targets. In addition, we will start with identification of targets for miR-22, miR-151, miR-221 and miR-335. Target identification for these miRNAs might result in identification of mechanisms or signalling pathways responsible for leukemic cell persistence and the role of the miRNAs in this process. Moreover, miRNA target identification might result in identification of genes or pathways easier to therapeutically target than the miRNA itself since many small-molecule inhibitors against signalling molecules are already available.

# Identification and characterization of potential ligand(s) for the C-type Lectin-like Receptor CLEC12A

**P. Noordhuis[1], S. Piersma[2], J. van Meerloo[1], C. Jimenez[2], G.J.Schuurhuis[1]**

**[1]Dept. of Hematology and [2]OncoProteomics Laboratory, VU University Medical Center, Amsterdam**

**Background**

**The CLEC12A receptor (CLL-1, MICL, KLRL1, DCAL-2) is frequently expressed on AML stem cells in contrast to its normal counter part (van Rhenen Blood 2007, Leukemia 2007).**
**Previously we showed that antibody-drug-conjugates targeting this receptor showed high activity towards AML progenitor cells in short term (24 hr) incubation assays and towards AML stem cells as determined after long term liquid culture and CFU assays. Also specific lysis of AML progenitor cells and leukemic stem cells could be demonstrated using bispecific BiTE antibody that redirects T-cells to CLEC12A expressing AML (stem) cells.**
**Little is known about the function of the inhibitory CLEC12A receptor both in healthy and leukemic cells. Identification of its endogenous ligand might provide more insight in the function of this receptor.**

**Aim**

**Identification and characterization of potential ligands of the C-type Lectin-like receptor CLEC12A.**

**Approach**

**Cell lines previously shown to express an endogenous ligand were used for pull-down experiments to identify potential ligands using proteomic analysis. Candidate proteins were tested for binding to CLEC12A in cell free systems as well to intact cells.**

**Results**

**An initial pull-down assay was performed with soluble ecto-domain of CLEC12A in A549 cells, previously shown to express an endogenous ligand of the receptor. Proteomic analysis of the pull-down samples, including a sample with competing antibody, showed a number of possible candidate ligands. Analysis of a second pull-down experiment in MCF-7 cells confirmed the results of the A549 experiment.**
**To validate the results of the pull-down assays several experiments were performed: solid-phase binding of a putative recombinant ligand to the soluble ecto-domain of CLEC12A and binding of FITC-labeled recombinant ligand to the CLEC12A expressing cell line HL60.**
**The solid-phase binding assay showed a standard receptor-ligand binding curve indicating specific binding. The estimated Kd of the ligand was in the low nM range. Fluorescence microscopy of FITC-labeled recombinant ligand showed that the putative ligand was bound to CLEC12A expressing cell line HL60 and binding could be inhibited by competitive antibodies for the C-type Lectin-like domain of receptor and the putative ligand.**
**In order to have functional confirmation of receptor-ligand binding a chimeric receptor construct was developed consisting of the transmembrane and the ectodomain of CLEC12A and the intra-cellular domain of CD3zeta. After transduction in a NFAT-GFP reporter cell line the putative ligand will be tested together with competing antibodies.**

**Conclusion**

**A putative ligand for the C-type Lectin-like receptor CLEC12A was identified. Specific binding was confirmed in a solid-phase binding assay and by binding to intact cells. Functional confirmation and characterization is in progress.**

# Predictive biomarker(s) and therapeutic target(s) in radiation-resistant head and neck squamous cell carcinomas (HNSCC)

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**Background and aim:** Radiation therapy is one of the main anti-cancer treatment approaches in HNSCC. Despite effective loco-regional management of malignancies, tumor recurrences can occur if not all tumor-initiating cells are eradicated by ionizing radiation. Unfortunately, the exact molecular characteristics of radiation-resistant cells capable of initiating tumor re-growth are still unknown. There is also limited knowledge about molecular features of tumors that have very low recurrence and metastatic potentials. Hence, it would be useful to predict which cancer patients could benefit from radiation therapy and who could suffer tumor relapse in time. This prediction of radiation therapy outcome can help to avoid either overtreatment or to enhance efficacy of ionizing radiation by application of concurrent chemotherapy or targeted therapy in cancer patients.

**Aims** Therefore, the aim of this research project is (1) to determine molecular mechanisms underlying radiation resistance and recurrent development in HNSCC patients; (2) to identify putative biomarkers and potential therapeutic targets to predict and overcome radiation resistance; (3) to evaluate molecular properties of carcinoma cells with enhanced metastatic activities in order to protect cancer patients from metastasis development or effectively combat metastatic spread.

**Approaches:** Radiation resistant IRR cells were derived from parental cells after repeated exposure to ionizing radiation ten times every two weeks at a single dose of 10 Gy, resulting in a total dose of 100 Gy. Protein profiling in parental and IRR cells was carried out using two-dimensional differential gel electrophoresis (2D-DIGE) followed by MALDI-TOF/TOF mass spectrometry. Additionally, migrated HNSCC cells were harvested from the non-migrated and their protein profilings were compared using nano-LC-MS/MS followed by computational analysis of the identified proteins. Other molecular biology methods (Western blot analysis, ELISA, G-LISA, cell viability assays, FACS, immunocytochemistry, immunofluorescent analysis, etc.) were also used to prove the mechanistical roles of the identified proteins of interest.

**Results:** Proteins identified using proteomic approach were analysed for their common targets and regulators. It was found that these either up- or down-regulated proteins are closely related to the overexpression and activation of Rac1 protein. It was also demonstrated that Rac1 protein is implicated in the formation and activation of carcinoma stem cell properties, enhancement of metastatic abilities of radioresistant carcinoma cells and development of HNSCC cell resistance to chemo- and targeted therapeutics (cisplatin, docetaxel, EGFR blockers). Inhibition of Rac1 expression and activity resulted in the (1) enhancement of HNSCC sensitivity to ionizing radiation and chemotherapeutics; (2) inhibition of the migratory activities of radio- and chemoresistant HNSCC cells; (3) suppression of angio- and vasculogenesis-related events. Pre-clinical results were confirmed in clinical samples. While Rac1 was poorly presented in normal mucosa, tumor tissues revealed increased Rac1 expression. The most pronounced Rac1 presence was observed in HNSCC patients with poor early or late responses to chemo-radiotherapy. Tissues taken at recurrence were characterized not only by enhanced Rac1 expression, but also increased nuclear Rac1 content.

**Conclusions and outlook:** Increased expression, activity and subcellular localization of Rac1 could be associated with lower early response rate and higher risk of tumour recurrences in HNSCC patients and warrants further validation in larger independent studies. Inhibition of Rac1 activity can be useful in overcoming treatment resistance and could be proposed for HNSCC patients with primary or secondary chemo-radioresistance.

*This research project is not yet finished. Thus, the research group by I.Skvortsova currently works on the protein profiling of the metastatically active carcinoma stem cells. Received results will be published in cooperation with the members of the OPL. Furthermore, based on the preliminary results obtained during collaboration with OPL, allowed to submit 3 research project proposals to the Austrian Science Foundation (FWF). One research project is already positively evaluated and was financially supported after its minor revision. Two additional projects are under review. Additionally, this research project is also positively evaluated by the Austrian Ion Beam Research Board (A-IONTREB) and proposed for further conducting in collaboration with ion beam centers in Austria (MedAustron, Wr.Neustadt) and Japan (NIRS, Chiba). It is suggested that further financial support by FWF and A-IONTREB will allow to intensify research collaboration between EXTRO-Lab and OPL.*

# Identifying interaction partners for the Hedgehog pathway regulator Smoothened

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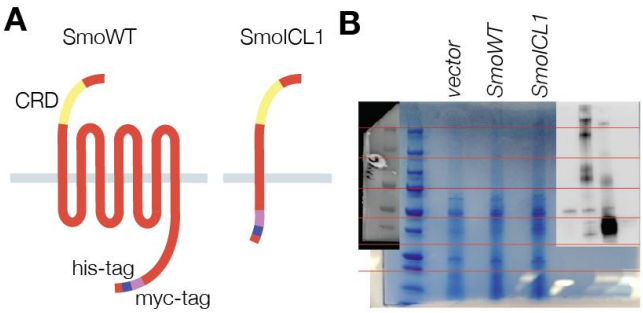
**Background**

Many patterning events in the developing embryo, as well as tissue homeostasis processes in adults, are driven by the *Hedgehog* pathway. In addition, deregulated Hedgehog signaling has been demonstrated to drive progression of tumors of the skin, lung, and pancreas. Unfortunately, our knowledge on the exact signaling mechanisms of this pathway remains incomplete. For instance, activation of the Hedgehog pathway through its positive regulatory receptor, *Smoothened* (Smo), is known to result in the activation of the pathway's transcription factors but my group has also identified an additional signaling mechanism through which the Hedgehog pathway can mediate cell motility and chemotaxis, independent of the pathways' transcription factors. This dichotomy and its functional consequences are robust, but the molecular basis underlying it remains unknown. Thus, the aim is to identify previously unknown interaction partners for Smo that can mediate and explain its dichotomous signaling actions.




**Methods**

For the first experiments, fibroblasts deficient for Smo (*Smo-/-*) were stably transfected with Myc-tagged forms of Smo indicated below, or vector control. SmoWT is the full-length receptor, and SmoICL1 are truncated forms (panel A) to allow binding only of those interactors that do not require Smo's heptahelical bundle. Following culture, cells were lysed, and coimmunoprecipitation was performed on Smo. Samples were run on SDS-PAGE (panel B) and immunoblotting was performed in parallel (panel B) to confirm expression of bait proteins and their molecular weights.

**Results**

First results have identified the bait proteins and previously known interactors for Smo like E3 ubiquitin ligases (top 10 hits shown below in Table, reads corrected for vector control).

| **Gene** | Fasta Header | Coverage (%) | MS/MS spectral count *vector* | MS/MS spectral count *SmoWT* | MS/MS spectral count *SmoICL1* |
|---|---|---|---|---|---|
| Smo | >sp\|P56726\| Smoothened homolog | 37.7 | 0 | 202 | 216 |
| Trim21 | >tr\|Q3U7K7\| E3 ubiquitin-protein ligase | 38.7 | 0 | 68 | 34 |
| Rpl5 | >sp\|P47962\| 60S ribosomal protein | 41.4 | 0 | 33 | 13 |
| - | >tr\|L7N202\| Uncharacterized protein | 45.1 | 0 | 24 | 14 |
| Rpl14 | >sp\|Q9CR57\|60S ribosomal protein | 25.8 | 0 | 18 | 5 |
| Rpl21 | >sp\|O09167\| 60S ribosomal protein | 16.2 | 0 | 17 | 1 |
| Ktn1 | >tr\|F8VQC7\| Kinectin | 12.6 | 0 | 17 | 3 |
| Rps15 | >sp\|P62843\| 40S ribosomal protein | 21.4 | 0 | 15 | 2 |
| Bag2 | >sp\|Q91YN9\| BAG family member 2 | 52.4 | 0 | 11 | 2 |
| Ide | >sp\|Q9JHR7\| Insulin-degrading enzyme | 10.4 | 0 | 11 | 3 |

Some other interesting and also novel proteins that could possibly prove to be *bona fide* interactors of Smo have come up, and these are currently being evaluated for possible follow-up experiments. However, the current list of candidate interactors is confounded by several hits indicative of overexpression artefacts and intracellular trafficking defects.

**Outlook**

We are currently optimizing methods that utilize novel Smo expression constructs to allow immunoprecipitation of the protein at the surface of the cell. The constructs required for this have been constructed, but the lysis and immunoprecipitation details need optimization to improve yield. This work is currently ongoing in my laboratory and will be pursued over the course of 2015.

# Proteomics analysis of HUVEC

Judy R. van Beijnum, Tse Wong, Arjan Griffioen – Angiogenesis Laboratory, Dept of Medical Oncology
Sander Piersma, Connie Jimenez – OncoProteomics Laboratory, Dept of Medical Oncology

**Background**
We have identified a panel of genes to be upregulated in tumor endothelium, and that may serve as therapeutic targets of anti-angiogenic therapy. One of these targets, vimentin, was previously considered an exclusively intracellular protein but has recently been suggested to also play roles outside of the cell. Preliminary investigations indicated vimentin is secreted by and deposited next to endothelial cells grown in culture.

**Aim**
To confirm the secretion and deposition of vimentin in human umbilical vein endothelial cells

**Approach**
HUVEC were cultured routinely. Conditioned medium, containing secreted factors, was collected. Cells were non-enzymatically detached and lysed. Proteins trapped in/on the growth substrate were collected by scraping these deposits off the culture dish using lysis buffer. Mass-spectrometry was performed to determine the identity and relative abundance of proteins in these three different cell culture fractions

**Results**
The results confirmed the abundant presence of vimentin in the cells, in the secretome as well as deposited on the growth substrate.

**Conclusions and outlook**
Analysis of putative splice variants of vimentin in these extracellular fractions is ongoing.

# Genome wide siRNA screen identifies the radiosensitizing effect of inhibition of MASTL and FOXM1 in NSCLC

Remco Nagel[1], Marijke Stigter-van Walsum[1], Marijke Buijze[1], Jaap van den Berg[2], Ida H. van der Meulen[3,4], Jasmina Hodzic[4], Sander R. Piersma[5], Thang V. Pham[5], Connie R. Jiménez[5], Victor W. van Beusechem[3,4] and Ruud H. Brakenhoff[1]

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**Background**

The kinase Microtubule Associated Serine/Threonine kinase-like (MASTL) has been identified as a regulator of mitotic progression. In recent reports Endosulphina alpha (ENSA) has been identified as a direct target for MASTL, and this target contributes to the progression through G2/M phase by downregulating the phosphatase PP2A. Recently, we identified a novel role for MASTL in the response to ionizing radiation (IR) in non-small cell lung cancer cells, where downregulation of this protein leads to radiosensitization. In our cells, however, we no regulation of ENSA by MASTL could be observed.

**Aim**

As the direct target(s) for MASTL in response to IR are unknown, the aim of the experiment was to gain insight in which targets were phosphorylated by MASTL.

**Approach**

In order to identify other candidate proteins that could be phosphorylated by MASTL, we performed a phosphoproteomics analysis on MASTL knockdown cells in the absence and presence of irradiation. For this purpose, control cells and MASTL knockdown cells were grown in six independent plates each, where three of these plates were irradiated with 2 Gy. One hour post treatment the cells were harvested and prepared for phosphoproteomics analysis.

**Results**

In total 8,410 peptides were identified in the phosphopeptide enriched samples, of which 7,603 were validated to be phosphopeptides. In total 8,263 phosphorylation sites were detected in the samples, of which 7,136 pSerine (86.4%), 1,082 pThreonine (13.1%) and 45 pTyrosine (0.5%)

In comparison to the control, 392 peptides corresponding to 254 unique proteins were at least 2-fold less phosphorylated in *MASTL* knockdown cells, including MASTL itself. A functional annotation clustering performed by DAVID software (http://david.abcc.ncifcrf.gov/home.jsp) showed that a number of differentially phosphorylated proteins were highly enriched in a few distinct functional clusters. Strikingly, a cluster involved in cell cycle regulation was significantly less phosphorylated after MASTL knockdown.

**Conclusions and outlook**

Using the phosphoproteomics analysis, a total of 254 potential MASTL targets were identified. A functional clustering analysis showed that a subset of these proteins plays a role in cell cycle regulation, which corresponds to the known function of MASTL. To identify critical targets of MASTL, subsequent follow up experiments are ongoing trying to rescue the effect of MASTL knockdown in response to radiation.

# Secretome proteomics of breast and colon cancer cell lines: Carcinoma origin dictates differential functional macrophage phenotype

Collaboration OncoProteomics lab (C. Jimenez), Pathology (R. Fijneman) and MCBI/Surgery (M. van Egmond)

***Background*** Macrophages are a more heterogenous group of cells than originally appreciated, performing distinct functions. Classically activated M1 macrophages act as pro-inflammatory effector cells that kill microorganisms and tumor cells. In contrast, alternatively activated M2 macrophages act as anti-inflammatory effector cells that promote tissue remodeling and repair[1]. Evidence is now emerging that tumor associated macrophages (TAM) predominantly act as alternatively activated macrophages, promoting tumor cell growth and suppressing immune responses[2-4]. Numerous studies reported on the ability of tumor cells to either downregulate cytotoxic activity of macrophages or directly stimulate macrophages to aid in tumor growth. The mechanisms and factors that tumors use to modulate macrophage effector functions are largely unknown. Interestingly, we observed a marked difference between effects of secreted colon carcinoma proteins versus proteins of mamma carcinoma on macrophage development. Whereas macrophages that were cultured in supernatant of colon carcinoma cells gained a M1 phenotype, mamma carcinoma supernatant induced an M2 phenotype.

**Aim**: Protein profiling of 4 mamma and 5 colon carcinoma secretomes to identify tumor type specific secreted proteins that may be involved in regulation of macrophage phenotype.

**Results** Comparison of secreted proteins by mamma versus colon carcinomas yielded ~500 and ~600 proteins that were differentially secreted by colon and mamma carcinoma, respectively. An internet search was performed for all differentially expressed proteins to investigate a potential link to monocytes or macrophages. For 16 proteins that were increased in colon carcinoma secretomes (Fold increase ranging from 2.4 to 195.9) and 12 proteins that were higher expressed in mamma carcinomas (Fold increase between 8.1 and 266.9) a reference with respect to monocyte/macrophage biology was found.

RT-PCR on all 28 proteins was performed to confirm differential protein expression. After combining secretome analysis, RT-PCR data and likelihood to be involved in monocyte/macrophage biology (based on literature search) we selected 6-8 candidate proteins as most interesting for further analysis (3-4 from mamma carcinoma secretome, and 3-4 from colon carcinoma secretome). Versican proved especially interesting, as it was exclusively produced by colon carcinoma cells (Bögels M. *et al*, in press), and shown to activate myeloid cells through TLR-2[5]. Moreover, pilot data demonstrated that IL-12, TNF-$\alpha$, and IL-6 production (markers of M1 macrophages) by monocytes, which had been incubated with colon carcinoma supernatant, was reduced in the presence of an anti-Versican monoclonal antibody. Transduction with Versican siRNA showed similar results.

**Conclusions and outlook** Versican was exclusively secreted by colon carcinoma cells. Furthermore, culture of monocytes in the presence of colon carcinomas resulted in the production of IL-12, TNF-$\alpha$, and IL-6 (M1 markers), which was decreased either in the presence of anti-Versican mAb or after transduction of colon carcinoma cellines with Versican siRNA. Thus, we hypothesise that Versican is involved in directing a M1 phenotype. Consequences for tumour development will be further investigated in mouse models. Additionally, the role of the other candidate proteins on macrophage phenotype and/ or tumour development still needs to be established.

**Publication**

Bogels M, Braster R, Nijland PG, Gul N, van de Luijtgaarden W, Fijneman RJA, Meijer GA, Jiménez CR, Beelen RHJ, van Egmond M. Carcinoma origin dictates differential skewing of monocyte function. OncoImmunology. 2012 Sept 1;1(6): 798-809.

Miscellaneous cancer proteomics

# Proteomics Profiling of cell lines from transgenic high-grade glioma mouse models

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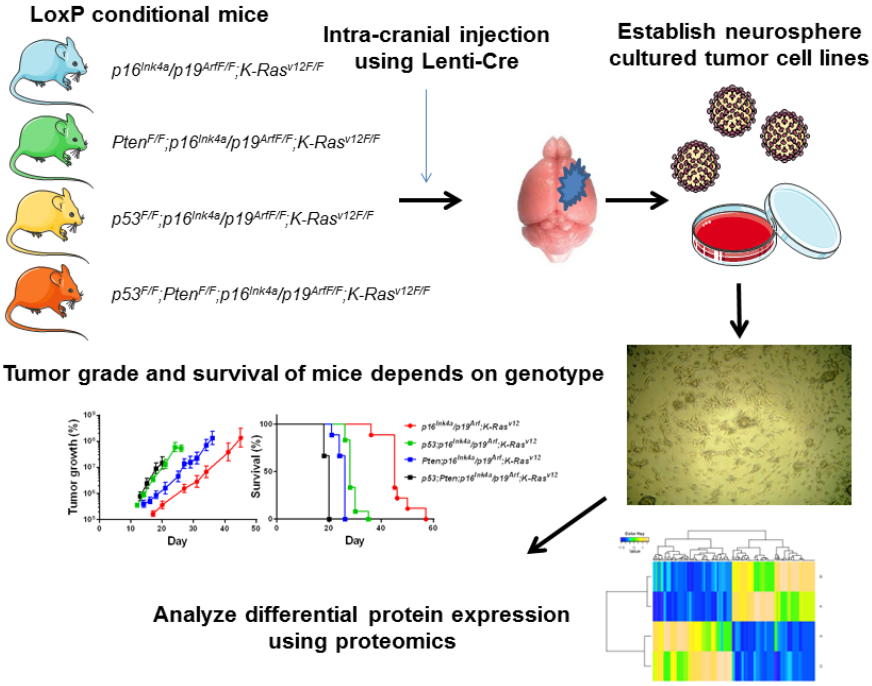
**Background:** Malignant gliomas are a group of brain tumors, which are traditionally classified grade II to IV based on histological features. Glioblastoma (GBMs) is the most aggressive subtype (WHO grade IV) and a devastating and uniformly lethal disease. Standard-of-care therapy consists of surgery and chemo-radiation and extends median survival only to about 12-15 months. Genetic analyses have demonstrated that GBMs frequently harbor alterations in the PI3K, MAPK or RB pathways. We have generated genetically relevant transgenic Cre-LoxP conditional glioma mouse models based on deletion of p16/p19, p53, and/or PTEN tumor suppressor genes. In depth histological analysis has demonstrated that these glioma models resemble many features human high-grade gliomas.

**Aim:** To determine the effects of relevant mutations in spontaneously developed gliomas at the proteome level and interrogate the correlation to WHO tumor grade and malignancy.

**Approach:** Tumors were induced in LSL-transgenic mice for kRAS$^{V12}$ also harboring loxP conditional alleles for tumor suppressor genes that are relevant for human GBM and firefly luciferase (Fluc) for non-invasive detection. Tumors develop within weeks following stereotactic intracranial injection of Cre lentivirus. Tumor cell lines were isolated from tumors of the various genetic backgrounds and cultured in serum-free medium. Lysates were prepared and subjected to total proteome analysis using MS based label-free proteomics. Bioinformatic approaches were then employed to discover correlations between tumor grade and protein expression patterns.

**Results:** Tumor grade, growth speed and overall survival depend on genetic background. *p16/p19;Kras$^{V12}$* were the slowest growing tumors and generally of grade III, whereas subtypes also lacking p53 and/or PTEN were mostly of grade IV. Survival ranked accordingly amongst the various genetic backgrounds. Proteomics revealed that the majority of differences in protein expression patterns are representative of an increase in metabolic demand to facilitate increased proliferation in higher graded gliomas, but distinct changes in specific protein networks for which we have no straightforward explanation (*e.g.* chromatin remodeling) have also been picked up. In depth bioinformatics analysis of the results is ongoing.

**Conclusions and outlook:** Increased tumor growth speed in higher graded gliomas is accompanied by protein wide adaptations to facilitate metabolic demand and high division rate. Targeting the protein networks involved may therefore impact glioma malignancy grade and growth speed. In depth bioinformatics analysis is ongoing and may shed light on which key players are predominantly affecting the observed proteomic changes in higher graded gliomas.



# Palmitoylation-dependent targeting of LMP1-TRAF2 complexes to endosomal membranes supports oncogenic NFκB activation and sorting into exosomes

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The Epstein Barr virus encoded oncoprotein LMP1 constitutively activates NFκB from intracellular membranes to promote cell growth and survival. Trafficking of LMP1 is mediated through association with CD63 explaining its release via exosomes. Whether TNF receptor-associated factors (TRAFs) that mediate downstream LMP1 signaling play a role in these processes has not been clarified.

Here we show that LMP1-TRAF2 signaling complexes localize to endosomes in a palmitoylation-dependent manner promoting LMP1-driven oncogenicity. We could recapitulate intracellular trafficking and endosomal membrane accumulation of activated LMP1-TRAF2 signaling complexes by ectopic LMP1 expression in HEK293 cells. Mutagenesis studies and chemical inhibition showed that LMP1-TRAF2 accumulation at endosomes is dependent on one single cysteine residue (C78), a known palmitoylation site of LMP1. Palmitoylation is a reversible post-translational modification and considered to function as a membrane anchor for proteins. Notably, growth assays in soft agar revealed that oncogenic properties of the C78 palmitoylation-deficient LMP1 mutant were diminished compared to wild type LMP1. Since LMP1 recruitment of TRAF2 and downstream NFκB signaling were not affected by palmitoylation, the specific localization of LMP1 at endosomal membranes appears crucial for its transforming potential. The importance of palmitoylation for trafficking to and signaling from endosomal membranes was not restricted to LMP1 as similar observations were made for cellular oncoproteins. Despite strong TRAF2 recruitment by LMP1 at endosomal membranes and prolific release of LMP1 via exosomes, TRAF2 was not detected in exosomes. Interestingly, point mutations in suspected TRAF-binding sites increased the sorting and release of LMP1 via exosomes.

These observations together reveal that LMP1-TRAF2 signaling complexes accumulate at endosomal membranes and that subsequent dissociation links LMP1 incorporation into luminal vesicles to downstream signaling .We propose that 'signaling endosomes' in EBV-infected tumor cells fuse with the plasmamembrane thereby having possible control over exosome-mediated cell-cell communication in the tumor microenvironment.

# Exosome sorting of virus-encoded 5'ppp-small RNA supports a host sensing mechanism of latent EBV infected B cells

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6Molecular Neurogenetics Unit, Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, MA
8OncoProteomics Laboratory, Dept. Medical Oncology, Cancer Center Amsterdam, , VU University Medical Center, de Boelelaan 1117HV Amsterdam, The Netherlands.

Epstein-Barr virus (EBV) establishes a persistent latent infection in virtually all humans avoiding uncontrolled immune responses despite massive production of inflammatory RNAs.
Here we show that latently EBV-infected B cells release exosomes that incorporate and transfer EBV-encoded small RNA (EBER1) into dendritic cells (DCs). Cell-secreted EBER1 is detectable in tonsillar exosomes and in skin lesions of cutaneous- and chronic discoid lupus erythematosus patients that are infiltrated with plasmacytoid (p)DCs. Primary pDCs express high surface levels of T-cell immunoglobulin mucin protein 4 (TIM4), a phosphatidylserine (PtdSer)-binding receptor that efficiently capture EBER1- carrying exosomes. Using an EBER-deficient EBV strain, enzymatic removal of 5'triphosphates (5'ppp) of exosome-RNA and in vitro transcribed EBER1 molecules, we established that exosome-delivered 5'pppEBER1 acts as the trigger of antiviral immunity in recipient DCs. Importantly, in latent EBV-infected cells, 5'pppEBERs shuttled into the cytoplasm are shielded from intrinsic RNA sensors by associating with ribonucleoproteins, including the major vault protein (MVP). Thus 5'ppp-EBER sorting and transfer via exosomes can exposes latent EBV that may trigger antiviral immunity in certain predisposed individuals.

# CSF biomarkers for early Alzheimer's disease

Davide Chasserini[1,2]*, Thorsten Mueller[3]*, Charlotte E. Teunissen[4], Thang V Pham[1], Sander R. Piersma[1], Silvina Fratantoni[1], Jaco C Knol[1], Lucilla Parnetti[2], Wiesje M. van der Flier[5], Philip Scheltens[5], Katrin Marcus[3], and Connie R. Jimenez[1]

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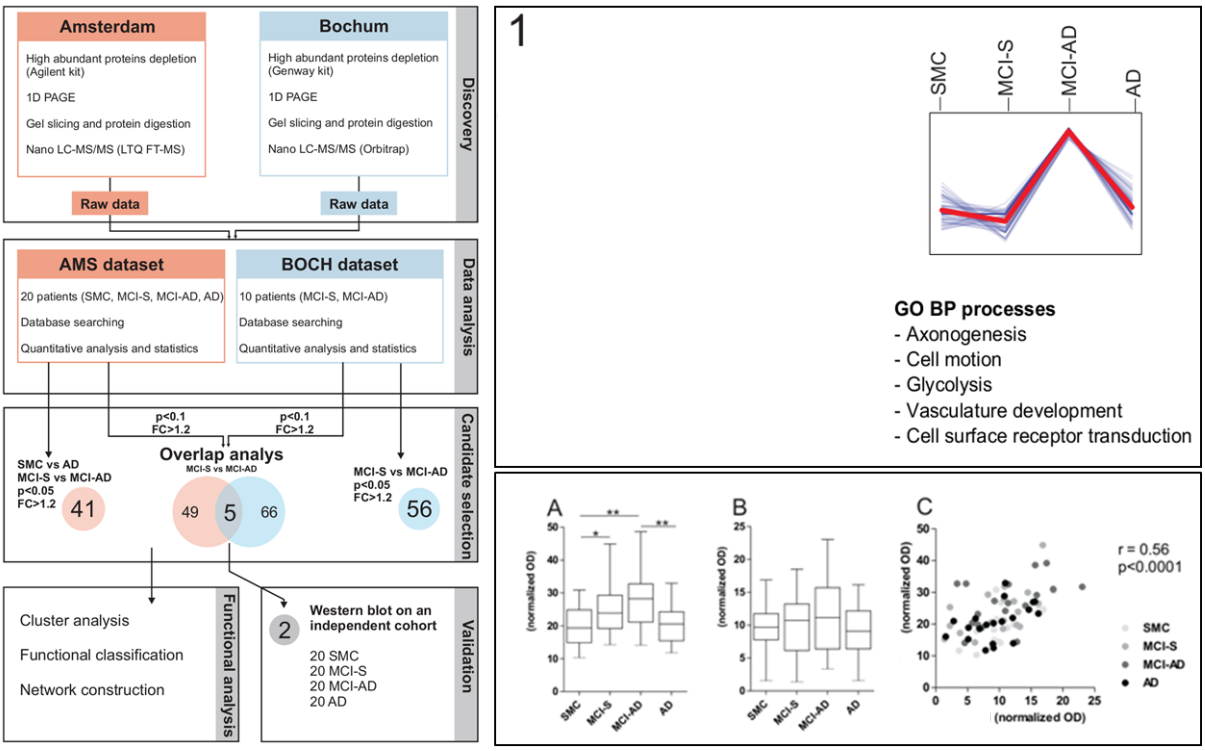
**Background:** Alzheimer's disease (AD) is the most common cause of dementia in the elderly, with a prevalence rate doubling every five years after 65 years of age. It is now established that the degenerative process in AD brain may begin 10 to 20 years before the clinical onset of the disease. During this preclinical phase there is a gradual loss of synapses and neurons, leading, later on, to the first cognitive symptoms. This condition, which does not reach clinical criteria for dementia, has been defined as mild cognitive impairment (MCI) and may be related to a multitude of pathogenetic factors besides AD . The hope for a disease-modifying treatment in the near future has attracted attention on biomarkers discovery for early detection of MCI converting to AD, ie, the so-called "pre-dementia phase of AD" or "MCI-AD" according to the Alzheimer's Association criteria.

**Aim:** the aim of this project was to discover new biomarkers for early AD in CSF, and to compare their performance with established AD biomarkers such as beta amyloid peptide 1-42 ($A\beta_{1-42}$), total tau (t-tau) and phosphorylated tau (p-tau).




**Approach:** In the context of sixth framework European project cNEUPRO (clinical NEUro PROteomics) we designed a two-center study for AD biomarker discovery and prioritization in CSF. We analyzed the CSF proteome from two high quality discovery cohorts of MCI patients recruited from two different European centers and prioritized the putative candidates using overlap analysis between the two datasets, expression clustering and functional annotations. CSF samples were analyzed in two laboratories (OPL, Amsterdam, and MPC, Bochum) using similar proteomic workflows, including depletion of high abundant proteins, mono-dimensional SDS-PAGE, label-free protein quantification and pathway analysis. Amsterdam cohort (AMS) was composed of patients diagnosed with mild cognitive impairment (MCI), patients with AD and control subjects and it was used as discovery

dataset. Top-candidates were then prioritized using overlap analysis with Bochum cohort (BOCH), composed of MCI-S and MCI-AD patients.

**Results:** Our analysis showed a significant overlap of identified proteins between AMS and BOCH whole datasets, together with a differential CSF protein profile in MCI-S and MCI-AD patients. Most part of the identified candidates were associated with cellular functions like neurogenesis, cell adhesion, axonal guidance, immune response and complement activation, globally showing an increase of neurogenesis related processes in early phases of AD paralleled by a down-regulation of immune and stress response pathways. Two candidates, Protein X and Protein Y, which showed an increase in the CSF of MCI-AD patients were also validated in an independent cohort of patients with western blot.

**Conclusions and outlook:** Protein X might represent a new biomarker for early AD, further validation using rigorous quantitative methods (ELISA, Selected reaction monitoring mass spectrometry) of this candidates is needed.

**Acknowledgements**: This research was supported by the European FP6 project: cNEUPRO

# Proteomic analysis of cerebrospinal fluid from early Alzheimer's disease, study 2

Davide Chasserini[1,2], Charlotte E. Teunissen[3], Thang V Pham[1], Sander R. Piersma[1], Jaco C Knol[1], Lucilla Parnetti[2], Philip Scheltens[4], and Connie R. Jimenez[1]

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**Background:** It is now established that the degenerative process in Alzheimer's disease (AD) begins several years before the clinical onset of the disease. During this preclinical phase there is a gradual loss of synapses and neurons, leading to the first cognitive symptoms. This condition, which does not reach clinical criteria for dementia, has been defined as mild cognitive impairment (MCI) and may be related to a multitude of pathogenetic factors besides AD. The hope for a disease-modifying treatment in the near future has attracted attention on the discovery of biomarkers for early detection of MCI converting to AD, i.e., the so-called "pre-dementia phase of AD". To this purpose, cerebrospinal fluid (CSF) represents the best compromise between accessibility and direct contact with central nervous system (CNS) structures. To date, three CSF biomarkers are able to predict progression to AD with relatively good sensitivity and specificity. Several studies have shown that CSF levels of beta amyloid peptide 1-42 (Aβ1-42), total tau (t-tau) and phosphorylated tau (p-tau) are able to detect AD not only in the overt phase of the disease, but also in the pre-dementia phase. However the performance of these three biomarkers for early diagnosis is still not optimal and current immunoassay methodologies lack of reproducibility among different labs. So, the search for biomarkers that might complement classical CSF AD biomarkers in early diagnosis is still open and it can be undertaken using different approach, either hypothesis driven or unbiased such as proteomics.

**Aim:** To search for new CSF candidate biomarkers of early AD in a well characterized cohort of AD and MCI patients with single-shot nanoLC-mass spectrometry.

**Approach:** A total of 80 patients were included in this study. Four different diagnostic groups were compared, a control groups composed of neurological controls (CTRL), a groups of MCI patients having a stable disease after at least two-years of follow-up (MCI-S), a group of MCI patients progressing to AD during follow-up (MCI-AD) and a group of AD patients. Each groups included 20 patients. CSF samples from patients were depleted of high abundant proteins, in-solution digested with trypsin and lysine-C and directly analyzed with QExactive mass spectrometer. Database search was performed with MaxQuant computational platform.

**Results:** a total of ~1400 proteins were identified across the eighty samples. Eighty-eight percent of the total proteins were identified in all the 4 diagnostic groups. According to beta-binomial statistics 45 proteins were differentially expressed in the CTRL vs AD comparison while 88 proteins showed altered levels between MCI-S and MCI-AD patients ($p<0.05$). Known candidate biomarkers of AD showed altered levels in AD CSF. Tau protein and heart fatty acid binding protein (HFABP) were increased in MCI-AD and AD patients when compared to MCI-S and CTRL groups. Interestingly several 14-3-3 protein isoforms, a known marker of neurodegeneration, showed increased levels in the MCI-AD and AD patients.

**Conclusions and outlook:** A number of already known and new candidate biomarkers for early AD has emerged from the high-throughput proteomic analysis of MCI patients. Bioinformatic analysis and immunochemical validation of top candidates on larger cohorts of AD patients is currently going on.

**Acknowledgements**: This research was supported by ISAO

# BRI2-BRICHOS is increased in human amyloid plaques in early stages of Alzheimer's disease

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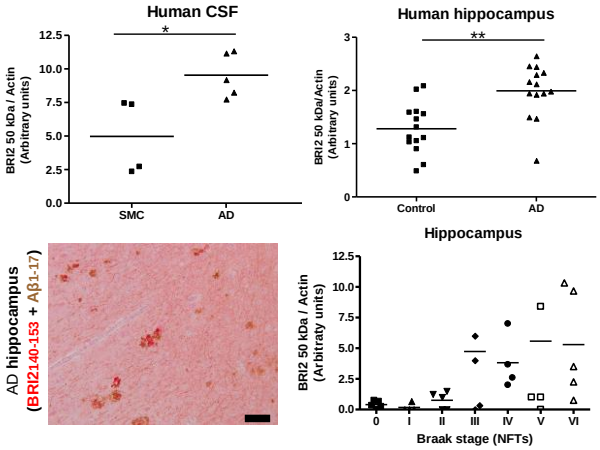
**Introduction** Alzheimer's disease (AD) is diagnosed in advanced stages of the disease. The current analysis of amyloid beta, tau and phosphorylated tau in cerebrospinal fluid (CSF) has limited value for early diagnosis. Using a proteomics approach, we found a significant increase of Integral membrane protein 2B (BRI2) levels in CSF from patients with AD and mild cognitive impairment (MCI) who later progressed to AD compared to controls and stable MCI patients. BRI2 protein binds amyloid precursor protein to halt amyloid-β production and inhibits amyloid-β aggregation via its BRICHOS-domain suggesting a link between BRI2 and Alzheimer's disease (AD). The relationship of BRI2 within human AD pathology is not known.

**Aim and approach** The aim of this study was to investigate the possible involvement of BRI2 in human AD pathogenesis. To this end, we analyzed BRI2 levels in human brain tissue from controls and AD-patients of different Braak stages. Moreover, the relationship between BRI2 and amyloid deposition was also analyzed.

**Methods** Polyclonal antibodies against a specific BRI2 peptide were produced by Biogenes (Germany). Immunohistochemistry analysis was performed on paraffin sections from AD patients (n = 17) and age-matched controls (n = 14)**.** Post-mortem human brain homogenates (HBH) from AD patients (HBH n = 14) and controls (HBH n=14) were analyzed by western blot.

**Results** BRI2 containing BRICHOS-domain was increased up to 3-fold in AD hippocampus ($p = 0.003$, n = 14/group). Immunohistochemistry showed BRI2 deposits associated with amyloid-β plaques in early pathologic stages (Braak-III; Thal-2/3). We observed a decrease in the protein levels of ADAM10 ($p = 0.02$) and furin ($p = 0.066$), as well as an increase in SPPL2b ($p < 0.0001$) in AD hippocampus. Because these enzymes are involved in BRI2 processing, their changes may lead to aberrant processing of BRI2 promoting its deposition and likely affecting BRI2 function. Loss of BRI2 function in AD was supported by the decreased presence of BRI2-amyloid precursor protein complexes in the hippocampus of AD patients compared with control subjects.




**Conclusions** In conclusion, our data obtained from human samples indicate that in early stages of AD there is an increased deposition of BRI2, which likely leads to impaired BRI2 function thereby influencing AD pathophysiology.

**Publication** Del Campo M, Hoozemans JJ, Dekkers LL, Rozemuller AJ, Korth C, Müller-Schiffmann A, Scheltens P, Blankenstein MA, Jimenez CR, Veerhuis R, Teunissen CE. BRI2-BRICHOS is increased in human amyloid plaques in early stages of Alzheimer's disease. Neurobiol Aging. 2014 Jul;35(7):1596-604.

**Neuroproteomics**

# Identification of novel diagnostic CSF protein biomarkers for FTD with high discriminatory power

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Background: Frontotemporal dementia (FTD) is the second commonest cause of early-onset dementia. Its underlying pathological spectrum, frontotemporal lobar degeneration (FTLD), can be divided in 2 main subtypes characterized by either tau or TDP-43 accumulations. Thus far, reliable biomarkers enabling the identification of FTD or distinguishing between its pathological subtypes are lacking. In the light of upcoming treatment options, such markers are highly needed.

Aim: The aim of this study was to identify cerebrospinal fluid (CSF) biomarkers for accurate diagnosis of the two main pathological subtypes of FTD using unbiased in-depth mass-spectrometry based proteomics.

Approach: FTD patients with TDP-43 (FTD-TDP-43, FTLD-TDP, n=12) or tau pathology (FTLD-tau, n=8), and asymptomatic controls with subjective memory complaints (SMC, n=10) were included. We analysed the CSF proteome after abundant protein depletion by 1D gel-nano-liquid chromatography coupled to tandem mass spectrometry. For validation, we carefully selected commercial ELISAs and tested their robustness before validation of these biomarkers in CSF of FTD patients in a partly overlapping larger cohort (FTLD-TDP, n=21, FTLD-tau, n=10, SMC, n=23) and of patients with other dementias (Alzheimer's disease (AD), n=20, dementia with Lewy bodies (DLB), n=20 and vascular dementia (VaD, n=18)).

Results: Out of a total dataset of 1914 CSF proteins, we identified 57 proteins that were differentially regulated (fold change>1.2, $p<0.05$) between the different patient groups: either between the two pathological subtypes (24 proteins), or between at least one of these FTD subtypes and controls (53 proteins). We confirmed the differential expression of 2 out of 4 candidate proteins for which commercial ELISA and Western blot were available, namely YKL-40 and FABP4, which are both known to be expressed in microglia. Further validation in a larger cohort showed that level of YKL-40 was 2-fold increased in both FTD pathological subtypes compared to controls, and that the levels in FTLD-tau we significantly higher compared to AD, DLB, and VaD patients. The CSF levels of FABP4 were significantly increased in FTLD-tau compared to controls, AD and DLB.

Conclusion: We performed high resolution CSF proteomics and identified 57 CSF biomarkers that appear very promising for both FTD diagnosis as well as subtyping of pathologies. The two positively validated biomarkers YKL-40 and FABP4 have a high potential for differential diagnosis, targeted treatment development and evaluation of FTD pathology-specific treatments.

Presented at International conference for Frontotemporal Dementia, Vancouver 2014

# 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.

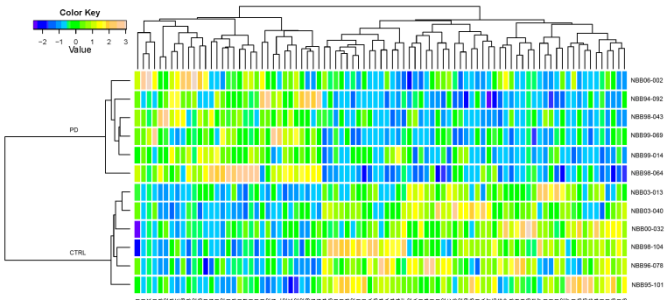


Figure: Heat map view of supervised cluster analysis using the differentially expressed proteins. Supervised clustering of 87 proteins (horizontal axis) that had significantly different expression levels between PD patients (PD) and controls (CTRL; vertical axis), with 33 upregulated and 54 downregulated proteins. The colors indicate the protein abundances.

**References**

Van Dijk KD, Berendse HW, Drukarch B, Fratantoni SA, Pham TV, Piersma SR, Huisman E, Brevé JJP, Groenewegen HJ, Jimenez CR*, van de Berg WDJ*. The proteome of the locus ceruleus in Parkinson's disease: relevance to pathogenesis. Brain Pathol. 2012 Jul;22(4): 485-98.

# Proteome of Cerebral Capillary Amyloid Angiopathy: relevance for amyloid clearance in Alzheimer's disease

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**Background**: Alzheimer's disease (AD) is characterized by progressive cognitive impairment associated with accumulation of amyloid β (Aβ) in the brain parenchyma as plaques and on the cerebral blood vessels as cerebral amyloid angiopathy (CAA). In 51% of AD cases Aβ accumulation is also found in and around cortical capillaries and it referred to as capillary CAA (capCAA). CapCAA cases exhibits a compromised blood-brain barrier (BBB) which could contribute to the impaired Aβ clearance observed in AD cases. Little is known about the molecular mechanism leading to capCAA in AD pathogenesis.

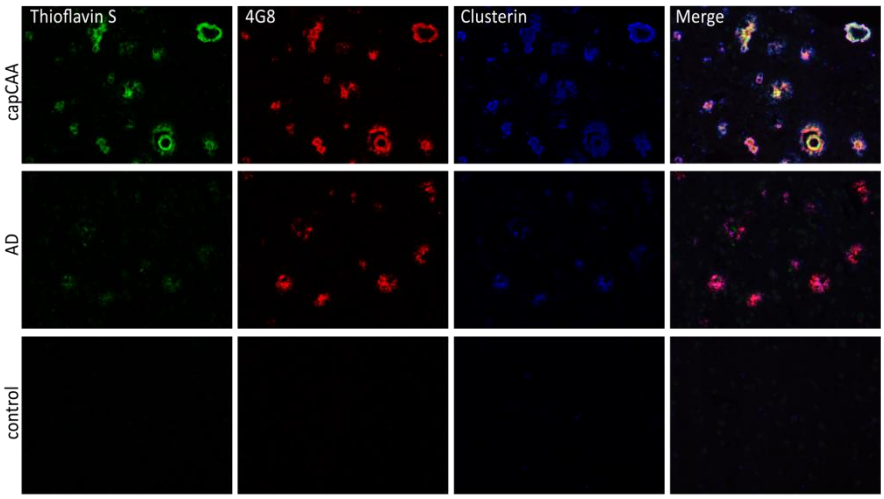
**Aims and approach** With this exploratory proteomic analysis on human post-mortem tissue to we aimed to identify differentially expressed proteins in cases that only show capCAA pathology compared to AD, in order to reveal specific insight in underlying molecular mechanisms resulting in disturbed clearance of Aβ across the BBB.
Furthermore by profiling the proteomes of capCAA patients and comparing them with AD cases, we aimed to identify proteins and processes that are involved in the pathogenesis of capCAA and possible biomarkers for the differential diagnosis of capCAA and AD.

**Methods** Protein lysates prepared from the occipital cortex of the brain were subjected to SDS-PAGE and in-gel polypeptide digestion with trypsin. Peptides were extracted and analysed by nanoLC-MS/MS using LTQ-FT mass spectrometry. Proteins were quantified using Spectral counting. Protein interaction networks and functional annotations were retrieved using String database and Ingenuity pathway analysis tool.

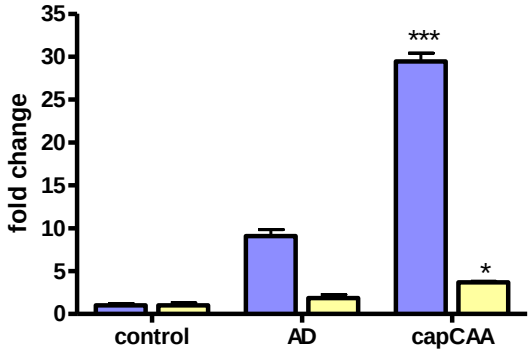
**Results** We have recently identified clinical AD cases that show extensive capCAA pathology without the common AD hallmarks like Aβ deposits in the parenchyma and neuronal accumulation of hyperphosphorylated tau (neurofibrillary tangles).
A proteomics analysis was performed using post-mortem brain tissue of patients with exclusive capCAA pathology, AD pathology (Braak stage 5) and age-matched non-demented control cases without AD pathology. Cases were selected on the basis of the clinical and neuropathological diagnosis and immunohistochemical characterisation of Aβ aggregates. For each condition 2 cases were selected (total of 6).
A total of 1547 proteins were identified among which a number of proteins that are known to have an altered expression profile in AD compared to controls, indicating the value of this approach. Interestingly, we also identified proteins that were differentially upregulated in capCAA compared to AD including laminin, serum amyloid P component (SAP) and clusterin (ApoJ). Immunohistochemical analysis was performed to validate these findings. Clusterin, a glycoprotein that binds amyloid-β peptides and is involved in the clearance of Aβ peptides and fibrils is localized in Aβ-laden capillaries, (Figure 1A). Clearance of Aβ peptides and fibrils occurs by binding to megalin receptors and enhancing endocytosis of fibrils into glial cells. Quantification of the immunohistochemical staining is in agreement with the results obtained from the proteomic analysis.

**Outlook** The availability of cases with capCAA only, i.e. without AD-related changes, enables us to identify novel proteins that may play a role in the clearance of Aβ from the brain. We could identify and validate proteins that were differentially upregulated in capCAA compared to AD, including clusterin (ApoJ), serum amyloid P component (SAP) and laminin β2. All these are proteins that are involved in the processes of Aβ aggregation and clearance from the brain and may provide markers and targets for follow-up analyses.

Figure 1




1A. Immunohistochemical analysis of brain tissue from capCaa, AD and control cases. Control, capCAA and AD brain tissue was stained using ThioflavinS (Abeta fibrils), 4G8 antibody (ABeta peptide (region 17-24)) and clusterin antibody. Far right panel (merge) shows an overlay of the three stainings.




1B Quantification of clusterin. Quantification was performed using Image G software and spectral counting for Immunohistochemical stainings (purple) and proteomics (yellow) respectively.

**Acknowledgements** This research was financially supported by the 'Internationale Stichting Alzheimer Onderzoek' (ISAO grant 09506).

# microRNA-124&137 regulate caspase-3 activity in neural stem cells by cooperatively fine-tuning BCL2L13

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**Background:** Neural stem/progenitor cells are activated after acute or chronic brain injuries and maybe critical in maintaining function and in repair of the injured tissue. In the dentate gyrus, a sharp increase in neural stem/progenitor cell proliferation is observed in several models of epilepsy. There, status epilepticus induces a gene expression profile characterized by enrichment in genes related to plasticity, proliferation and fate determination, possibly contributing to network alterations associated with epileptogenesis and, at the same time, mitochondrial dysfunction.

**Aim and approach:** By means of proteomics, transcriptomics and microRNAomics we investigated differential gene expression after KA-induced status epilepticus. We investigated the molecular changes induced in the dentate gyrus shortly after kainic acid-induced status epilepticus, with particular focus on mitochondria-related processes.

**Results:** Using comparative proteomics we identified a group of significantly downregulated mitochondrial proteins including the BCL2 family member BCL-RAMBO. BLC-RAMBO expression was regulated by the cooperative action of two microRNAs, microRNA-124 and -137 and controlled caspase-3 activity in neural stem/progenitor cells.

**Conclusion and outlook:** Our observations suggest that mitochondria-dependent pathways may be fine-tuned by the cooperative actions of miRs on specific targets to disfavor apoptosis in neural stem/progenitor cells of the dentate gyrus shortly after status epilepticus.

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# Mass spectrometric detection of amyloid beta-peptide fragments in CSF of Alzheimer's disease patients and in mild cognitive impairment

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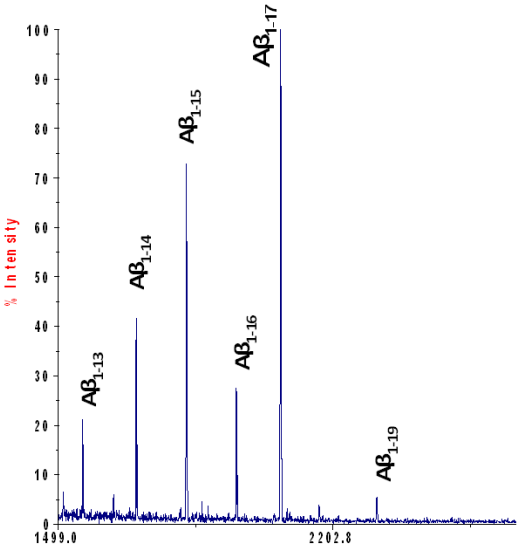
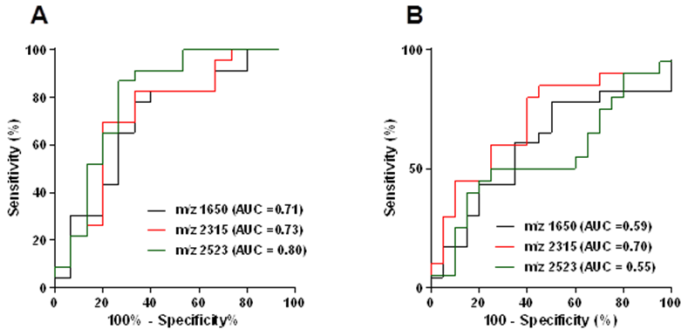
**Background:** A striking pathological feature of Alzheimer's disease (AD) is the deposition of extracellular plaques, mainly consisting of proteinaceous material. One of the most abundant peptides enriched in the plaques is the Aβ peptide, which is produced by sequential cleavage of the amyloid precursor protein (APP). Of major pathological importance is the 42 aminoacids form of the Aβ peptide (Aβ42), which is generated by sequential cleavage of β- and γ-secretases and has intrinsic aggregation potential.
Cerebrospinal fluid (CSF) levels of Aβ42 are extensively used as AD biomarkers together with total tau protein (t-tau) and phosphorylated tau at threonine 181 (p-tau). In recent years several other amyloid fragments have been studied in CSF. These fragments are produced by differential endoproteases cleavages and their presence is physiological in CSF and brain. The functional importance of these peptides is not yet understood, but some of the peptides, such as the the Aβ15 and Aβ16 showed altered levels in AD CSF in studies performed in relatively small cohorts of patients.

**Aim:** In this study we explored the hypothesis that specific combinations of Aβ fragments can be used to detect progression to AD from a mild cognitive impairment condition.

**Approach:** A mass spectrometry analysis of immunoprecipitated amyloid fragments (IP-MS) was used to measure six short Aβ fragments (Aβ13, Aβ14, Aβ15, Aβ16, Aβ17 and Aβ19) in the CSF of two well-characterized cohorts of MCI and AD patients, coming from two different European centres, Amsterdam (AMS) and Perugia (PG).
Moreover, the performance of the amyloid fragments profile was compared with that of the classical AD biomarkers, to verify if the short fragments may improve their global diagnostic performance.




| Cohort | Peaks (m/z) | Sensitivity | Specificity |
|---|---|---|---|
| Perugia | 1650 | 78 | 66 |
| | 2315 | 69 | 80 |
| | 2523 | 86 | 73 |
| Amsterdam | 1650 | 78 | 50 |
| | 2315 | 85 | 55 |
| | 2523 | 50 | 75 |

**Results:** In the PG cohort Aβ15 and Aβ16 were quantitatively increased in AD patients when compared to controls (p= 0.032 for Aβ15 and p = 0.014 for Aβ16). No significant changes were present for the MCI groups. On the other hand, in the AMS group, the normalized areas of the Aβ15 and Aβ16 peptides were not significantly different among the diagnostic groups. No significant changes of the normalized abundances for Aβ13, Aβ14, Aβ17 and Aβ19 were noticed when the groups were compared using multiple corrections testing, either in PG cohort or AMS cohort.

CSF levels of shorter isoforms did not correlate with any of the classical CSF biomarkers either in the whole sample or in the separate groups. Instead a significant correlation among the short fragments (especially Aβ14, Aβ15, Aβ16 and Aβ17) was found both within the PG and AMS cohort.
In the Perugia cohort the diagnostic performance of Aβ1-16 in distinguishing AD patients from controls subjects using Receiver operator characteristic analysis showed a sensitivity of 83% (95% CI: 67.19% to 93.63%) and a specificity of 55% (95% CI: 31.53% to 76.94%, Area under the Curve, AUC = 0.70, cut-off value=106).

**Conclusions and outlook:** The dysregulation of the short amyloid isoforms we found in one of the two cohorts may support the role of these peptides in AD. The different results in PG and AMS cohort may depend on differences in the control groups and/or pre-analytical conditions between the two centers.
Interestingly, the correlation between the levels of the short c-terminal Aβ peptides in the CSF may support a common mechanism for their production. Further studies are needed to extensively clarify the mechanism of physiological production of these peptides in CSF and brain and their role as AD biomarkers.

**Acknowledgements:** This research is supported by the Hersenstichting

# Search for CSF biomarkers for DISC1opathies

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**Background:** Biological diagnostics of mental illnesses is urgently needed for 1. Defining diagnostic categories based on objective markers rather than self-reporting, and, 2. Being able to identify biologically defined subsets of mental illnesses for future biological-based causal treatment. The authors proposed that protein pathology of the Disrupted-in-schizophrenia 1 protein (DISC1) could be used to define a subset of mental illnesses, termed DISC1opathies (Korth, 2012 Prion 6:134)

**Aim:** To identify cerebrospinal fluid (CSF) biomarkers for DISC1opathies

**Approach:** A transgenic rat model was generated that mimicks characteristics of DISC1opathies and phenotypes of schizophrenia. A characteristic signature of uniquely increased or decreased CSF proteins is sought that would define DISC1opathies. This signature would be validated by immunological assays in the rat CSF and in patient CSF. The proteomics workflow consisted of abundant protein depletion coupled to single band GeLC-MS/MS.

**Results:** Two experiments were performed, the first on individual CSF samples (N=5 per group) the second on pooled CSF samples (2 pools per group). The first experiment yielded a dataset of over 700 CSF proteins, the second a dataset of only ~350 CSF proteins. These two rounds of CSF proteomics have revealed some candidate proteins. However, overlap between the two independent analyses was not satisfying. Technical problems with low albumin depletion was present in experiment 2, in part due to the lack of established protocols for handling rat CSF.

**Conclusions and outlook:** Validation of proteins obtained so far in independent samples is ongoing. Likely, to obtain more inter-test reliability, and with improved albumin depletion, a third round of proteomics will be performed on the rat model in the near future.

## Protein network disturbance in response to oxidative stress

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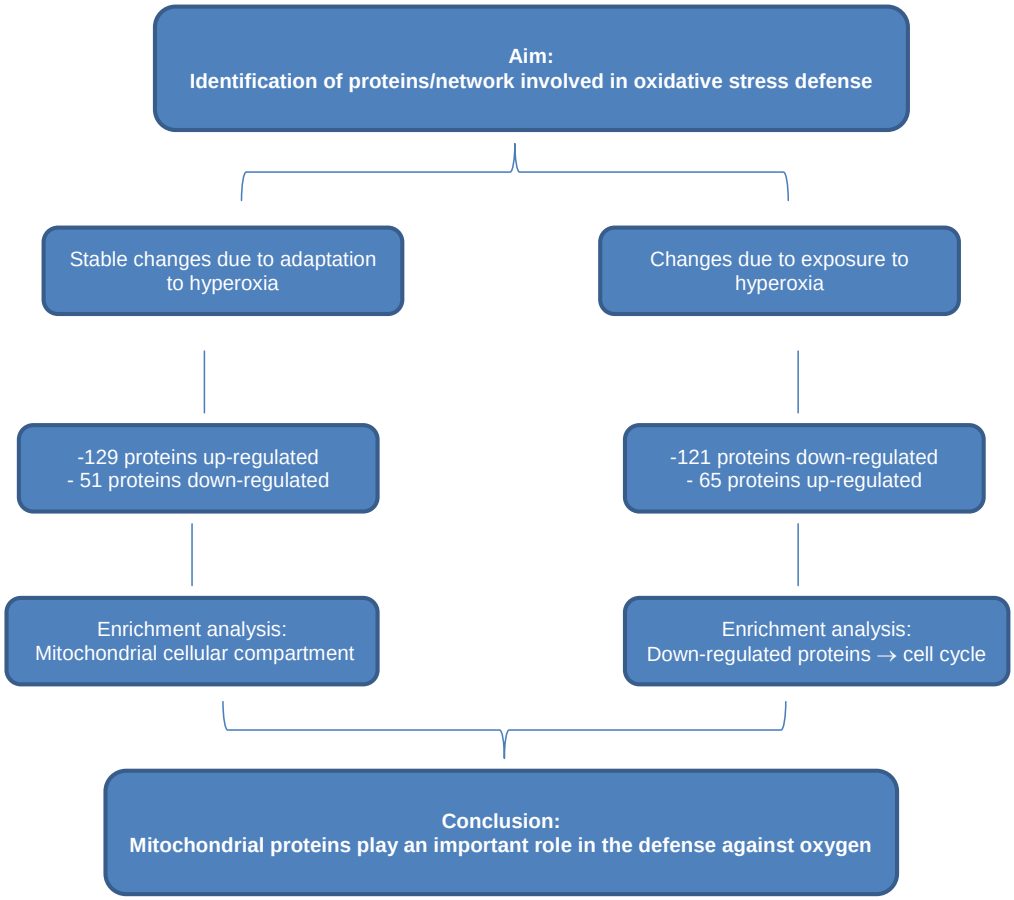
***Background:*** Oxygen derivatives generated during normal metabolisms can induce oxidative stress which has been linked to aging and age-related degenerative processes such as cancer. The crucial mechanisms underlying defense against oxidative stress have, nevertheless, not been studied in an unbiased manner in human cells. To fill this gap, we have generated a unique panel of oxygen-resistant human cell lines.

***Aim:*** We plan to identify proteins/networks that are deregulated due to exposure to normobaric hyperoxia, a physiological mimic of oxidative stress.

***Approach:*** To determine stable alterations due to hyperoxia adaptation, the proteome of two oxygen-resistant HeLa cell lines was compared to their non-resistant counterparts. The hyperoxia effect on the proteome was also determined, via comparing the non-tolerant cell lines under normoxia (20% oxygen) versus non-tolerant cell lines under hyperoxia. Subsequently, a selection was made for proteins that displayed differential expression with a p-value of ≤ 0.05 and a log fold change of ≥ 4.

***Results:*** The comparison of the tolerant HeLa cell lines versus their sensitive counterparts revealed a total 129 up-regulated proteins and 51 down-regulated proteins. The proteins identified were enriched for the mitochondrial cellular compartment. Proteins down-regulated in the non-tolerant cell lines due to hyperoxia exposure were involved in cell cycle.

***Conclusion:*** The observed enrichment for proteins functioning within the mitochondria is in line with the fact that mitochondria are the main site of oxygen derivatives generation. However, further studies are needed to determine the relevance of these proteins in the defense against oxidative stress



# Secretome analysis of TLR2-stimulated FLT3L bone marrow cultures

Ida H. Hiemstra[1], Sander R. Piersma[2], Jaco C. Knol[2], Meike de Wit[2], Kim Vrijland[1], Gerd Bouma[3], Georg Kraal[1], Connie R. Jimenez[2], Joke M.M. den Haan[1]

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2. Oncoproteomics laboratory, VU University Medical Center, Amsterdam, The Netherlands
3. Department of Gastroenterology, VU University Medical Center, Amsterdam, The Netherlands

**Background and aim**
Bone marrow cells contain dendritic cell precursors that can be stimulated in vitro with Flt3L to differentiate into dendritic cells. We previously established that inclusion of the TLR2 ligand Pam3CysK4 in these cultures results in the generation of monocytes by the induction of a soluble factor. Our aim was to identify this unknown factor by secretome analysis using mass spectrometry.

**Approach**
Serum-free supernatants of bone marrow cultures were concentrated and proteins were separated by SDS gel electroforesis. Gels were cut in pieces and digested by trypsin. NanoLC was performed and analysed by a Q-Exactive mass spectrometer. Significantly altered proteins with larger than 3 fold change between stimulated and unstimulated supernatants were selected.

**Results**
732 proteins were identified to be more than 3 fold changed between stimulated and unstimulated cultures. These belonged to 796 upregulated biological processes and 97 downregulated processes as defined by the gene ontology program BINGO. 122 proteins were only detected in the stimulated supernatant, whereas 46 proteins could only be detected in unstimulated supernatant. We tested one of these proteins for activity to induce monocytes in these cultures, but this factor did not induce monocyte differentiation.

**Conclusion**
Secretome analysis of bone marrow cultures revealed 732 proteins to be significantly changed upon inclusion of TLR2. Further analysis of candidates may reveal factors that drive monocyte differentiation.

# Identification of novel signalling partners of the HCMV encoded viral GPCR US28

Erik Slinger1,3*, Ellen V. Langemeijer1,4*, Sabrina de Munnik1, Andreas Schreiber1, David Maussang1, Henry Vischer1, Sander R. Piersma2, Connie R. Jiménez2, Hessam Tabeian1, Folkert Verkaar1, Rob Leurs1, Marco Siderius1 and Martine J. Smit1

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**Introduction**

The genomes of many herpesviruses carry genes that encode G-protein-coupled receptor (GPCR) homologues. It is assumed that the viral GPCRs (vGPCRs), which are homologous to chemokine receptors, play a role in deceiving the host's immune system. Although the molecular mechanism for vGPCR-mediated immune-evasion still is elusive, these proteins may prove to be important drug targets to prevent virus-related pathologies. US28, one of four HCMV-encoded chemokine receptors, has been shown to constitutively activate signalling pathways linked to proliferation and inflammation and to promote tumor growth in vivo.

**Aim**

To elucidate the oncogenic signalling pathways activated by the HCMV-encoded chemokine receptor US28 and to identify novel signalling partners of US28.

**Approach**

Immunoprecipitation of the HA-tagged US28 in mammalian cells transfected with US28 to isolate US28-complexes for proteomics analysis

**Results**

Using the approach described above, over 400 proteins were identified to co-precipitate with US28. The amount of co-precipitating proteins underlines the importance of proper negative controls. Amongst the identified proteins absent in the controls, are a number of scaffolding proteins that may prove to be important novel signalling partners for US28. Also, a number of chaperone proteins were specifically precipitated with US28 that may be interesting in view of oncogenic signalling initiated by US28. Furthermore, a number of signalling factors that are involved in pathways known to be altered in cancer are also found to co-precipitate with US28. In addition, proteins specifically co-precipitating with the $R^{129}A$ G-protein uncoupled mutant were identified.

**Outlook**

US28 interacting proteins have been identified that are potentially involved in both G-protein dependent and independent signalling pathways. Functional validation experiments are on-going.

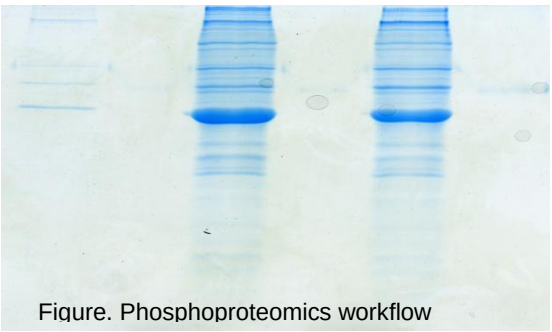


Figure. Applications of phosphoproteomics (left) and enrichment methods (figures above and

# Threonine-594 of the Estrogen Receptor Alpha F domain is a phosphorylated residue involved in down-regulation of receptor activity.

A.H. de Boer[1], Sander R. Piersma[2] and Connie R. Jiménez[2]

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**Background** The Estrogen Receptor Alpha (ERα) is a nuclear receptor, involved in many breast cancers. Therapeutic strategies to treat these tumors rely on selective ER modulators, aromatase inhibitors or drugs that target ERα/DNA or ERα/cofactor interactions. One essential step in the ERα activation cascade, namely receptor dimerization, is currently unexploited in drug design. The finding that the small molecule Fusicoccin reduces 17β-estradiol, resulted in the discovery of an as yet unknown regulation mechanism of ERα activity that acts by reducing receptor dimerization.

**Aim** The aim of this study was to demonstrate that the pen-ultimate residue of ERα, Threonine-594, is a phospho-site.

**Approach:** Fusicoccin stabilizes the interaction between ERα and 14-3-3 proteins[1]. Because the interaction motif of 14-3-3 proteins almost always involves a phospho-site, we generated an phospho-antibody that specifically recognizes the pThr$^{594}$ of the C-terminal tip of ERα (Fig. 1). Treatment of tumor cells with Fusicoccin showed a strong increase in Thr$^{594}$ phosphorylation by Western blotting with the pThr$^{594}$-antibody (Figure 1).

To demonstrate unequivocally that Thr$^{594}$ is phosphorylated, we used immuno-precipitation in combination with mass-spectrometry.

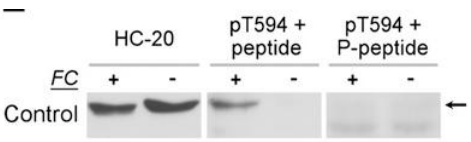


Fig. 1. Western blot analysis with the HC-20 and pThr$^{594}$ antibodies of cell lysate from MCF-7 cells treated with and without FC (30 µM). The pThr$^{594}$ antibody was used in the presence of the nonphosphorylated (Center) and the Thr$^{594}$ phosphorylated ERα peptide (Right).

**Results:**

In order to demonstrate Thr$^{594}$ phosphorylation, cells were pre-treated with Fusicoccin for 1 day. Cell lysate was prepared in the presence of phosphatase inhibitors and the cell lysate was digested with trypsin. To enrich for the peptide that contains pThr$^{594}$, the lysate was digested with trypsin, desalted and subsequently incubated with pThr$^{594}$ rabbit polyclonal antibody. Immuno-precipitated peptides were further purified and finally analyzed by LC-MS/MS. Mass-spectrometry analysis of this fraction identified the C-terminal ERα peptide (14 aa) with Thr$^{594}$ phosphorylated (Figures 2 and 3).

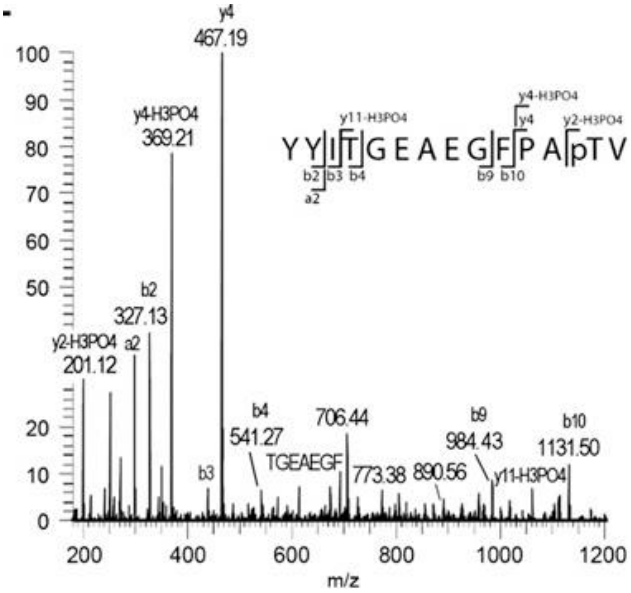


Fig. 2. Mass-spectrometry analysis of the C-terminal ERα peptide purified from a trypsin digested MCF-7 cells lysate with the pThr$^{594}$ antibody. Shown is the tandem MS (MS2) spectrum of the C-terminal ERα tryptic peptide showing modification by phosphorylation at Thr$^{594}$.

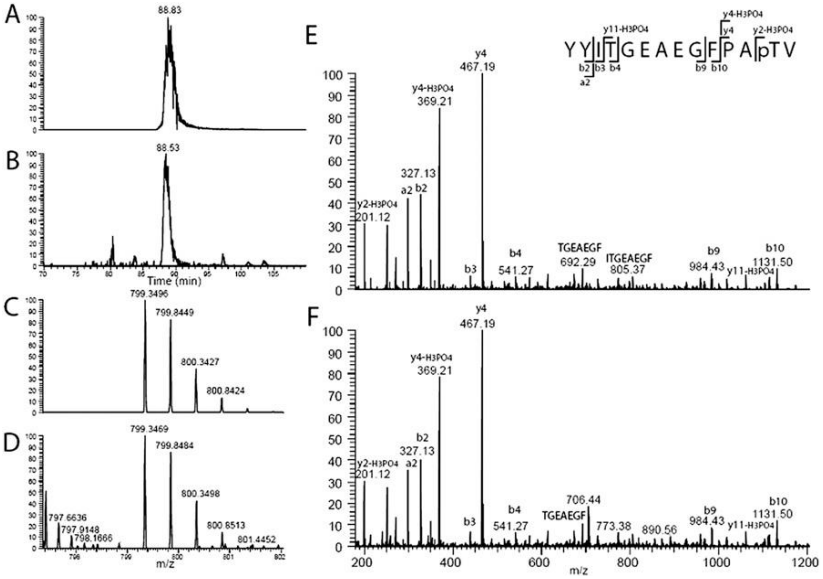


Fig. 3. Mass-spectrometry analysis of the C-terminal ERα peptide shows phosphorylation of the penultimate threonine, $Thr^{594}$. (A) Extracted ion chromatogram of synthetic ERα C-terminal tryptic phosphopeptide YYITGEAEGFPApTV phosphorylated at $Thr^{594}$. (B) The corresponding peptide from MCF-7 digest, after IP is shown. In A and B coelution at 89–90 min is observed. (C and D) Accurate intact mass of the peptide, corresponding to the [M+2H]2+ monoisotopic peak at m/z 799.3447, for the synthetic and MCF7-derived phosphopeptide, respectively. (E) MS/MS spectrum of synthetic YYITGEAEGFPApTV.

## Conclusions and outlook

The experiments with the $pThr^{594}$ antibody in combination with the LC-MS/MS analysis conclusively demonstrated that $Thr^{594}$ is a phosphorylated residue in MCF-7 cells and that FC "protects" the $Thr^{594}$ phosphosite from dephosphorylation resulting in increased phosphorylation.

Phosphorylation of $Thr^{594}$ is now subject of different studies: i) Immuno-histochemistry with the $pThr^{594}$-antibody using patient material to detect phosphorylation differences in tumors and correlate that with survival, ii) identification of the kinase/phosphatase that determine the level of $Thr^{594}$ phosphorylation and thus ERα activity, and iii) screening small molecule libraries to find novel molecules with improved pharmacokinetic properties that can stabilize 14-3-3 interaction with $pThr^{594}$-ERα.

### References

1. De Vries-van Leeuwen et al. (2013) Interaction of 14-3-3 proteins with the Estrogen Receptor Alpha F domain provides a drug target interface. PNAS 110, 8894-8899.

# Accurate quantitation of MHC-bound peptides by application of isotopically labeled peptide MHC complexes.

Hassan C[1], Kester MG[2], Oudgenoeg G[3], de Ru AH[1], Janssen GM[1], Drijfhout JW[1], Spaapen RM[4], Jiménez CR[3], Heemskerk MH[2], Falkenburg JH[2], van Veelen PA[5].

**Background** Knowledge of the accurate copy number of HLA class I presented ligands is important in fundamental and clinical immunology. Currently, the best copy number determinations are based on mass spectrometry, employing single reaction monitoring (SRM) in combination with a known amount of isotopically labeled peptide. The major drawback of this approach is that the losses during sample pretreatment, i.e. immunopurification and filtration steps, are not well defined and must, therefore, be estimated. In addition, such losses can vary for individual peptides.

**Aim an approach** To develop a new approach for accurate determination of copy number of HLA class I presented ligands. To this end, isotopically labeled peptide-MHC monomers (hpMHC) are prepared and added directly after cell lysis, i.e. before the usual sample processing. Using this approach, all losses during sample processing can be accounted for and allows accurate determination of specific MHC class I-presented ligands.

**Results** Our study pinpoints the immunopurification step as the origin of the rather extreme losses during sample pretreatment and offers a solution to account for these losses. Obviously, this has important implications for accurate HLA-ligand quantitation.

**Conclusions** The strategy presented here can be used to obtain a reliable view of epitope copy number and thus allows improvement of vaccine design and strategies for immunotherapy.

**Publication**

Hassan C, Kester MG, Oudgenoeg G, de Ru AH, Janssen GM, Drijfhout JW, Spaapen RM, Jiménez CR, Heemskerk MH, Falkenburg JH, van Veelen PA. Accurate quantitation of MHC-bound peptides by application of isotopically labeled peptide MHC complexes. J Proteomics. 2014 Jul 19;109C:240-244.

# A proteomic analysis of the cardiac sodium channel macromolecular complex

R.F. Marsman[1], S.P. Podliesna[1], S. Piersma[2], B.P. Scicluna[1], M.L. Westerveld[1], E.M. Lodder[1], C.R. Jimenez[2], C.R. Bezzina[1], P. Barnett[3]

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**Background**

The cardiac sodium channel Na$_V$1.5 is a critical mediator of normal cardiac conduction. Multiple proteins, such as regulatory kinases and calcium-sensing molecules, have been shown to regulate and interact with Na$_V$1.5, some of which have also been linked to familial arrhythmia syndromes. While some of these interactions have been uncovered, knowledge on the sodium channel macromolecular complex remains a largely untapped area in cardiac electrophysiology.

**Methods and Results**

We undertook an unbiased proteomics-based analysis of Na$_V$1.5 protein interactions using an approach entailing affinity-purification coupled to mass spectrometry. Protein complexes were purified from transiently transfected H10 cells (a rat cardiomyocyte cell line) using the tandem affinity purification (TAP) methodology or FLAG-based immunoprecipitation, and subsequently analyzed by liquid chromatography followed by tandem mass spectrometry (LC-MS). This identified an extensive set of candidate Nav1.5 interaction partners which were prioritized for validation studies by bio-informatic analysis. The interaction of Nav1.5 with one of the candidate proteins, myoferlin, was validated by co- immunoprecipitation and colocalization studies.

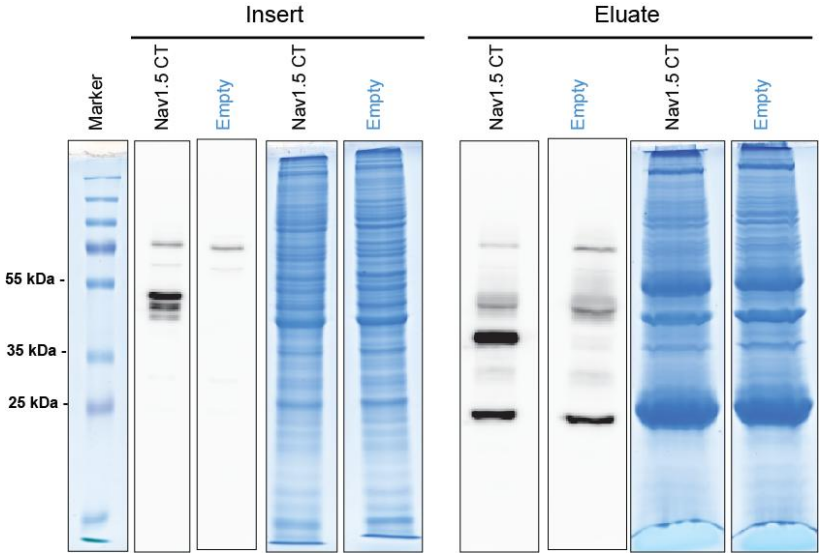



**Figure: Validation of candidate Protein X. 2A:** FLAG co-immunoprecipitation experiments confirm the association of flag-Nav1.5 C-terminus (CT, 35 kDa) and flag-full-length Nav1.5 (hH1, 260 kDa ) with HA-Protein X (180 kDa). Lysates from transiently transfected HEK293 cells were

**Conclusion**

Our study reveals putative novel cardiac sodium channel interacting proteins, providing a resource for future studies on the molecular mechanisms underlying Na$_V$1.5 expression, subcellular localization and function.

# A quantitative phosphoproteomics study reveals a role for kinase X in cardiomyocyte proliferation

Amin Damanafshan[1*], Ies Elzenaar[1*], Sander R. Piersma[2], Benoit C. Samson-Couterie[1], Maarten M. van den Hoogenhof[1], Nina E. de Groot[1], Jolanda van der Velden[3], Connie R. Jimenez[2], Yigal M. Pinto[1], Ralph J. van Oort[1]

[1]*Department of Experimental Cardiology, Academic Medical Center, Amsterdam, The Netherlands.*
[2]*OncoProteomics Laboratory, Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands.*
[3]*Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands*

**Background:** The pathology of heart failure is characterized by poorly contracting and dilated ventricles, which is associated with lengthening of individual cardiomyocytes and loss of sarcomeres. We have previously identified an alternative splice variant of kinase X as important mediator of sarcomere degeneration. To date, however, the physiological role of kinase X in the heart has not been investigated and the cardiac phosphorylation substrates are unknown. Here, we used a quantitative phosphoproteomics approach to characterize the --- -dependent phosphoproteome in cardiomyocytes.

**Methods and Results:** Isolated neonatal rat cardiomyocytes (NRCMs) were infected with adenoviruses expressing kinase X, a control kinase dead mutant, or control GFP for 24 hours. Protein lysates from these cells were trypsinized and phosphopeptides were enriched by titanium dioxide capture. Subsequent analysis by quantitative mass spectrometry identified 129 specific phosphorylation events in 109 proteins that are kinase X dependent. The kinase X dependent phosphoproteome was subjected to further bioinformatic analyses to screen for enriched phosphorylation motifs (motif-x), to test for enriched gene ontologies (DAVID) and to perform network analysis (STRING). Interestingly, we found an overrepresentation of nuclear substrates implicated in chromatin assembly and the cell cycle, indicating a possible role for kinase X in cell proliferation. Indeed, immunostainings for the proliferation marker Ki-67 revealed a drastic increase in proliferation of NRCMs upon kinase X overexpression.

**Conclusion:** We have identified a set of kinase X -dependent phosphorylation events in cardiomyocytes. Furthermore, our data revealed a previously unknown role for kinase X in cardiomyocyte proliferation. Future work will include experiments to determine whether kinase X-induced loss of sarcomeres results from cell cycle activation and cardiomyocyte dedifferentiation.

# PKCα-specific phosphorylation of the troponin complex in human myocardium: a functional and proteomics analysis

Viola Kooij[1,2], Pingbo Zhang[2], Sander R. Piersma[3], Paul J.M. Wijnker[1], Nicky M. Boontje[1] Connie R. Jiménez[3], Kornelia E. Jaquet[4], Cris dos Remedios[5], Anne M. Murphy[6], Jennifer E. Van Eyk[2], Jolanda van der Velden[1], Ger J.M. Stienen[1]

[1]Laboratory for Physiology, Institute for Cardiovascular Research, VU Medical center, Amsterdam, Netherlands; [2]Johns Hopkins Bayview Proteomics Center, Department of Medicine, School of Medicine, Johns Hopkins University, Baltimore, USA; [3]OncoProteomics Laboratory, Department of Medical Oncology, VU Medical center, Amsterdam, Netherlands; [4]St Josef-Hospital/Bergmannsheil, Clinic of the Ruhr-University of Bochum, Bochum, Germany; [5]Muscle Research Unit, Institute for Biomedical Research, The University of Sydney, Sydney, Australia; [6]Institute of Molecular Cardiobiology, Department of Pediatrics, School of Medical, Johns Hopkins University, Baltimore, USA

**Aims:** Protein kinase Cα (PKCα) is one of the predominant PKC isoforms that phosphorylate cardiac troponin. PKCα is implicated in heart failure and serves as a potential therapeutic target, however, the exact consequences for contractile function in human myocardium are unclear. This study aimed to investigate the effects of PKCα phosphorylation of cardiac troponin (cTn) on myofilament function in human failing cardiomyocytes and to resolve the potential targets involved.

**Methods and results:** Endogenous cTn from permeabilized cardiomyocytes from patients with end-stage idiopathic dilated cardiomyopathy was exchanged (~69%) with PKCα-treated recombinant human cTn (cTn (DD+PKCα)). This complex has Ser23/24 on cTnI mutated into aspartic acids (D) to rule out in vitro cross-phosphorylation of the PKA sites by PKCα. Isometric force was measured at various [Ca(2+)] after exchange. The maximal force (Fmax) in the cTn (DD+PKCα) group (17.1±1.9 kN/m(2)) was significantly reduced compared to the cTn (DD) group (26.1±1.9 kN/m(2)). Exchange of endogenous cTn with cTn (DD+PKCα) increased Ca(2+)-sensitivity of force (pCa50 = 5.59±0.02) compared to cTn (DD) (pCa50 = 5.51±0.02). In contrast, subsequent PKCα treatment of the cells exchanged with cTn (DD+PKCα) reduced pCa50 to 5.45±0.02. Two PKCα-phosphorylated residues were identified with mass spectrometry: Ser198 on cTnI and Ser179 on cTnT, although phosphorylation of Ser198 is very low. Using mass spectrometry based-multiple reaction monitoring, the extent of phosphorylation of the cTnI sites was quantified before and after treatment with PKCα and showed the highest phosphorylation increase on Thr143.

**Conclusion:** PKCα-mediated phosphorylation of the cTn complex decreases Fmax and increases myofilament Ca(2+)-sensitivity, while subsequent treatment with PKCα in situ decreased myofilament Ca(2+)-sensitivity. The known PKC sites as well as two sites which have not been previously linked to PKCα are phosphorylated in human cTn complex treated with PKCα with a high degree of specificity for Thr143.

**Publication**

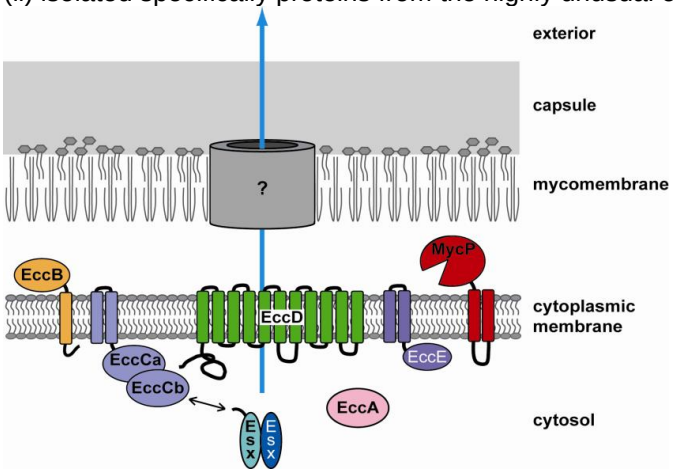
Kooij V, Zhang P, Piersma SR, Sequeira V, Boontje NM, Wijnker PJ, **Jiménez CR**, Jaquet KE, Dos Remedios C, Murphy AM, Van Eyk JE, van der Velden J, Stienen GJ. PKCα-Specific Phosphorylation of the Troponin Complex in Human Myocardium: A Functional and Proteomics Analysis. PLoS One. 2013 Oct 7;8(10): e74847.

# Proteomics of Mycobacterial Pathogens

Edith Houben, Aniek van der Woude, Theo Verboom, Roy Ummels, Astrid van der Sar, Esther Stoop, Eveline Weerdenburg and Wilbert Bitter

Department of Medical Microbiology VU medical center Amsterdam; Department of Molecular Microbiology, VU University Amsterdam

**Background** Gram-negative bacteria are surrounded by an outer membrane, which protects them against harmful compounds. Proteins within this outer membrane play important roles in the interaction of (pathogenic) bacteria with their environment. Recently, it has become clear that also Mycobacteria, such as Mycobacterium leprae and Mycobacterium tuberculosis, possess an outer membrane. However, the nature and the composition of this membrane are completely different from the classical Gram-negative outer membrane. Whereas the lipid composition and the biogenesis of this special outer membrane are reasonably well understood, our knowledge of mycobacterial outer membrane proteins, surface proteins and secretome is rudimentary. This deficiency in our knowledge not only limits our understanding of these important bacteria, but is also a missed opportunity to identify new target molecules for the development and improvement of vaccines and antibiotics. Recently, we have (i) identified two major secretion pathway called type VII secretion and SecA2 and (ii) isolated specifically proteins from the highly unusual outer membrane.




Identification of these proteins by LC-MS/MS

**Aims and approach** In various projects, we aimed to identify and characterize proteins from the outer membrane, cell surface and secretome of different pathogenic mycobacteria. In addition, we have also characterized the effects of several mutations, such as secretion or cell wall mutants, on the proteome. Also the composition of macromolecular structures isolated from these fractions have been analyzed. These studies led to several publications that are listed below.

**Publications**

Daleke-Schermerhorn MH, Felix T, Soprova Z, Ten Hagen-Jongman CM, Vikström D, Majlessi L, Beskers J, Follmann F, de Punder K, van der Wel NN, Baumgarten T, Pham TV, Piersma SR, Jiménez CR, van Ulsen P, de Gier JW, Leclerc C, Jong WS, Luirink J. Decoration of outer membrane vesicles with multiple antigens by using an autotransporter approach. Appl Environ Microbiol. 2014 Sep 15;80(18):5854-65.

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Van der Woude AD, Mahendran KR, Ummels R, Piersma SR, Pham TV, Jiménez CR, de Punder K, van der Wel NN, Winterhalter M, Luirink J, Bitter W, Houben EN. Differential detergent extraction of mycobacterium marinum cell envelope proteins identifies an extensively modified threonine-rich outer membrane protein with channel activity. J Bacteriol. 2013 May;195(9): 2050-9.

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Daleke MH, van der Woude AD, Parret AH, Ummels R, de Groot AM, Watson D, Piersma SR, Jiménez CR, Luirink J, Bitter W, Houben EN. Specific chaperones for the type VII protein secretion pathway. J Biol Chem. 2012 Sep14;287(38): 31939-47

# Proteomic profiling of the *Mycobacterium tuberculosis* identifies nutrient starvation responsive toxin-antitoxin systems

Jakob Albrethsen[1], Jeppe Agner[1], Sander R. Piersma[2], Peter Højrup[3], Thang V. Pham[2], Karin Weldingh[1], Connie R. Jimenez[2], Peter Andersen[1] & Ida Rosenkrands[1]*
[1]Department of Infectious Disease Immunology, Statens Serum Institut, Copenhagen, Denmark; [2]OncoProteomics Laboratory, Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands; [3]Department of Biochemistry and Molecular Biology, University of Southern Denmark

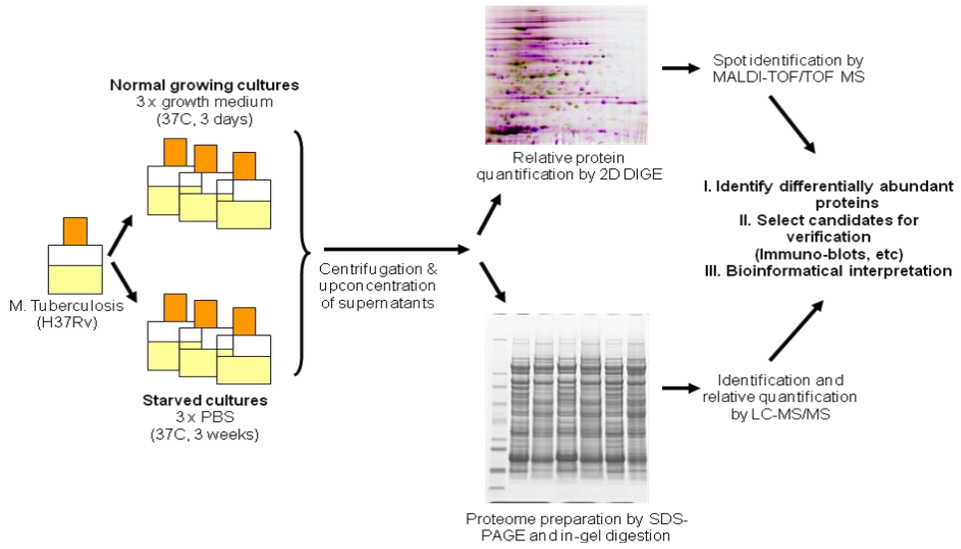
**Background** Mycobacterium tuberculosis is a very successful pathogen which has the ability to persist in humans for decades without symptoms. Latent tuberculosis (TB) infection can reactivate and progress into active, clinically apparent TB disease. To enter the latent stage, M. tuberculosis must adapt to conditions such as nutrient limitation and hypoxia.

**Aim and Approach** In vitro models which mimic latent infection are valuable tools to describe the changes in metabolism which occur when the bacterium exists in a non-growing form. We used two complementary proteomic approaches, label-free LC-MS/MS analysis and two-dimensional DIGE, to determine the expression profile of extracellular proteins from M. tuberculosis cultured under nutrient starvation.

**Results** By label-free LC-MS/MS analysis of fractionated samples, 947 proteins were identified from culture filtrates of log phase and nutrient starved cultures, and the protein levels of 239 proteins were increased in nutrient starved culture filtrates, while 204 were decreased. Notably, members of the toxin-antitoxin systems were present in much larger quantities in nutrient starved cultures supporting a role for these global modules as M. tuberculosis switches its metabolism into dormancy. Further analysis of the dataset identified increased abundance of the phospholipases and lipoproteins and decreased abundance of Esx proteins. Results from 2D-DIGE based proteomics demonstrated an overall agreement with the LC-MS/MS data and added complementary insights about protein degradation and modification.

**Conclusions and Outlook** We focused on the secretome of M. tuberculosis as extracellular proteins play an important role in the host-pathogen interactions of M. tuberculosis and furthermore

Workflow:




*Albrethsen J, Agner J, Piersma SR, Højrup P, Pham TV, Weldingh K, Jimenez CR, Andersen P, Rosenkrands I. Proteomic profiling of Mycobacterium tuberculosis identifies nutrient-starvation-responsive toxin-antitoxin systems. Mol Cell Proteomics. 2013 May;12(5): 1180-91.*