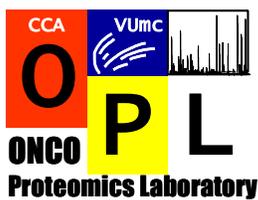
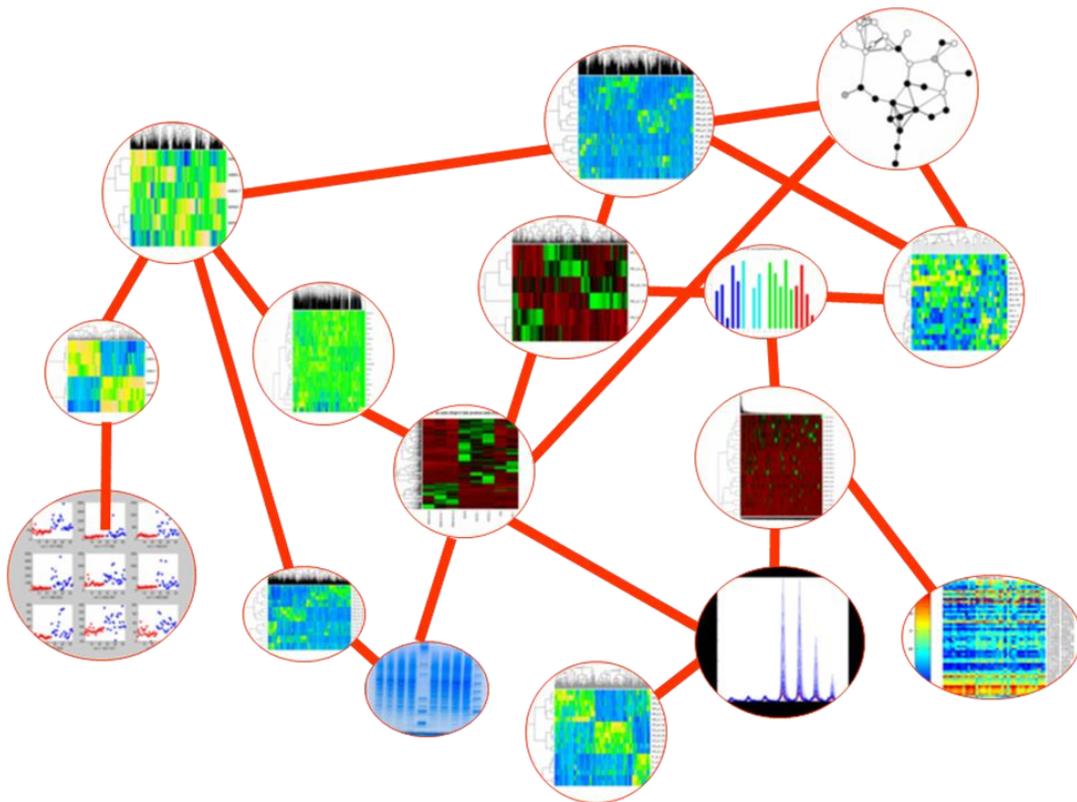


# OncoProteomics Laboratory

## Progress Report

### 2010-2011-mid2012



# Progress Report 2010-mid2012

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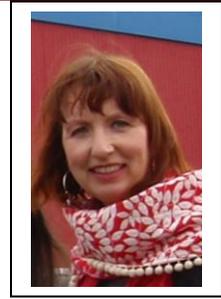
*Cover: Co-production Thang, Silvina and Connie*

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## Foreword

The progress report 2010-mid2012 covers the 4<sup>th</sup> - 6<sup>th</sup> year of the OncoProteomics Laboratory (OPL) that was established in April 2006 at the VUmc department of Medical Oncology with support from the Cancer Center Amsterdam. In this report, you can read about our mass spectrometry infrastructure and workflows, as well as about our biomarker discovery research, including summaries of running core and collaborative projects.



In the past 2 years, we consolidated and extended several research lines and published several successful cancer applications, both in model systems and human material. A brief overview of the highlights:

- Workflows for sputum and faeces proteomics have been set up and successfully applied to the identification of non-invasive lung and colon cancer biomarker candidates (context CTMM Airforce and DeCoDe projects).
- Progress was made in the area of targeted mass spectrometry. A dedicated mass spectrometrist, Dr. Gideon Oudgenoeg joined the OPL in 2011 to enable validation in stool of a promising panel of colon cancer biomarkers for early detection and screening.
- I am proud of the publication of the first papers on novel colon cancer markers in the nr. one proteomics journal (Molecular and Cellular Proteomics, IF 8) and in Clinical Cancer Research. The novel candidate markers for non-invasive early detection of CRC are now being validated in the context of the CTMM-DeCoDe project. More work in collaboration with Dr. Remond Fljneman and Prof. Gerrit Meijer, VUmc-Dept. Pathology) is in the pipeline including the development of new stool-based markers that have been identified in this difficult biomaterial. This work was nominated for the Amsterdam Invention Motor Award in 2010.
- Several invited reviews were written on proteomics technology for signaling pathway proteomics, label-free proteomics for biomarker discovery, and colon cancer proteomics as well as a conference report on the Cancer Proteomics meeting in Dublin of which I was a co-organizer.
- Proteomics of a colon cancer stem cell model revealed new drug targets associated with stemness and drug resistance (work in collaboration with Dr. Onno Kranenberg, UMCU, published in MCP and in preparation).
- Proteomics of a genetic mouse model for human BRCA1 deficient breast cancer in conjunction with network-based analysis has identified a new BRCAness signature that can identify human breast tumors with defects in the homology DNA repair pathway (work in collaboration with Dr. Jos Jonkers and Dr. Sven Rottenberg, NKI). This work was honoured as a highly rated abstract at the AACR 2010 meeting, received the poster prize at the EMBO Cancer Proteomics and Systems Biology meeting in Dublin 2011 and was recently published in MCP.
- Phosphoproteomics was set up and applied in several studies using model systems and promising results were obtained. We envision that this new exciting line of research in collaboration with Prof. Henk Verheul will enable patient stratification for targeted therapy.
- Last but not least: the LTQFTMS was replaced for a new generation Orbitrap platform (QExactive), in part with support from CCA/V-ICI, and a 2<sup>nd</sup> discovery system dedicated to clinical studies of the Dept. of Medical Oncology was obtained as well. Therefore, we are again excellently equipped for the coming exciting years of cancer proteomics!

Finally, I hope you will enjoy reading this report on OPL research activities with high end proteomics 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, March. 2012

**Head, Associate Professor:** Dr. Connie R Jimenez

**Research associates:**

Dr. Sander Piersma (OPL-core: discovery nanoLC-MS/MS)  
Dr Thang V. Pham (OPL core: data analysis)  
Dr. Gideon Oudgenoeg (OPL-core: targeted mass spectrometry)

**Research technicians**

Dr. Jaco C. Knol (OPL core: MALDI-TOF/TOF, back-up nanoLC-MS/MS, data mining)  
Ing. Inge de Reus (lung cancer sputum project)

**Post-doctoral fellows:**

Dr. Tienke Schaaïj-Visser (lung cancer biomarkers)  
Dr. Meike de Wit (colon cancer biomarkers)  
Dr. Davide Chasserini (CSF biomarkers)

**PhD students:**

Marc Warmoes (breast cancer biomarkers)  
Joey Lam (breast cancer biomarkers)

**Visiting scientists**

Kasia Wojcik (2010)  
Ira Skvortsov (Innsbruck Medical University, Austria; 2010-2011)

**Internship students**

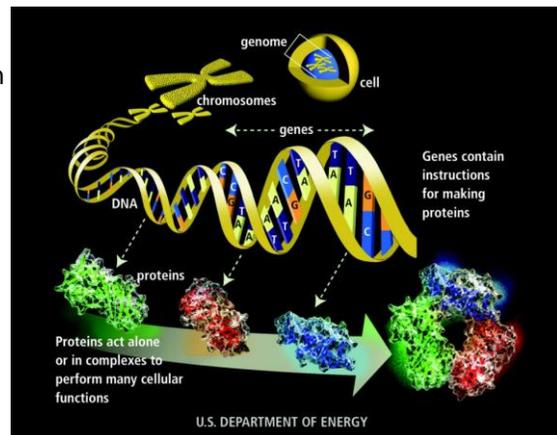
Carolien van Alphen (proteomics HPV progression model, 2010)  
Mehrdad Lavaei (exosome proteomics 2008-2010)  
Leticia Leon (bioinformatics, 2010)  
Richard Jaarsveld (colon cancer cell surface and exosome markers, 2011)  
Barath Kumar (phosphoproteomics panel of colon cancer cell lines, 2010-2011)  
Burcin Colak (lung cancer sputum project, 2011)  
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 creates a link between genomic information and biological function through large-scale analysis of protein expression, protein modification and protein-protein interactions

Advances in key proteomics tools such as mass spectrometry (MS) and (bio)informatics provide tremendous opportunities for biomarker-related clinical applications. Current MS platforms allow for the detection, quantitation and identification thousands of peptides and proteins in complex biological and clinical samples. Proteomics applications in cancer include 1. discovery of novel cancer (subtype) and treatment (outcome)-related protein profiles and candidate protein biomarkers, 2. insight into oncogenesis mechanisms and 3. discovery of novel protein targets for therapeutic intervention.



### Mission of the OPL

The mission of the OPL is to develop and implement innovative proteomics technologies and data analysis methods to improve (early) diagnostics and treatment of cancer.

To this end, we have developed and implemented robust label-free mass spectrometry-based strategies for biomarker discovery in tumor tissue and biofluids including workflows for phosphoproteomics, sub-nuclear, cell surface and secretome proteomics as well as workflows for sputum and faeces proteomics and multi-affinity depletion of biofluids. In-depth proteomics for comprehensive global protein identification and quantification is performed using nano-liquid chromatography on-line coupled to tandem mass spectrometry of gel-fractionated samples. Targeted analysis of panels of biomarker candidates for large scale biomarker validation is performed on a dedicated tandem mass spectrometry system.

### OPL research activities

The projects of the OPL broadly can be subdivided into 3 categories: 1. OPL core research (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<sup>nd</sup> and 4<sup>th</sup> Tuesday): journal club
- All OPL people attend and present their projects in the weekly Medical Oncology Dept. seminars.
- Through our website ([www.oncoproteomics.nl](http://www.oncoproteomics.nl))

## OPL Research & Collaboration Diagram

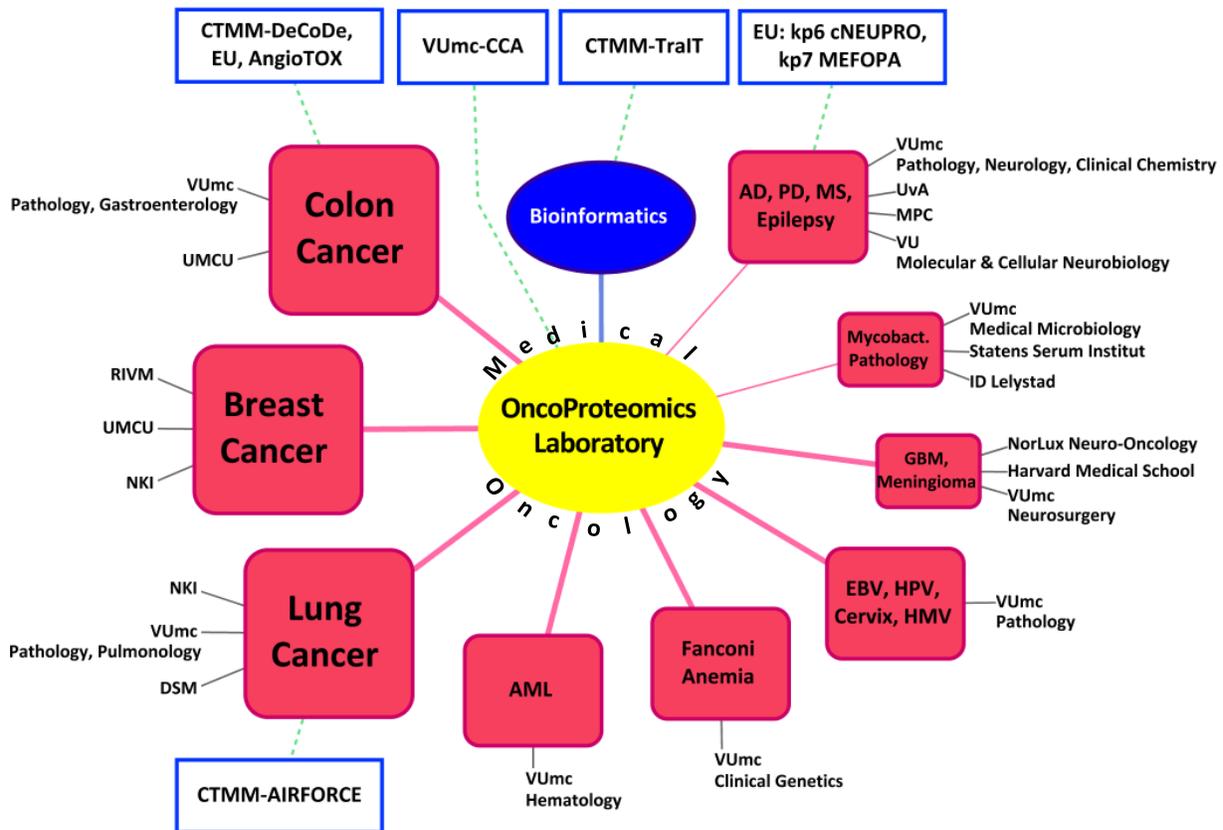


Figure 1. Areas of OPL proteomics activities and collaborating departments and institutes. The size of the boxes is an approximate indication of the magnitude of the effort.

### 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 (4 fte, two mass spectrometrists, Dr. Sander Piersma and Dr. Gideon Oudgenoeg; 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/V-ICI, VUmc as well as at the national (CTMM projects) and international level (EU projects) (see figure 1). The main focus is on oncogenesis / early (non-invasive) detection of cancer and on patient selection for targeted therapy and therapy 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 adequate in 2010-2012 though the wet lab is at times very crowded. The only minor point is that the MS lab is very noisy. Ear plugs have provided a solution (though not ideal). The OPL office space has been sufficient to accommodate everyone together on the first floor.

**PLATFORMS** The mass spectrometry lab houses four tandem mass spectrometers on-line coupled to nanoLC systems : Three discovery systems for global profiling: two next generation Orbitrap platforms (QExactive) (figure 2, left picture) and a low-resolution tandem MS (LTQ), and one state-of-the-art system for targeted mass spectrometry: the QTrap 5500 (right picture).

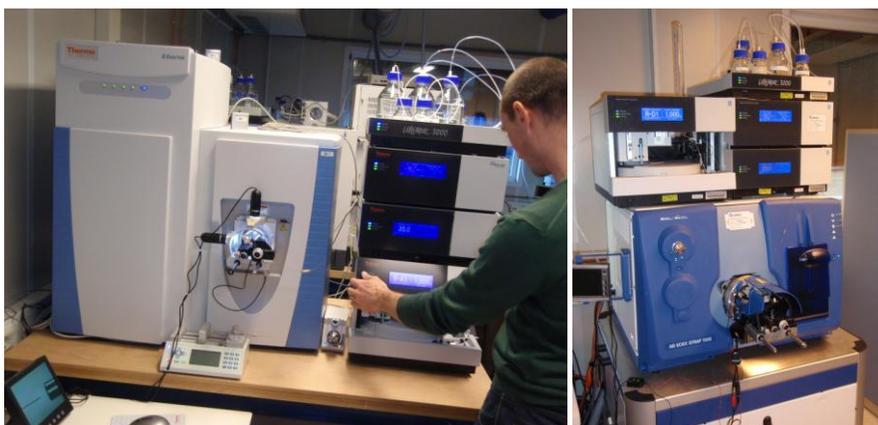


Figure 2. Tandem mass spectrometers at the OPL (CCA 1-47). Left: next generation Orbitrap, the QExactive (ThermoFischer). Right: nanoLC system (Ultimate3000) coupled to a QTrap 5500 platform (Applied Biosystems).

### 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 and database searching, all peptide identification and quantification data from the different runs are imported into the software tool 'Scaffold' and more recently in Proteome Discoverer or MaxQUant. These tools aid in confidence assignment to protein identification as well as in data organisation and visualization. So far this process has been manual and therefore labour intensive. The CTMM TraIT project in which the OPL participates may facilitate development of streamlined pipelines for data (pre)processing and analysis. Data exports to excel are used to perform further dedicated statistical analyses, which is also facilitated by the OPL.

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 switch (see figure 3). In addition to the servers, multiple external 1Tb hard-discs are used for raw data back-up. We will set-up a -much needed- database in conjunction with a Laboratory Information Management System in the context of the newly launched CTMM-TraIT project.

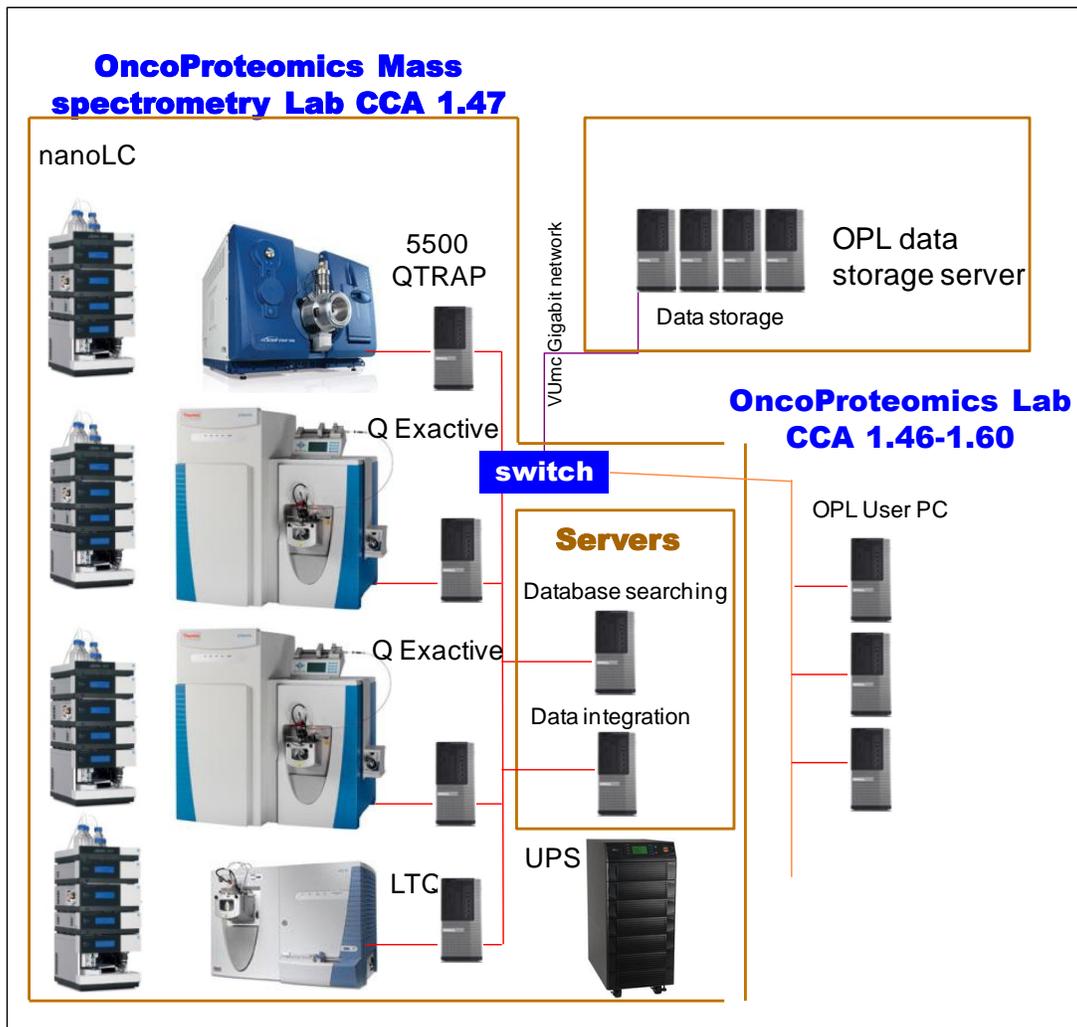


Figure 3. Proteomics infrastructuur OncoProteomics Laboratory VUmc Connectivity of proteomics and IT infrastructure.

## Research strategy and projects

### Introduction

The lack of reliable, robust and easily assessable biomarkers greatly hampers cancer management. 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 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.

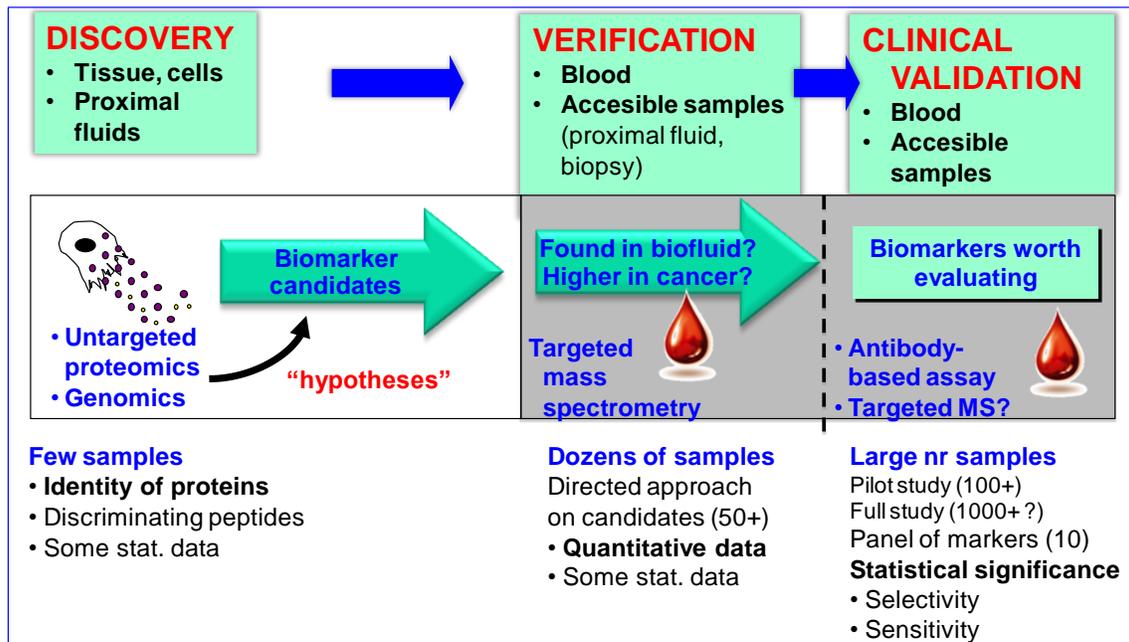


Figure 4. Complementary mass spectrometry-based proteomics approaches for discovery and validation of cancer signatures and biomarkers at the OncoProteomics Laboratory.

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. 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
- In-depth proteomics of organelles/subcellular compartments, biofluids, stool, platelets
- Biofluid (CSF) proteomics
- High-throughput biofluid peptide profiling
- Kinome profiling and phosphoproteomics
- Mining high-dimensional proteomics data

#### 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 ( $\sim 10^6$ ) at intermediate throughput (2 hrs- 15 hrs per sample).

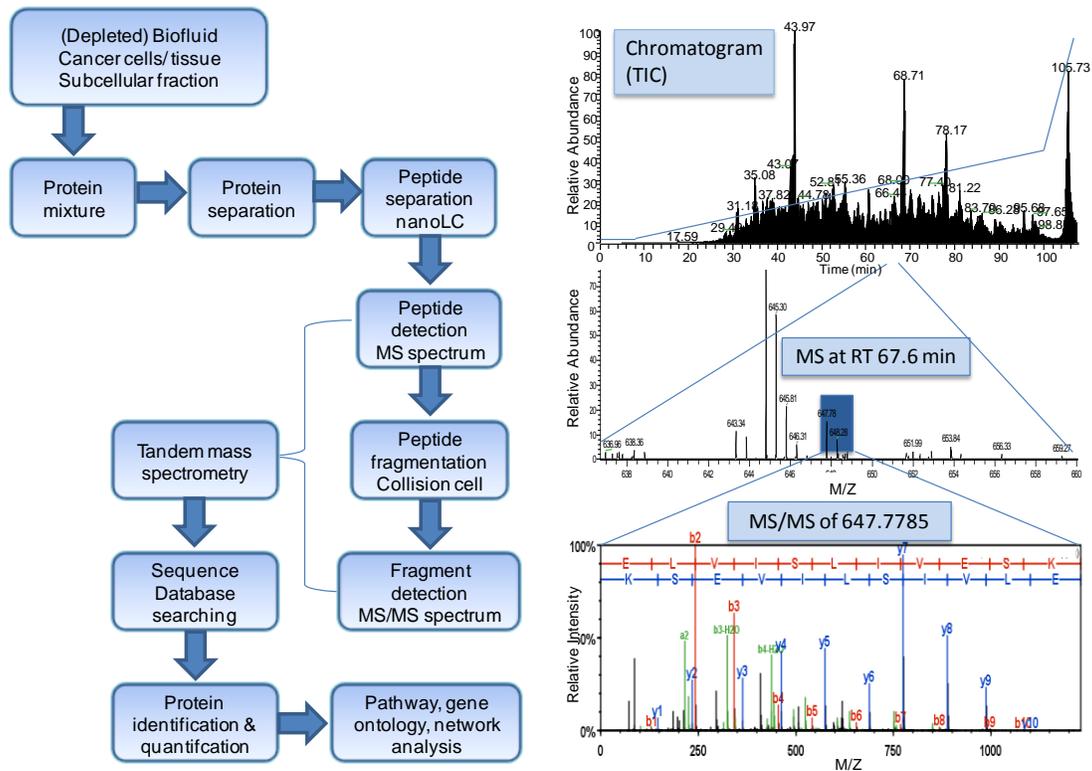
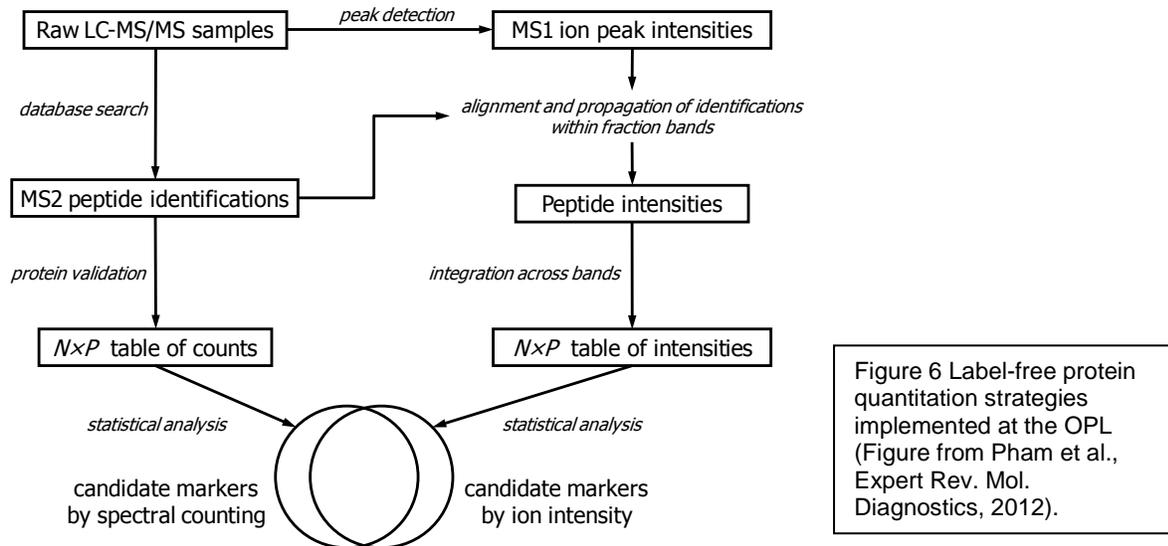


Figure 5. NanoLC-MS/MS-based proteomics workflow of implemented at the OPL (figure from Pham et al., Expert Rev. Mol. Diagnostics 2012).

Label-free 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 (eg., Piersma et al., J Proteome Res. 2010; Albrethsen et al., Mol. Cell. Proteomics 2010) (appendix pages 30 and 51). 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., submitted). 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., 2009). 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).



### Candidate-based targeted mass spectrometry

A challenge in biomarker discovery projects is the task to differentiate true from false biomarker candidates derived from discovery experiments. Follow-up analyses are needed to test a large number of candidates in relatively large sets of patient and control samples for initial verification (Figure 4 and 7). Unfortunately, a pressing lack of suitable antibodies has hampered the necessary validation efforts. Moreover, antibody-based methods do not have high multiplexing potential.

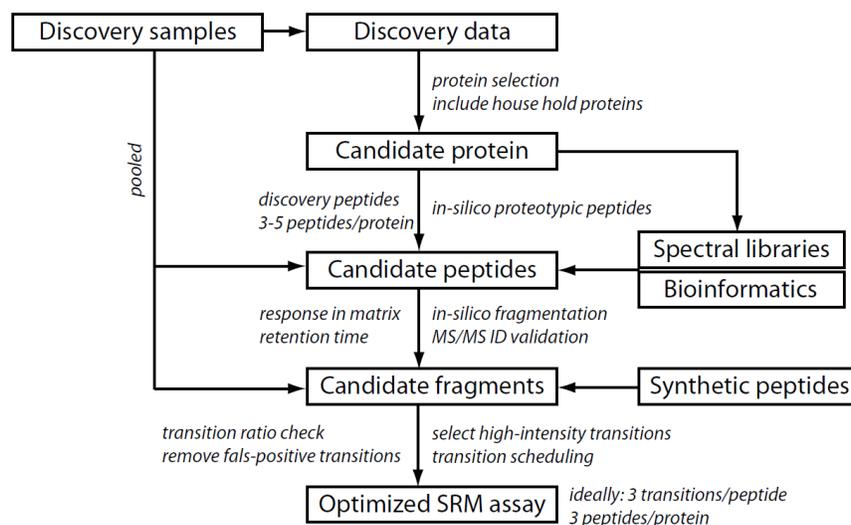


Figure 7. Workflow for making targeted mass spectrometry assays for proteins of interest (candidate biomarkers). (Figure from Pham et al., Expert Rev. Mol. Diagnostics, 2012)

Recently, robust MS-based methods that allow for multiplexed, high-throughput, and sensitive biomarker validation (multiple-reaction monitoring mass spectrometry, MRM-MS; figure 7) have emerged to overcome this problem. Peptide detection and quantification is based on the combination of two consecutive stages of mass filtering, selecting a peptide precursor mass in the 1<sup>st</sup> quadrupole (Q1) and, after CID, a fragment ion mass in the 3<sup>rd</sup> quadrupole (Q3) (figure 7). The OPL has a dedicated platform for targeted MRM-MS (the QTrap 5500, see picture in figure 2). Despite the increased sensitivity of targeted MS, some level of fractionation and enrichment is still required to reach the required sensitivity (low ng/ml range) to detect tissue leakage proteins in plasma/ biofluids.

In the context of the CTMM DeCoDe project, we have developed a multiplex assay for 38 biomarkers candidates for colon cancer screening that are currently being validated in a series of > 200 stool samples. Based on these analyses a small discriminatory panel will be selected for further antibody-based assay development and testing for complementarity to the FIT test. The latter test that will be used for population-based screening in the Netherlands in 2013.

### **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 thousands of identified and quantified proteins.

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 65. 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., Gastroenterology 2011). The project with Prof. Onno Kranenburg at the UMCU has yielded a novel drug resistance protein enriched in the stem cells (Van Houdt et al., Mol. Cellular Prot. 2011) (see abstracts, pages 58). The project with Dr. Simone Niclou, Prof. Bjerkvig and Uros Rajicevic is described in the abstract section on page 57. In both projects, proteins associated with stemness have been identified.

Other projects that successfully employed total cancer cell lysate proteomics have identified predictive candidate biomarkers for cisplatin sensitivity and resistance in a panel of NSCLC lines (appendix page 71) and candidate biomarkers to predict risk for cervical cancer progression in a HPV progression model (appendix page 73). Finally, an exciting new development that will enable many biomarker projects in the coming years are new methods that allow for protein profiling of FFPE material. We recently implemented a protocol for protein lysis from FFPE tissues in order to investigate protein expression changes in colon adenoma to carcinoma progression.

### **Targeted biomarker discovery strategies: In-depth proteomics of organelles/subcellular compartments, secretion media, stool, platelets**

For in-depth quantitative analysis of peptides and proteins in (pre)clinical samples, whenever possible, we focus on sub-proteomes (looking at less to see more) and proximal fluids. 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. cell surface/ plasma membrane to provide candidate biomarkers for molecular imaging and drug targeting and 2. 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. 3 In vitro generated tumor secretomes and exosomes to identify candidate biomarkers that have an increased chance to be detected in serum. Other biofluid avenues with potential for biomarker discovery that are being explored are platelets and platelet-derived releasates (Piersma et al., J. Proteomics 2009) and plasma microparticles/ exosomes.

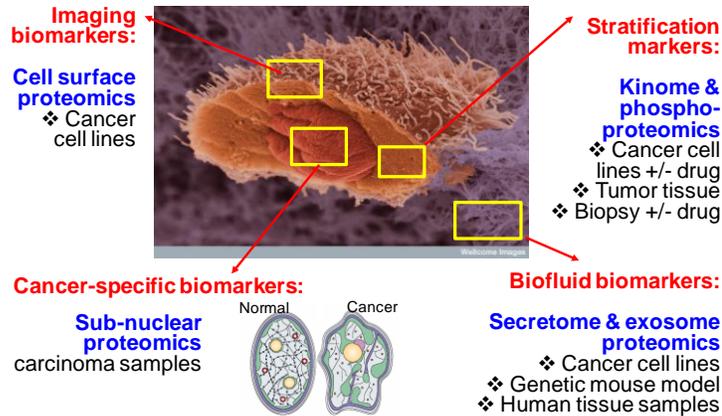


Figure 8. Targeted biomarker discovery strategies of biomarker-rich subproteomes

The feasibility of secretome proteomics to identify serum-based markers was first explored in a mouse model system (Piersma et al., *J. Proteome Res.* 2010; page 30). In hybrid core-collaborative projects, we have explored the above listed subproteomes often using colorectal tumor tissue as the model. For this cancer, fresh frozen tissue is available for proteomics together with extensive knowledge of the chromosomal and transcriptome aberrations in adenoma to carcinoma progression (on-going work in the Tumor Profiling Unit of Prof. Gerrit Meijer). The strategy from secretome proteomics to a novel serum marker for colon cancer early detection was published recently in the journal *Clinical Cancer Research* (Fijneman et al., 2011) and is further described in the research appendix (pages 48). In addition, cell surface proteomics of a panel of colorectal cell lines integrated with transcriptomics has identified novel biomarkers for molecular imaging (De Wit et al., *Gut* 2011; appendix page 53).

### In-depth biofluid (CSF) proteomics

Proximal fluids in contact with the diseased organ provide an enriched source for biomarker discovery. For example, CSF provides an attractive source for biomarker discovery in brain and neurological diseases (Figure 9).

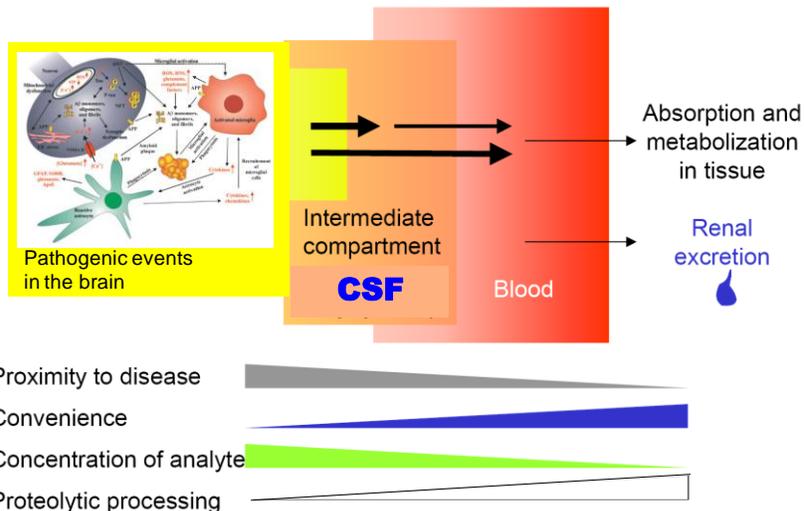


Figure 9. Proximal fluids provide an enriched source of in vivo pathogenic events and candidate biomarkers for biofluid-based diagnostics. (Modified from Schulz-Knappe and Schrader, 2003)

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 evaluated affinity-based abundant

protein depletion (Top14) to enrich for low abundant target proteins (in CSF, typically brain-derived plasmamembrane and secreted proteins (Fratantoni et al., *Prot. Clinical Applic.* 2010) (Figure 10, appendix page 87).

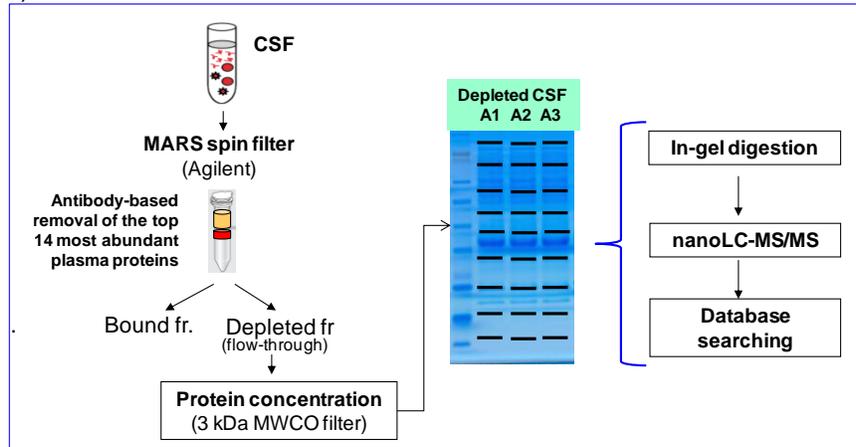


Figure 10. Workflow for in-depth CSF proteomics (figure modified from Fratantoni and Jimenez, 2010). The depleted CSF fraction is enriched for brain-derived proteins.

In 2010-2012 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 (EU cNeuPRO project) (appendix page 88). Currently we are applying this approach to the analysis of genetic Parkinson's Disease (context EU Mefopa project, pag. 91) and to sporadic Parkinson's disease in collaboration with Dr. Wilma van den Berg (VUmc), to frontotemporal dementia and to multiple sclerosis (appendix page 93; collaboration with Dr. Charlotte Teunissen, VUmc).

### High-throughput biofluid peptide profiling

We and others have successfully combined serum peptide profiling by mass spectrometry with bioinformatics to establish distinctive serum polypeptide mass spectral patterns that correlated with clinically relevant outcomes. Peptides making up these signatures are derived from differential processing of abundant blood and clotting proteins, thereby providing a direct link to cancer differential protease activity (Villanueva et al., *JCI*, 2007, Voortman et al., *Proteome Science* 2009). Therefore these serum peptide patterns may have clinical utility as surrogate markers for detection and classification of cancer.

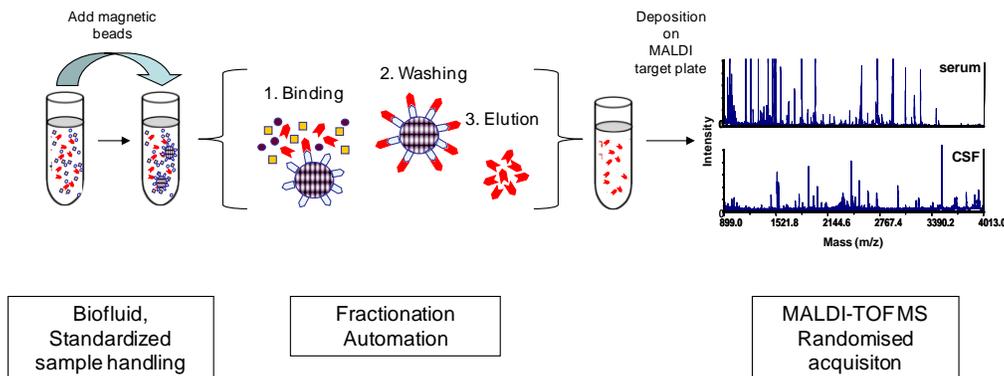


Figure 11. High-throughput serum peptide profiling MALDI-TOF-MS-based workflow automated at the OPL (Figure from Jimenez and Knol, *Nanoproteomics:Methods and Protocols*, Humana Press, 2010). The biofluid MS spectra are subjected to bioinformatics analysis to identify discriminatory patterns between clinical groups.

We have implemented an automated magnetic-particle-assisted peptide capture coupled to MALDI-TOF-MS (Figure 11) that provides a fast and reproducible profiling platform for measuring peptides in the low molecular mass range of the serum and CSF proteomes (Jimenez et al., *Proteomics Clin. Applic.* 2007a

and 2007b). Advantages of the method are: 1. the high throughput nature: ~100 samples can be processed and measured in less than a day, and 2. the low sample consumption (for blood-serum 20  $\mu$ l is enough). For MALDI-TOF-MS data, MarkerView in conjunction with the OPLAnalyzer tool box, developed at the OPL (Pham et al., 2008), has proven to be a reliable data preprocessing and visualization tool. We applied serum peptide mass profiling to in a phase I trial and identified peptide signatures associated with drug response and clinical outcome (Voortman et al., Proteome Science 2009). This prognostic value of the signature was recently validated in an independent study in collaboration with Prof. Egbert Smit (see appendix page 76). Finally, we applied CSF peptide profiling to clinical subtype analysis in multiple sclerosis (collaboration Dr. Charlotte Teunissen and Dr. Marleen Koel-Simmelink). From this study several peptides were identified with altered levels in one or more sub-groups and one peptide was validated using an ELISA (see appendix page 98).

### Kinome profiling and phosphoproteomics

Kinome profiling and phosphoproteomics provide powerful targeted approaches to analyze kinase and phosphatase signalling pathways, that may yield therapeutic targets and predictive markers (Figure 12).

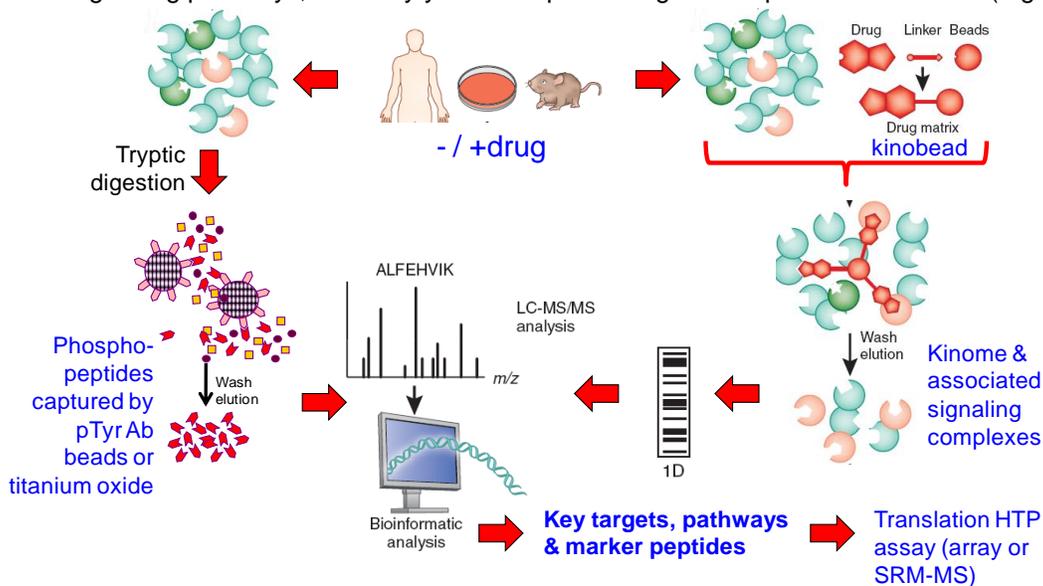


Figure 12. Kinome profiling and phosphoproteomics workflows at the OPL to enable personalized medicine.

Tyrosine kinases play a prominent role in human cancer, yet the oncogenic signalling pathways driving cell proliferation and survival have been difficult to identify, in part because of the complexity of the pathways, and in part because of low cellular levels of tyrosine phosphorylation. Two affinity-based phosphopeptide enrichment methods have been implemented at the OPL: 1. A titanium-oxide-based capture method that can enrich for all phosphopeptides containing pTyr, pThr, Pser and that works at low level (see page 40) and 2. An antibody-based method that can specifically capture phosphopeptides containing a phosphotyrosine residues (see page 39). The captured phosphopeptides are subsequently identified and quantified by nanoLC-MS/MS. We recently successfully applied this method in several studies using model systems. In collaboration with Dr. Henk Broxterman, we explored sunitinib-induced alterations in the phosphotyrosine proteome of colon cancer cells and identified promising new kinase targets. Phosphoproteomics in a glioblastoma model system with mutant EGFR (collaboration Prof. Verheul, Dr. Tom Wurdinger) identified the largest dataset to date (see page 44). 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.

For targeted kinome profiling, we have implemented chemical proteomics: a powerful affinity chromatography approach coupled to tandem mass spectrometry for identifying proteome-wide small molecule-protein interactions (see page 45).

The preliminary studies show that label-free kinome and phosphoproteomics provides a promising avenue for the identification of (hyper)activated protein kinases as candidate therapeutic targets and diagnostic markers enabling stratification for targeted therapy.

## 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 and commercial 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 Ingenuity Pathway Analysis, FatiGO, David, STRING, Cytoscape and sequence motif tools like SecretomeP and SignalP. 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.

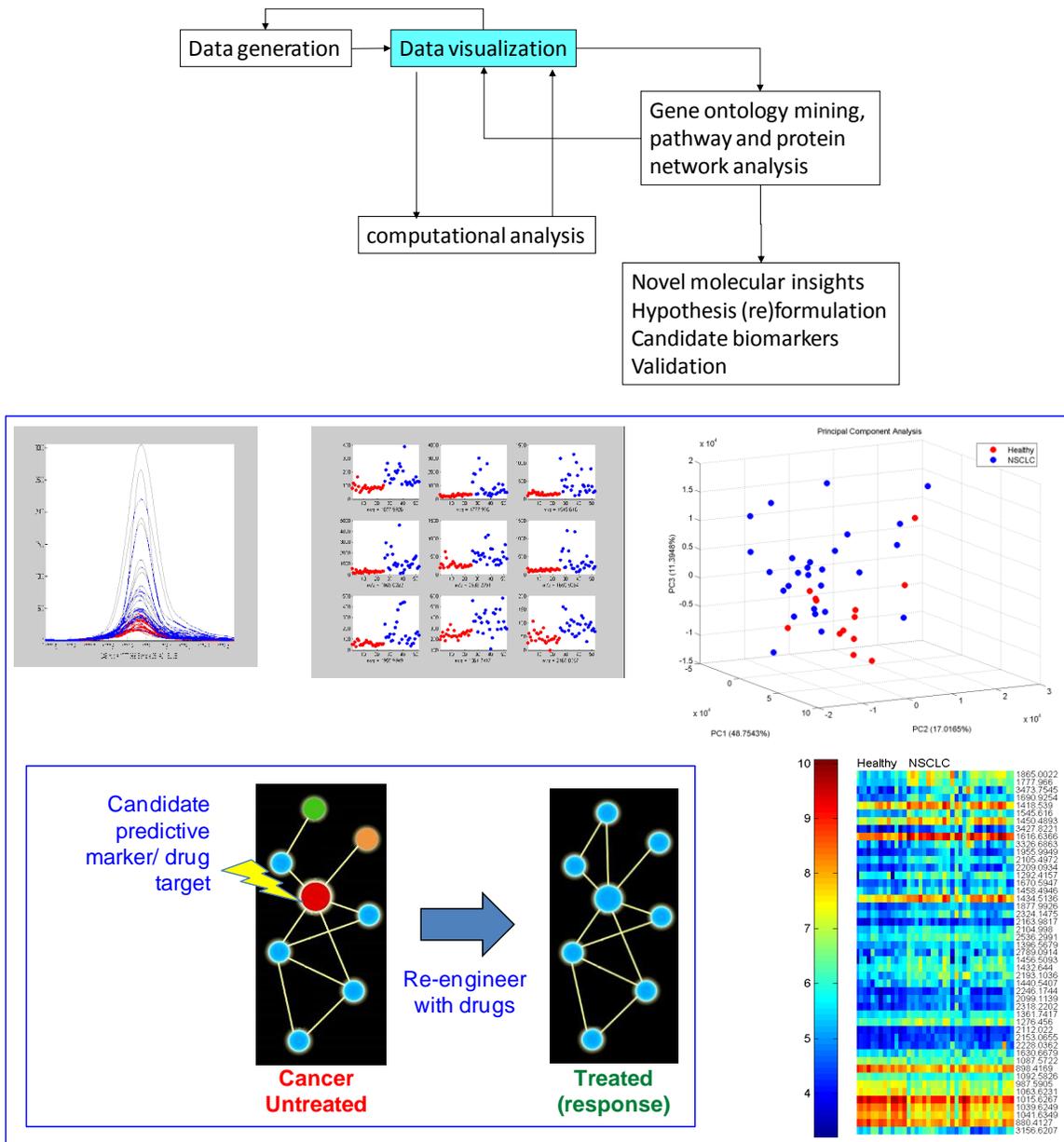


Figure 13. Computational analysis and data visualization is key in the analysis of highthroughput data. Upper panel: General scheme of OMICs data mining. Lower panel: Data may be visualized at different levels: raw data, peptide ion, spectral overlay, scatter plot, boxplot, clustering, heatmap, PCA plot and at an even higher analysis level: protein-protein interaction network with overlaid 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. Targeted proteomics strategies for discovery of biomarkers, drugable targets and immune-modulators: secretome, exosome, cell surface proteome, platelets** (pag. 29)

- Sub-Project 1: Label-free quantitative secretome proteomics for discovery of serum-based cancer biomarkers Workflow evaluation and proof-of-concept analysis in model system (pag. 30)
- Sub-project 2: Secretome proteomics of breast and colon cancer cell lines: Carcinoma origin dictates differential functional macrophage phenotype (pag. 32)
- Sub-project 3: Proteomics of exosomes secreted by cancer cell lines and primary cells reveals oncogenic signaling and biomarker potential (pag.33)
- Sub-project 4: Novel identified proteins in cancer derived exosomes can change the behavior of healthy prostate cells (pag. 34)
- Sub-project 5: Exosomes proteomics yields new insights into the endosomal-exosomal pathway of B cells during infection and malignant transformation (pag.35)
- Sub-project 6: Cell surface proteomics of osteosarcoma cells (pag. 36)
- Sub-Project 7: Platelet proteomics for cancer biomarker discovery (pag. 37)

### **2. Signaling pathway proteomics for insight into cancer signaling, and identification of drug targets and biomarkers for patient stratification** (pag. 38)

- Sub-project 1: Phosphotyrosine peptide immunoprecipitation for phosphoproteomics of tyrosine kinase signaling events (pag. 39)
- Sub-project 2: Titanium-oxide-based phosphoproteomics for analysis of global protein phosphorylation (pag. 40)
- Sub-project 3: Deciphering global kinase signaling patterns in a panel of colorectal cancer cell lines in relation to Aurora kinase A by phosphoproteomics (pag. 42)
- Sub-project 4: Phosphoproteomics of glioblastoma cells:  $\Delta$ EGFR targets and erlotinib effects (pag. 44)
- Sub-project 5: Development of kinobeads for comprehensive capture of the kinome (all protein kinases) prior to analysis by proteomics (pag. 45)

### **3. Colorectal cancer (CRC) proteomics** (pag. 46)

- Sub-project 1: Proteome profiling of mouse colon tumor proximal fluids revealing candidate biomarkers for colorectal cancer screening (pag. 48)
- Sub-project 2: Proteome profiling of proximal fluids from human colon cancer tissue reveals Novel candidate biomarkers for early diagnosis (pag. 50)
- Sub-project 3: Sub-nuclear proteomics in colorectal cancer: Identification of proteins enriched in the nuclear matrix fraction and regulation in adenoma-carcinoma progression (pag. 51)
- Sub-project 4: Label-free LC-MS/MS profiling of chromatin-associated proteins in colorectal adenoma and carcinoma tissues (pag. 52)
- Sub-project 5: Cell surface proteomics identifies glucose transporter type 1 and prion protein as candidate biomarkers for colorectal adenoma-to-carcinoma progression (pag. 53)
- Sub-project 6: Protein biomarker discovery in stool for the early detection of colorectal cancer (pag. 54)
- Sub-project 7: Proteomics of tumorigenic stem-like cells in colon cancer spheroids (pag. 56)
  - Sub-project 7a: Proteomics of tissue-based spheroids in comparison to colorectal cancer resection material (pag. 57)
  - Sub-project 7b: Comparative proteomics of colon cancer stem cells and differentiated tumor cells identifies BIRC6 as a potential therapeutic target (pag. 58)
  - Sub-project 7c: The secretome of colon cancer stem cells contains drug-metabolizing enzymes (pag. 59)
- Sub-project 8: Proteomics of CRC liver metastases: High expression of serpinB5 (Maspin) is associated with poor survival in stage III and IV colorectal cancer (pag. 60)

## 5. Brain cancer proteomics

- Sub-project 1: Proteomics of meningiomas in correlation with growth rate (pag. 61)
- Sub-project 2: Proteomics reveals minichromosome maintenance proteins as novel tumor markers for meningiomas (pag. 62)
- Sub-project 3: miR-200a inhibits meningioma cell migration and tumor growth by directly targeting non-muscle heavy chain IIb (pag. 63)
- Sub-project 4: Comparative proteomics of brain neural stem cells and brain cancer stem cells (pag 64)

## 5. Breast cancer proteomics

- Sub-project 1: Proteomics of murine *Brca1*-deficient breast tumors identifies DNA repair proteins with prognostic value in human breast cancer. (pag. 66)
- Sub-project 2: Immunohistochemical validation in human breast cancer of selected protein members of the mouse 45 protein BRCA1 deficiency signature (pag. 67)
- Sub-project 3: Exploring differences in short-term response to cisplatin in sensitive and non-sensitive mouse mammary tumors (pag. 69)
- Sub-project 4: Secretome proteomics of BRCA1 deficient and proficient cell lines reveals candidate markers for non-invasive BRCAness detection (pag 70)

## 6. Lung cancer proteomics

- Subproject 1: Novel candidate biomarkers for cisplatin response prediction and monitoring in non-small-cell lung carcinoma (NSCLC) (pag. 71)
- Sub-project 2: Exploration of sputum to develop protein-based assays for early detection, prognosis and drug response of lung cancer (pag. 72)

## 7. Cervical cancer proteomics

- Sub-project 1: Proteomic unravelling of protein expression changes during HPV-induced transformation in an *in vitro* model of human keratinocytes (pag. 73)

## 8. Acute Myeloid Leukemia proteomics

- Sub-project 1: Identification of protein profiles of the bone marrow microenvironment associated with apoptosis-resistance of acute myeloid leukemia (pag. 74)

## 9. High-throughput body fluid peptide profiling by MALDI mass spectrometry for patient stratification by pattern diagnostics (pag. 75)

- Sub-project 1: Mass spectrometric serum peptide profiling for drug response prediction: signature validation (pag. 76)
- Sub-project 2: Serum peptide profiling in non-small cell lung cancer patients treated with sorafenib and erlotinib (pag. 77)

## 10. Mining high-dimensional proteomics data (pag. 78)

- Sub-project 1: On the beta binomial model for comparative analysis of spectral count data in label-free tandem mass spectrometry-based proteomics (pag. 78)
- Sub-project 2: Strategies for analyzing spectral count data in label-free tandem mass spectrometry-based proteomics (pag. 79)
- Sub-project 3: Network-based data analysis serving marker prioritization for targeted mass spectrometry validation (pag. 80)
- Sub-project 4: Computational analysis of phosphoproteomics data (pag. 81)

## Other core/ collaborative projects

### 1. Neuroproteomics (pag. 86)

- Sub-project 1: In-depth analysis of the cerebrospinal fluid proteome for biomarker discovery: abundant protein depletion sample pretreatment method (pag. 87)
- Sub-project 2: Novel candidate CSF protein biomarkers for mild cognitive impairment and Alzheimer's disease (pag. 88)
- Sub-project 3: CSF proteomics reveals novel biomarker BRI2 that is increased in amyloid plaques in Alzheimer's disease (pag. 89)
- Sub-project 4: CSF biomarkers for Parkinson's disease patients with LRKK2 mutations (pag. 90)
- Sub-project 5: Proteomics of CSF-derived microvesicles (pag. 91)
- Sub-project 6: Proteomics discovery of novel CSF biomarkers for Multiple Sclerosis (pag. 93)
- Sub-project 7: Proteomics analysis of the locus ceruleus in Parkinson's disease (pag. 94)

- Sub-project 8: Identification of new proteins associated with cerebral capillary amyloid angiopathy: relevance for amyloid clearance in Alzheimer's disease (pag. 95)
- Sub-project 9: Proteomics analysis of kainic acid-induced changes in the mouse hippocampus reveals microRNAs as regulators of neural stem cell apoptosis (pag. 97)
- Sub-project 10: Identification of CSF biomarkers for diagnosis and progression of multiple sclerosis by MALDI-TOF mass spectrometry (98)
- Sub-project 11: Diagnosis and prognosis of Alzheimer's by mass spectrometric detection of specific amyloid-beta peptide fragments in CSF in combination with pattern analysis (pag 99).

## **2. Proteomics of signaling protein complexes**

- Sub-project 1: FANCM phosphorylation in response to topoisomerase I inhibitor camptothecin (pag. 101)
- Sub-project 2: Fishing for new Fanconi genes by proteomics of protein complexes (pag. 102)
- Sub-project 3 PPIB mutations cause severe osteogenesis imperfecta (pag. 103)
- Subproject 4: Identification of novel signalling partners of the HCMV encoded viral GPCR US28 (pag. 104)
- Subproject 5: Cancerous inhibitor of PP2A, CIP2A as target for fusiciccin and 14-3-3 proteins (pag. 105)
- Subproject 6: Identification of novel molecular interactions of the cardiac sodium channel carboxyl-terminal domain (pag. 106)
- Subproject 7: In vitro PKC $\alpha$ -phosphorylated cardiac troponin reduces maximal force and increases Ca<sup>2+</sup>-sensitivity in failing human cardiocytes (pag 108)

## **3. Proteomics of mycobacterium**

- Subproject 1: Proteomics of mycobacterial pathogens (page 109)
- Subproject 2: Proteomics profiling of mycobacterium tuberculosis identifies nutrient starvation responsive toxin-antitoxin systems (page 110)

## **Five-year view**

With ever-increasing sensitivity, resolution and speed of LC-MS/MS platforms, the sample size of clinical biomarker discovery experiments will increase in years to come, enabling identification of more reliable biomarker candidates. In addition, sample input levels will go down, enabling biopsy-level measurements. We also expect that the coming years will show a shift towards discovery in sample-limited but highly defined samples, such as microdissected FFPE material, or cells obtained by Laser Capture Microdissection or FACS-selected cell populations. With these minute amounts of material, label-free approaches are the only viable option. Moreover, software tools for robust MS intensity-based quantification and visualization will be consolidated, and improved statistical methods will be developed that can reliably translate peptide peaks into protein level quantification. Finally, label-free SRM is certainly expected to be the method of choice for initial protein biomarker verification in biofluids because of its cost-effectiveness and successful validation as an intermediate method in recent years.

**We look forward to continuing our discovery journey at a new and unprecedented level and apply our newly installed next generation tandem mass spectrometers to our running projects and new avenues in FFPE biobanked material and in tumor biopsies obtained in clinical trials.**

## EDUCATION

In 2010-mid2012 several lectures were given to bachelor, master and PhD students. We participated in the VU bachelor and master courses 'Integrative tumor biology' and 'bioinformatics for translational medicine'; and in the VUmc Bachelor Toegepaste/Experimentele immunologie; in the VUmc Master Oncology (in the courses 'Tumor Biology' and 'Therapeutics'); and in the AIO cursus 'Pharmacogenomics' of LACDR. Furthermore, in 2010 we organized a practical 3-day lab course 'Protein identification by mass spectrometry and database searching', to train collaborators to prepare their own samples for mass spectrometry. In addition, the master course 'Biomedical Proteomics' was set up for more in-depth education of students and collaborators (start in 2011). In addition, several VU bioinformatics master students and master oncology students performed their internship at the OPL in 2010 - mid2012 (for a listing see page 5).

## SCIENTIFIC OUTPUT 2010-present

### 2012

1. Kranenburg O, Emmink BL, Knol J, Borel Rinkes IHM, Jimenez CR. Proteomics in studying cancer stem cell biology. *Expert Reviews Proteomics*. In press.
2. Bogels M, Braster R, Nijland PG, Gul N, van de Luijngaarden W, Fijneman RJA, Meijer GA, Jimenez CR, Beelen RHJ, van Egmond M. Carcinoma origin dictates differential skewing of monocyte function. *Oncol Immunology*. In press.
3. Pham TV, Piersma SR, Oudgenoeg G, Jimenez CR. Label-free mass spectrometry-based proteomics for biomarker discovery and validation. *Expert Rev Mol Diagn*. 2012 May;12(4):343-59.
4. Warmoes M, Jaspers JE, Pham TV, Piersma SR, Oudgenoeg G, Massink MP, Waisfisz Q, Rottenberg S, Boven E, Jonkers J and 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). [Epub ahead of print]
5. 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 and Jimenez CR. Proximal fluid proteome profiling of mouse colon tumors reveals biomarkers for early diagnosis of human colorectal cancer. *Clin. Cancer Res. Clin Cancer Res*; 18(9); 2613-24. (2012).

### 2011

6. Bosch LJ, Carvalho B, Fijneman RJ, Jimenez CR, Pinedo HM, van EM and Meijer GA. Molecular tests for colorectal cancer screening. *Clin. Colorectal Cancer* 10: 8-23 (2011).
7. de Wit M, Jimenez CR, Carvalho B, Belien JA, Delis-van Diemen PM, Mongera S, Piersma SR, Vikas M, Navani S, Ponten F, Meijer GA and Fijneman RJ. Cell surface proteomics identifies glucose transporter type 1 and prion protein as candidate biomarkers for colorectal adenoma-to-carcinoma progression. *Gut* (2011).
8. Emmink BL, Van Houdt WJ, Vries RG, Hoogwater FJ, Govaert KM, Verheem A, Nijkamp MW, Steller EJ, Jimenez CR, Clevers H, Borel Rinkes IH and Kranenburg O. Differentiated human colorectal cancer cells protect tumor-initiating cells from irinotecan. *Gastroenterology* 141: 269-278 (2011).
9. Gast MC, Zapatka M, van TH, Bontenbal M, Span PN, Tjan-Heijnen VC, Knol JC, Jimenez CR, Schellens JH and Beijnen JH. Postoperative serum proteomic profiles may predict recurrence-free survival in high-risk primary breast cancer. *J. Cancer Res. Clin. Oncol.* 137: 1773-1783 (2011).
10. Knol JC and Jimenez CR. MALDI-TOF serum profiling using semiautomated serum peptide capture with magnetic reversed phase (C18) beads. *Methods Mol. Biol.* 790: 3-16 (2011).
11. Skvortsov S, Jimenez CR, Knol JC, Eichberger P, Schiestl B, Debbage P, Skvortsova I and Lukas P. Radioresistant head and neck squamous cell carcinoma cells: intracellular signaling, putative biomarkers for tumor recurrences and possible therapeutic targets. *Radiother. Oncol.* 101: 177-182 (2011).

12. Teunissen CE, Koel-Simmelink MJ, Pham TV, Knol JC, Khalil M, Trentini A, Killestein J, Nielsen J, Vrenken H, Popescu V, Dijkstra CD and Jimenez CR. Identification of biomarkers for diagnosis and progression of MS by MALDI-TOF mass spectrometry. *Mult. Scler.* **17**: 838-850 (2011).
13. Teunissen CE, Tumani H, Bennett JL, Berven FS, Brundin L, Comabella M, Franciotta D, Federiksen JL, Fleming JO, Furlan R, Hintzen RQ, Hughes SG, Jimenez CR, Johnson MH, Killestein J, Krasulova E, Kuhle J, Magnone MC, Petzold A, Rajda C, Rejdak K, Schmidt HK, van P, V, Waubant E, Wolf C, Deisenhammer F, Giovannoni G and Hemmer B. Consensus Guidelines for CSF and Blood Biobanking for CNS Biomarker Studies. *Mult. Scler. Int.* **2011**: 246412 (2011).
14. van Dijk KD, Berendse HW, Drukarch B, Fratantoni SA, Pham TV, Piersma SR, Huisman E, Breve JJ, Groenewegen HJ, Jimenez CR\* and van de Berg WD\*. The Proteome of the Locus Ceruleus in Parkinson's Disease: Relevance to Pathogenesis. *Brain Pathol.* (2011).
15. Van Houdt WJ, Emmink BL, Pham TV, Piersma SR, Verheem A, Vries RG, Fratantoni SA, Pronk A, Clevers H, Borel Rinkes IH, Jimenez CR\* and Kranenburg O\*. Comparative proteomics of colon cancer stem cells and differentiated tumor cells identifies BIRC6 as a potential therapeutic target. *Mol. Cell Proteomics.* **10**: M111 (2011).

## 2010

16. Albrethsen J, Knol JC, Piersma SR, Pham TV, de Wit M, Mongera S, Carvalho B, Verheul HM, Fijneman RJ, Meijer GA and Jimenez CR. Subnuclear proteomics in colorectal cancer: identification of proteins enriched in the nuclear matrix fraction and regulation in adenoma to carcinoma progression. *Mol. Cell Proteomics.* **9**: 988-1005 (2010).
17. Fratantoni SA, Piersma SR and Jimenez CR. Comparison of the performance of two affinity depletion spin filters for quantitative proteomics of CSF: Evaluation of sensitivity and reproducibility of CSF analysis using GeLC-MS/MS and spectral counting. *Proteomics. Clin. Appl.* **4**: 613-617 (2010).
18. Jimenez CR, Knol JC, Meijer GA and Fijneman RJ. Proteomics of colorectal cancer: overview of discovery studies and identification of commonly identified cancer-associated proteins and candidate CRC serum markers. *J. Proteomics.* **73**: 1873-1895 (2010).
19. Jimenez CR and Slijper M. Current status of cancer proteomics, how far are we from clinical applications? *J. Proteomics.* **73**: 1787-1789 (2010).
20. Pham TV, Piersma SR, Warmoes M and Jimenez CR. On the beta-binomial model for analysis of spectral count data in label-free tandem mass spectrometry-based proteomics. *Bioinformatics.* **26**: 363-369 (2010).
21. Piersma SR, Labots M, Verheul HM and Jimenez CR. Strategies for kinome profiling in cancer and potential clinical applications: chemical proteomics and array-based methods. *Anal. Bioanal. Chem.* **397**: 3163-3171 (2010).
22. Piersma SR, Fiedler U, Span S, Lingnau A, Pham TV, Hoffmann S, Kubbutat MH and Jimenez CR. Workflow comparison for label-free, quantitative secretome proteomics for cancer biomarker discovery: method evaluation, differential analysis, and verification in serum. *J. Proteome. Res.* **9**: 1913-1922 (2010).
23. Sani M, Houben EN, Geurtsen J, Pierson J, de PK, van ZM, Wever B, Piersma SR, Jimenez CR, Daffe M, Appelmek BJ, Bitter W, van der Wel N and Peters PJ. Direct visualization by cryo-EM of the mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. *PLoS. Pathog.* **6**: e1000794 (2010).
24. Saydam O, Senol O, Schaaïj-Visser TB, Pham TV, Piersma SR, Stemmer-Rachamimov AO, Wurdinger T, Peerdeman SM and Jimenez CR. Comparative protein profiling reveals minichromosome maintenance (MCM) proteins as novel potential tumor markers for meningiomas. *J. Proteome. Res.* **9**: 485-494 (2010).
25. Spitzer P, Klafki HW, Blennow K, Buee L, Esselmann H, Herruka SK, Jimenez C, Klivenyi P, Lewczuk P, Maler JM, Markus K, Meyer HE, Morris C, Muller T, Otto M, Parnetti L, Soininen H, Schraen S, Teunissen C, Vecsei L, Zetterberg H and Wiltfang J. cNEUPRO: Novel Biomarkers for Neurodegenerative Diseases. *Int. J. Alzheimers. Dis.* **2010**: (2010).
26. van Dijk KD, Teunissen CE, Drukarch B, Jimenez CR, Groenewegen HJ, Berendse HW and van de Berg WD. Diagnostic cerebrospinal fluid biomarkers for Parkinson's disease: a pathogenetically based approach. *Neurobiol. Dis.* **39**: 229-241 (2010).

## In submission

1. PosthumaDeBoer J, Piersma SR, Pham TV, Knol JC, van Beusechem VW, Kaspers GJL, van Royen BJ, Jiménez CR, Helder MN. Surface proteomic analysis of osteosarcoma identifies EPHA2 as receptor for targeted drug delivery. Submitted to Mol Cell Proteomics.
2. Houben ENG, Bestebroer J, Wilson L, Piersma SR, Jiménez CR., Ottenhoff THM, Luirink J, Bitter W. Composition of the mycobacterial type VII secretion system membrane complex. Submitted to Plos Pathogens.
3. Albrethsen J, Agner J, Piersma SR, Højrup P, Pham TV, Weldingh K, Jimenez CR, Andersen P, Rosenkrands I. Proteomic profiling of the Mycobacterium tuberculosis identifies nutrient starvation responsive toxin-antitoxin systems. Submitted to Mol Cell Proteomics.

## Book chapters:

1. Fratantoni, S. and Jimenez, CR. Abundant protein depletion sample pretreatment method for in-depth analysis of the cerebrospinal fluid proteome and biomarker discovery. Springer Protocols. Neuroproteomics issue, 2011.
2. Pham,TV., Piersma, SR., Warmoes, M., Jimenez, CR.. Statistical analysis of spectral count data in label-free tandem mass spectrometry-based proteomics. Springer Protocols. Neuroproteomics issue, 2011.
3. Knol. JC. and Jimenez, CR. MALDI-TOF Serum Profiling Using Semi-Automated Serum Peptide Capture With Magnetic Reversed Phase (C18) Beads. In: 'Nanoproteomics: Methods and Protocols'. Humana Press, 2010.
4. Fratantoni, S. and Jimenez, CR. Abundant protein depletion sample pretreatment method for in-depth analysis of the cerebrospinal fluid proteome and biomarker discovery. Springer Protocols. Neuroproteomics issue, 2010.
5. Pham,TV., Piersma, SR., Warmoes, M., Jimenez, CR.. Statistical analysis of spectral count data in label-free tandem mass spectrometry-based proteomics. Springer Protocols. Neuroproteomics issue, 2010.
6. Altelaar, AFM. and Piersma, SR. Cellular imaging using matrix-enhanced and metal-assisted SIMS. Methods Mol. Biol. Mass Spectrometry Imaging 656 197-208, 2010.

## INDICATORS of ESTEEM

### Obtained grants

- 2010 EU kp7 MEFOPA (300K, WP, Dept. Med. Onc, Project leader: Dr. Connie R. Jimenez)  
2010 Life Science Validation Fund (55K, WP, Dept. Med. Onc, Project leader: Dr. Connie R. Jimenez)  
2011 Hersenstichting Nederland (35K, NWP, Dept. Med. Onc, Project leader: Dr. Connie R. Jimenez)  
2011 200K CCA, apparatuurronde

### Invited lectures at (inter)national conferences

*Invited lectures Dr. Connie R Jimenez*

- 23 march, 2010, Seattle, USA, International Cancer Biomarker Consortium Conference, Title: "Targeted Mass Spectrometry-based Proteomics Strategies for Biomarkers Discovery and Validation in Colorectal Cancer"
- 13 april, 2010, Aberdeen, Ireland. Symposium "Advanced Proteomic Applications in Clinical and Life Sciences". Title: 'Targeted mass spectrometry-based proteomics strategies for biomarker discovery and validation in colorectal cancer'.
- 4 june, 2010, Assisi, Italy. "Workshop on Biomarkers in the early diagnosis of neurodegenerative disorders". Title: "CSF proteomics for biomarker discovery in mild cognitive impairment and Alzheimer's disease"
- 8 Oct, 2010, Heraklion, Greece. "1st International Proteomics Conference on Crete – IPCC01". Title: 'Label-free proteomics targeted to sub-cellular compartments for candidate biomarker discovery and early detection of colorectal cancer'
- 28 Oct, 2010, Amsterdam, The Netherlands. Annual meeting Nederlandse Vereniging voor

Massspectrometrie: 'Mass spectrometry in aMsterdam'. Title: "Label-free mass spectrometry-based proteomics for biomarker discovery and validation in tissues and biofluids"

- 11 feb. 2011. SEPROT 2011, Segovia. 'Label-free mass spectrometry-based proteomics for biomarker discovery and validation in tissues and biofluids'
- 30 mei 2011. EndoNeuroPsycho meeting, ENP 2011. Lunteren. CSF proteomics reveals pathogenesis and novel candidate biomarkers for mild cognitive impairment and Alzheimer's Disease
- 20 juni 2011, Cancer Proteomics 2011: Systems Biology, Developmental Models and Data Integration, Dublin. "Label-free mass spectrometry-based proteomics for biomarker discovery in murine and human cancer"
- 20 juli 2011. AAICAD 2011, Paris. Progress in CSF biomarker discovery for mild cognitive impairment and Alzheimer's Disease by label-free quantitative proteomics
- 25 sept. 2011. Multidisciplinary European Cancer Congress, Stockholm: Label-free mass spectrometry-based proteomics for biomarker discovery and validation in tissues and biofluids
- 29 sept. 2011. Annual CTMM meeting Utrecht. Protein biomarkers for cisplatin treatment response prediction in non-small cell lung cancer
- 10 okt. 2011. Freiburg. Symposium: Integrative OMICS approaches to disease mechanisms. "Label-free mass spectrometry-based proteomics for biomarker discovery in cancer"
- 5 March. 2012, Utrecht. NBIC, Hot topics day: 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"
- 8 juni 2012, Assisi, Italy. 2nd workshop Biomarkers in the early diagnosis of neurodegenerative disorders. "New CSF biomarkers for AD: report from the cNEUPRO discovery phase"
- 15 juni 2012, NUBIN2012 symposium, Amsterdam. "New CSF biomarkers for AD: report from the cNEUPRO discovery phase"
- 26 June, 2012. The 19<sup>th</sup> Arbeitstagung Micromethods in Protein Chemistry, Bochum. "Cancer biomarker discovery and validation: Label-free mass spectrometry-based proteomics"

*Invited lecture Dr. Sander R Piersma*

- 28 okt. 2011. Fall meeting Netherlands Proteomics Platform, Leiden. 'Collagen prolyl 3-hydroxylation in Osteogenesis Imperfecta: a rare modification in a rare disease'

*Invited lectures Dr. Thang V. Pham*

- 20 September, 2010, Sydney, Australia, "Human Proteome Organisation (HUPO) 9<sup>th</sup> Annual World Congress". Title: "Progress in CSF biomarker discovery for mild cognitive impairment and Alzheimer's disease by label-free quantitative proteomics"
- 15 October, 2010, Utrecht, The Netherlands. "Fall Meeting Netherlands Proteomics Platform 2010". Title: "Strategies for analyzing spectral count data in label-free tandem mass spectrometry-based proteomics".

*Invited lectures Dr. Gideon Oudgenoeg*

- 29 March 2012, Rolduc, NVMS-BSMS International conference on mass spectrometry. "Label-free Selected Reaction Monitoring for Biomarker verification"
- June 2012, Applied Biosystems, User meeting, Eindhoven. "Label-free Selected Reaction Monitoring for Biomarker verification"

## **Other academic activities**

### **(Inter)national functions (Dr. Connie R. Jimenez):**

- Coördinator Netherlands Proteomics Platform
- General Council Member European Proteomics Association (EuPA)

### **Memberships of editorial boards (Dr. Connie R. Jimenez):**

- Journal of Proteomics
- Molecular and Cellular Proteomics

- Proteomics
- Guest Editor, special issue, aug. 2010, 'Cancer Proteomics', Journal of Proteomics

#### Organisation of congresses (Dr. Connie R. Jimenez):

- Co-organisor, Fall meeting Netherlands Proteomics Platform, Oct 15, 2010, Utrecht
- Conference co-organiser: Cancer Proteomics 2011: Systems Biology, Developmental Models and Data Integration. Dublin. 20-23 juni 2011.
- Conference session organizer, 30 mei 2011, ENP 2011, Lunteren: CSF proteomics.
- Co-organisor, Fall meeting Netherlands Proteomics Platform, Oct 28, 2011, Leiden

#### News

- Jan 2010. Installation of new state-of-the-art tandem mass spectrometer (ABI-QTrap 5500) for multiplexed targeted detection of panels of protein biomarker candidates
- June 2010. Nomination for Amsterdam Invention Award. Invention title: 'Novel panel of candidate biomarkers for early detection of colon cancer'. Inventors: Dr. CR. Jimenez (Dept. Med. Onc.), Dr. R.J.A. Fijneman (Dept. PA), Prof. G.A. Meijer (Dept. PA).
- Dec 2011. Two next generation tandem mass spectrometers ordered
- Poster prize to Dr. Thang V. Pham at conference: Systems Biology, Developmental Models and Data Integration. Dublin. 20-23 juni 2011. Title: Proteomics of mouse BRCA1-deficient mammary tumors identifies DNA repair proteins with diagnostic and prognostic value in human breast cancer

#### Media attention

- Parool, feb. 2010. Interview met CRJ. Publication title: "Sneller speuren naar kankereiwit"
- Tracer, feb. 2010. Interview met CRJ. Publication title: "Nieuwe massaspectrometer gaat jaren tijdswinst opleveren"
- Ziekenhuiskrant, feb. 2010, Pag. 3. Interview met CRJ. Title: "Tijdswinst van jaren in kankeronderzoek".
- Diario Medico 19 feb. 2011. 'Ahora es el turno de las proteínas'
- Tracer 9 sept. 2011. Eiwitten verraden darmkanker
- Ad Valvas 15 sept. 2011. Eiwitten en darmkanker
- OncoPost 25 sept. 2011, the daily journal of The 2011 Multidisciplinary European Cancer Congress. The language of proteins: understanding proteins today can change cancer therapy tomorrow

#### Societal Impact

- Lekenpublicatie 'Eiwitten op darmcellen markeren gevaarlijke voorstadia darmkanker' Oncologie Up-to-date | 2011 • vol 2 • nummer 6
- Lekenpublicatie: Oncoproteomics: systeembio, ontwikkelingsmodellen en data-integratie. Oncologie Up-to-date | 2011 • vol 2 • nummer 5
- Lekenpublicatie: Kankerbreed (2012, maart issue) 'OncoProteomics voor ontdekken van biomarkers en drug targets'

#### Patent applications

- Patent 2010: PROTEIN-BASED METHODS AND COMPOSITION FOR THE DIAGNOSIS OF COLORECTAL CANCER. Subject of the invention: The present invention relates to contrast agents, diagnostic markers and methods for detecting colorectal adenocarcinomas. Inventors: Dr. Remond JA. Fijneman, Drs. Meike de Wit, Dr. CR Jimenez, Prof. Gerrit A. Meijer
- Protein biomarkers for detection of colorectal cancer (CRC). Inventors: L.J.W. Bosch, M. de Wit, B. Carvalho, R.J.A. Fijneman, G.A. Meijer, C.R. Jimenez, S.R. Piersma, T. V. Pham, G. Oudgenoeg
- Protein markers and BRCAness signature for identification of patients with homology repair deficiencies and stratification for DNA damaging therapy. Inventors: C.R. Jimenez, M.O. Warmoes, T.V. Pham, S. Rottenberg, J. Jonkers

## Collaborations

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- Prof. Christine Sers (Charité, Berlin, Germany)
- Dr. Thorsten Muller, Prof. Katrin Marcus, Prof. Helmut Meyer (Medizinische Proteom Center, Bochum, Germany)
- Dr. Matthew Fitzgibbon, Fred Hutchinson Cancer Research Center, Seattle, USA
- Dr. Frode Berven, Proteomics Unit, University of Bergen, Bergen, Norway
- Dr. Uros Rajicevic and Simone Niclou, NorLux Neuro-Oncology Laboratory, Luxembourg
- Dr. Okay Saydam (Harvard Medical School, Boston, USA)
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- Dr. Henk Broxterman, Prof. Henk Verheul, Prof. Epie Boven, Dr. Victor van Beusechem, Dr. Tanja de Gruijl (VUmc-Dept. Medical Oncology)
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- Dr. Johan de Winter, Dr. Josephine Dorsman, Dr. Quinten Waisfiz (Dept. Clinical Genetics)
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## **APPENDIX: PROJECT SUMMARIES**

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



## Targeted proteomics strategies for discovery of biomarkers, drugable targets and immune-modulators

The analysis of changes in plasma or serum protein repertoire in search for diagnostic and prognostic markers of human disease, such as cancer, has evolved rapidly from single to multi-analyte approaches by recent developments in mass spectrometry. The classical approach in shot-gun proteomics studies involves digestion of complex protein mixtures into peptides followed by liquid chromatography coupled to tandem mass spectrometric and database searching and has provided a powerful means to identify hundreds to thousands of proteins in biological samples. Still, plasma and serum are complex mixtures spanning a broad dynamic range of concentrations, which makes it difficult to analyse and identify low abundant proteins and peptides with a role in the disease process. Therefore, emerging strategies focus on pre-fractionation of the proteome of complex matrices such as serum and cancer cells/ tissues to enrich for sub-proteomes of interest. Which approach is used depends on the clinical question and on the amount of available material, as analysis of sub-proteomes often requires higher amounts of starting material.

**AIM:** To use proteomics of subcellular fractions as tools for discovery of candidate biomarkers for cancer detection and drug-response. Analyzed fractions include: cancer cell surface fractions, cancer cell conditioned media (cancer cell secretomes and exosomes derived thereof), tumor tissue-derived media and platelet releasates

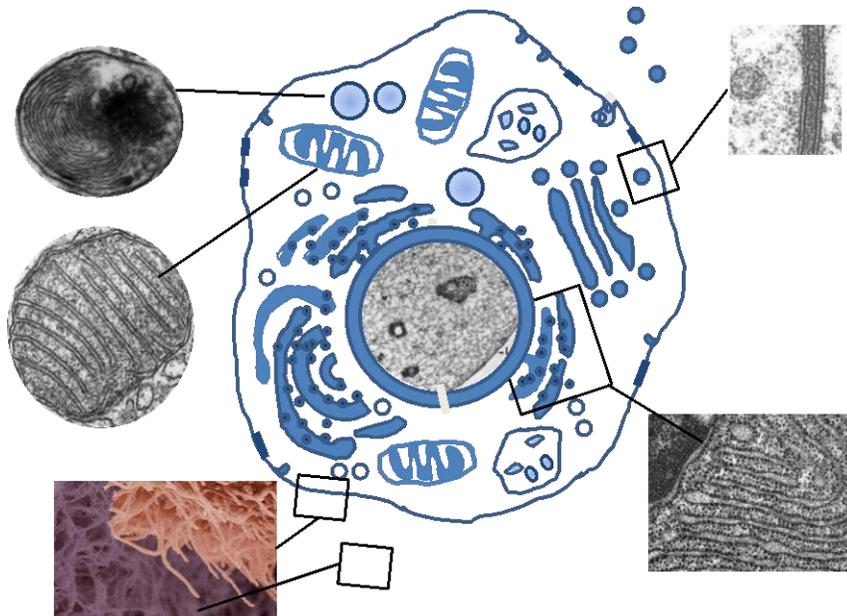


Figure. Cell map with main organelles and subcellular structures (figure from Jimenez, Editorial, special issue *Journal of Proteomics* 2009 'Proteomics of organelles and subcellular compartments')

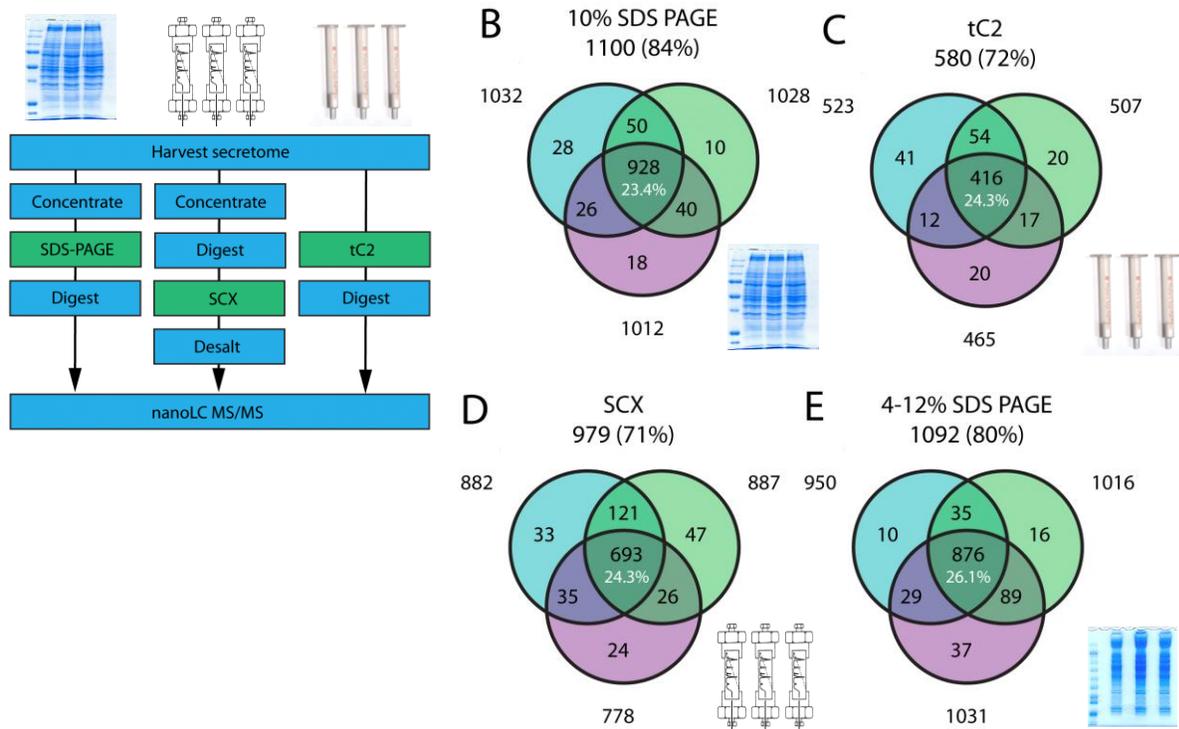
## Label-free quantitative secretome proteomics for discovery of serum-based cancer biomarkers: a proof-of-concept study

Sander R. Piersma<sup>1#</sup>, Ulrike Fiedler<sup>2#</sup>, Simone Span<sup>1</sup>, Andreas Lingnau<sup>2</sup>, Thang V. Pham<sup>1</sup>, Steffen Hoffmann<sup>2</sup>, Michael HG Kubbutat<sup>2</sup> and Connie R. Jiménez<sup>1\*</sup>

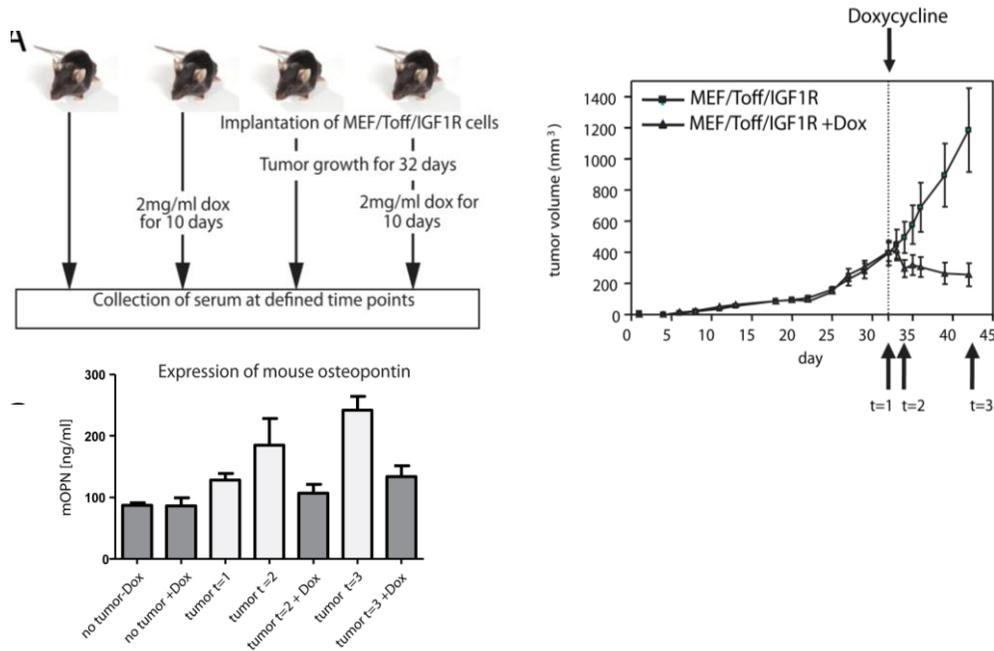
<sup>1</sup>Oncoproteomics Laboratory, Dept of Medical Oncology, VUmc-Cancer Center Amsterdam, VU University Medical Center, Amsterdam, the Netherlands. <sup>2</sup>ProQinase GmbH, Freiburg, Germany

**Background** Cell secretome (cell-conditioned medium) is composed of proteins that are found in the extracellular growth medium. The secretome consists of proteins that are secreted, shed from the cell surface and intracellular proteins released into the supernatant due to cell lysis, apoptosis and necrosis. These proteins may end up in the bloodstream, and thereby may have a potential use as non-invasive biomarkers. Moreover, the secretome has the added analytical advantage of being of medium-complexity compared to total cell lysate or plasma/serum. For these reasons, the cancer cell secretome has emerged as an attractive starting point for biomarker discovery

**Aim** A comprehensive evaluation of proteomics workflows for secretome analysis, including analysis of reproducibility, to choose the best performing workflow and application in model system.



**Results** We assessed the performance of three first-dimension separation strategies prior to nanoLC-MS/MS analysis of H460 cell line secretome: 1. 1D gel electrophoresis (1DGE), 2. peptide SCX chromatography and 3. tC2 protein reversed phase chromatography. 1DGE using 4-12% gradient gels outperformed the SCX and tC2 methods with respect to number of identified proteins (1092 vs. 979 and 580, respectively) and reproducibility of protein identification in three biological replicates (80% vs 70% and 72%, respectively). Reproducibility of protein quantitation based on spectral counting was similar for all 3 methods (CV: 26% vs 24% and 24%, respectively).



As a proof-of-concept of secretome proteomics for blood-based biomarker discovery, the gradient 1DGE workflow was subsequently applied to identify IGF1R-signalling related proteins in the secretome of mouse embryonic fibroblasts transformed with human IGF1R (MEF/Toff/IGF1R). VEGF and osteopontin were verified in secretomes by ELISA. Follow-up in serum of mouse bearing MEF/Toff/IGF1R tumors showed an increase of osteopontin levels, paralleling tumor growth and reduction, in the serum of mice in which IGF1R expression was shut-off and tumor regressed.

**Conclusions** Although chromatographic-based approaches have become popular fractionation methods, 1DE provides the best results in our head-to-head comparison. Furthermore, we showed that cell secretome can be used as a starting point for serum biomarker discovery.

#### Reference

Piersma SR, Fiedler U, Span S, Lingnau A, Pham T, Hoffmann S, Kubbutat MH, Jimenez CR. Workflow comparison for in-depth, quantitative secretome proteomics for cancer biomarker discovery: Method evaluation, differential analysis and verification in serum. *J Proteome Res.* 2010 Apr 5;9(4):1913-22.]

*This research was supported by the VUmc Cancer Center Amsterdam and EU kp6 Angiotargeting project*

## 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 heterogeneous 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<sup>1</sup>. Evidence is now emerging that tumor associated macrophages (TAM) predominantly act as alternatively activated macrophages, promoting tumor cell growth and suppressing immune responses<sup>2-4</sup>. 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<sup>5</sup>. 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.

### Reference

Marijn Bögels, Rens Braster, Philip Nijland, Nuray Gül, Wendy van de Luitgaarden, Remond J.A. Fijneman, Connie R. Jimenez, Robert H.J. Beelen, Marjolein van Egmond. Carcinoma origin dictates differential functional macrophage phenotype. *Oncoimmunology*, 2012 in press.

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

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

<sup>1</sup>Dept. Pathology, <sup>2</sup>Dept. Medical Oncology, <sup>3</sup>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.

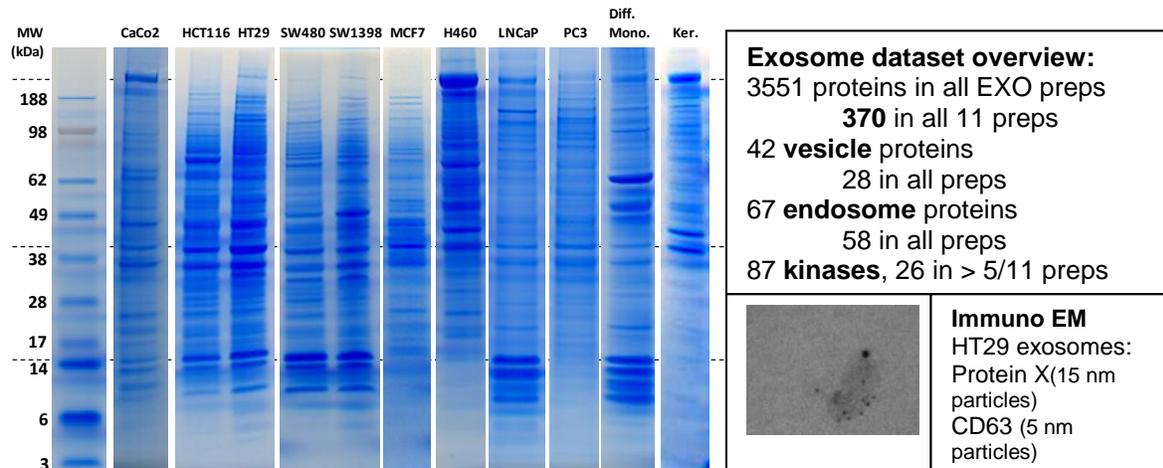


Figure Overlapping but different protein patterns in exosomes derived from different cancer cell lines and primary cells

**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 prediction and monitoring in biofluids, we will employ targeted detection methods (SRM mass spectrometry and antibodies).

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

## Novel identified proteins in cancer cell derived exosomes can change the behavior of healthy prostate cells

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**Background** Prostate cancer is the most common type of cancer in males and the leading cause of cancer-related death for European and North American men. Our recent findings indicate that cancer cells may change the protein expression of adjacent healthy prostate cells. The intercellular communication might run through exosomes, small secreted vesicles that contain various potential signaling molecules, such as proteins and (small)RNAs which are known to be able to regulate various physiological processes.

**Aim** In the present study we determined the role of various identified proteins in exosomes that were secreted by non-aggressive (LNCaP) and aggressive (PC3) prostate cancer cells on the behavior of normal prostate epithelial and stromal cells *in vitro*.

**Approach and methods** For this, healthy prostate epithelial and stromal cells (Lonza) and prostate cancer cell lines LNCaP and PC3 were used. Conditioned media (which includes exosomes) were collected after 72 h of LNCaP and PC3 cell culturing in serum free medium. The effect of this conditioned medium on epithelial and stromal cell proliferation was determined by the SRB assay, while migration and invasion were determined by the transwell assay. Protein profile in the secreted exosomes was determined by proteomics analysis. Proteomics data were evaluated using String and David websites. Proteins were selected based on expression level and differences between LNCaP and PC3 cells. Protein expression in exosomes was verified by Western blotting. Exosomal uptake into epithelial and stromal cells was determined by FACS analysis

**Results** 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. After analysis, 5 proteins enriched in PC3 exosomes were selected for further functional experiments. These proteins were selected based on differential expression and function in migration/invasion. Protein expression of these selected proteins was confirmed by Western blotting. Exosomes were taken up by the epithelial and stromal cells. This uptake was at a higher level for the epithelial cells than for the stromal cells, and also much higher for exosomes derived from PC3 than LNCaP cells. To evaluate whether inhibition of these proteins could decrease the effects on proliferation, migration and invasion, blocking antibodies were used. These blocking antibodies could decrease the effect found in the epithelial cells, indicating that these proteins play a function in proliferation, migration and invasion.

**Conclusions and outlook** In conclusion, proteins that are present in exosomes which are secreted by prostate cancer cells can change the behavior of healthy prostate epithelial cells and to some extent that of the stromal cells. These data provide new perspectives on the biological function of exosomes and on the role of the healthy prostate cells in order to find new biomarkers that may better predict time to progression or prostate cancer aggressiveness.

## Protein Composition of exosomes yields new insights into the endosomal-exosomal pathway of B cells during infection and malignant transformation

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\*Shared corresponding authors

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**Introduction:** Exosomes are nano-sized vesicles secreted by various cell types and have a function in intracellular communication. Exosomes are formed as intraluminal vesicles in multi-vesicular bodies (MVBs) that are part of the late endosomes. Exosomes from activated antigen presenting B cells have a distinct protein compositions and are derived from multiple MVB compartments. Most notably it was shown previously that B cell-secreted exosomes contain antigen presenting MHC class II molecules and co-stimulatory molecules such as CD86 and as a consequence have potent T-cell stimulatory properties. Interestingly, we have shown recently that EBV-driven activated B cells secrete exosomes that hold functional (small)RNA molecules that can be delivered to recipient cells. These findings raise many questions on their origin and biogenesis, host-pathogen interaction and provide novel opportunities for diagnostics as exosomes can be isolated from most human body fluids.

**AIM:** Define the protein composition of exosomes secreted by healthy proliferating B cells, EBV-infected blasts and tumor cells. These studies are expected to give important new insights into the role of the endosomal-exosomal pathway in (oncogenic) cell-signaling regulation and intracellular communication via exosomes.

**Methods:** We used differential ultra-centrifugation and sucrose gradients to obtain highly purified fractions of B cell secreted exosomes that were analyzed by Mass Spectrometry (MS)-based proteomics.

**Results:** We have performed Gel- nanoLC-LTQ-FTMS with exosome fractions that were isolated from B cells in culture, including exosomes from diffuse large B cell lymphoma, EBV-driven B cells (LCLs) and CD40L-stimulated proliferating B cells. We detected an unprecedented 2129 exosomal proteins including highly abundant B cell exosomal markers such as MHC class I and II molecules, CD19, HSP90, HSP70, CD81, CD82, CD9, flotillin-1 and alix an ESCRT protein. Proteins such as Cytochrome C were not detected and the presence or absence of these proteins were confirmed by western blotting. Extensive analysis has yielded multiple new insights into the endosomal-exosomal pathway that is used by B cells for communication and how malignant transformation or viral infection effects this pathway.

Of notable interest was the observation that many different proteins in exosomes are secreted by several (7) activated B cell types we analyzed. As expected a large proportion of the most abundant molecules are involved in immune responses. Unsupervised hierarchical clustering of the complete profiles indicated however a clear separation between exosomes from malignant and healthy B cells. Although virus infection increases B cell proliferation, the exosomes released more closely resemble exosomes secreted from healthy B cells stimulated by antigen.

In addition, we determined by gene ontology that large proportion of the exosomal proteins are related to protein translation/modification. This suggest that exosomes from activated B cells are not only derived from intracellular compartments (MVBs) involved in antigen-presentation but also other processes such as post-transcriptional gene regulation. These profiles were particularly abundant in the exosome populations derived from the malignant DLBCL cells, strongly suggesting that these pathways are disturbed in the cancerous cells.

**Conclusions** These findings thus suggest that proteomics on circulating exosomes could be a useful tool to discover circulating biomarkers for cancer diagnostics. We found multiple viral proteins in exosomes that are related to intracellular signaling and are currently investigating the biological relevance of their secretion in the context of immune evasion. Further research is directed to the function and mechanisms of these non-immune related exosomes secreted by B cells and how secretion of this material effects gene regulation in the cell that produces them and their effect on surrounding cells upon transfer.

## Cell surface proteomics of osteosarcoma identifies EPHA2 as receptor for targeted drug delivery

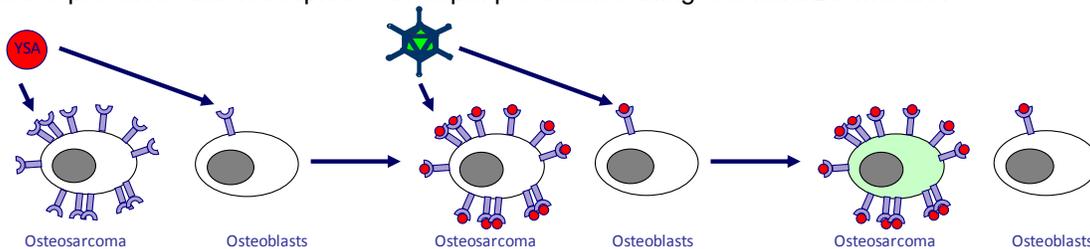
J. PosthumaDeBoer<sup>1</sup>, S.R. Piersma<sup>2</sup>, T.V. Pham<sup>2</sup>, J.C. Knol<sup>2</sup>, V.W. van Beusechem<sup>3</sup>, G.J.L. Kaspers<sup>4</sup>, B.J. van Royen<sup>1,5</sup>, C.R. Jiménez<sup>2,§</sup>, M.N. Helder<sup>1,5,§§</sup> *Shared corresponding authors*

1) Department of Orthopaedic Surgery, 2) Oncoproteomics Laboratory, 3) Department of Medical Oncology, 4) Paediatric Oncology/Haematology, VU University Medical Center, Amsterdam

**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.



**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.

**Conclusions** Cell surface biotinylation combined with nanoLC-MS/MS successfully 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

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<sup>1</sup>Angiogenesis Laboratory and <sup>2</sup>OncoProteomics Laboratory, Dept. Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands

**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 releasate 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 releasate proteins. 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.

We recently extended these observations to a pilot analysis of platelet releasate and platelet lysate of control subjects and cancer patients. 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.

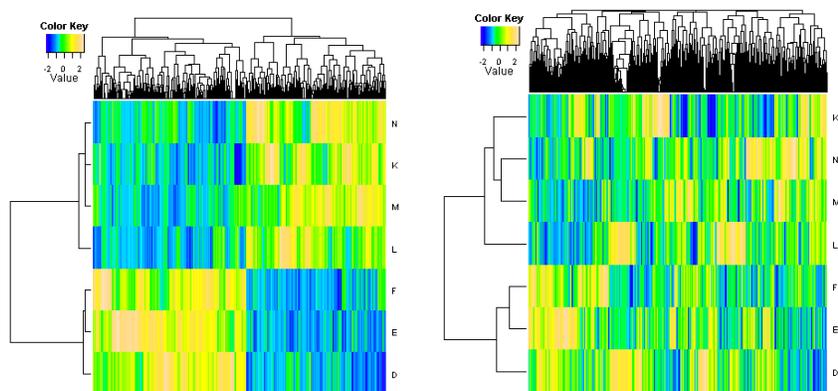


Figure. Heat maps of the platelet proteome in cancer patients and control subjects. Supervised (left) and unsupervised (right) cluster analysis using significantly regulated ( $p < 0.05$ ) and all platelet proteins, respectively. In each case two clusters are obtained with a perfect separation of controls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

**Outlook** 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.*

## Signaling pathway proteomics for insight into cancer signaling, identification of drug targets and biomarkers for patient stratification

Kinases are key enzymes involved in deregulated signal transduction associated with cancer development and progression. To enable personalized medicine, new biomarkers and diagnostic tools for patient stratification and therapy selection are needed. Since deregulation of kinase-mediated signal transduction is implied in tumorigenesis, the analysis of all kinases (the kinome) active in a particular tumor may yield tumor-specific information on aberrant cell signalling pathways and candidate markers for patient selection for targeted therapy. This line of research is in close collaboration with members from the VUmc Dept. Medical Oncology, i.e., Dr. Henk Broxterman and Prof. Henk Verheul.

**Global aim** Mass spectrometry-based kinome profiling and phosphoproteomics to enable insight into aberrant cancer signaling pathways, and a rationale for (combination) therapy selection.

**Reference** Piersma SR, Labots M, Verheul HM and Jimenez CR. Strategies for kinome profiling in cancer and potential clinical applications: chemical proteomics and array-based methods. *Anal. Bioanal. Chem.* **397**: 3163-3171 (2010).

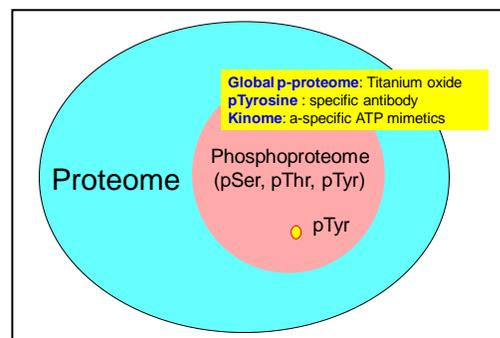
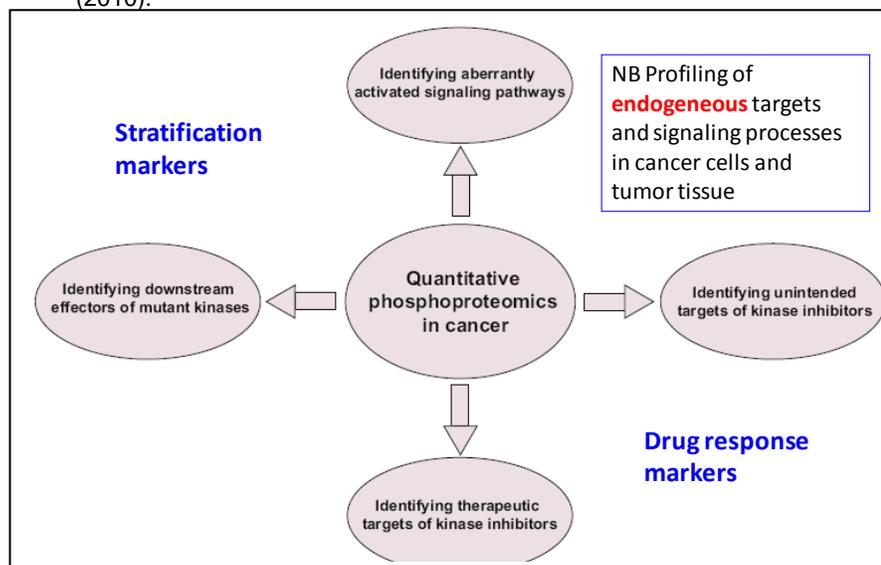


Figure. Applications of phosphoproteomics (left) and enrichment methods (figures above and below) to enable comprehensive analysis of the kinome and phosphoproteome (for further details and results see abstracts in the following pages)

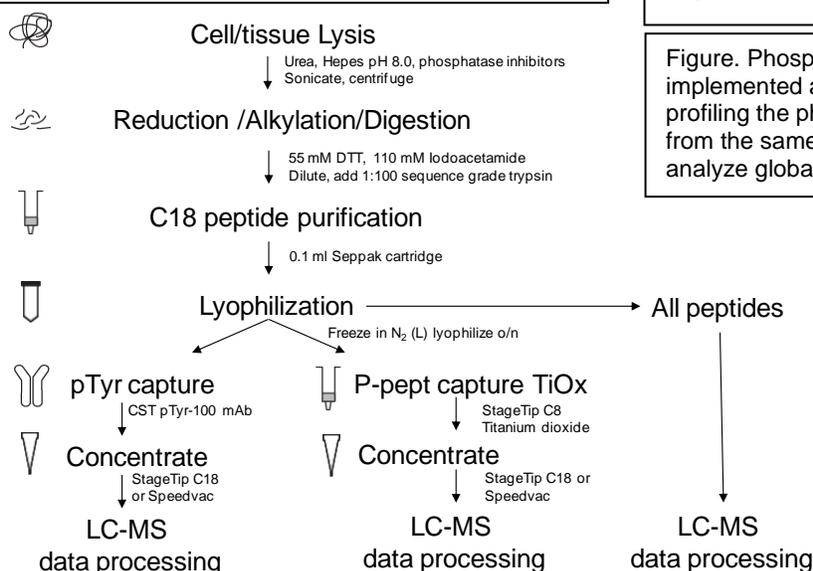


Figure. Phosphoproteomics workflow implemented at the OPL. In parallel to profiling the phosphoproteome, an aliquot from the same sample can be used to analyze global protein expression as well.

### Phosphotyrosine immunoprecipitation for phosphotyrosine-based phosphoproteomics

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<sup>1</sup>OncoProteomics Laboratory, <sup>2</sup>Angiogenesis Laboratory, Department of Medical Oncology, VU University Medical Center, Amsterdam, the Netherlands

**Background** Phosphoproteomics allow for profiling of aberrant signaling pathways in cancer cells and tissues. As such, comparative phosphoproteomics can be used to identify candidate drivers of cancer progression. Analysis of the phosphoproteome remains a challenge because of the low intensity of phosphorylated peptides detected by LC-MS/MS as compared to 'normal' peptides and the lability of phosphopeptides and the substoichiometric phosphosite occupancy. Strategies are needed for enrichment of phosphopeptides in order to analyse the phosphoproteome by LC-MS/MS. Protein phosphorylation occurs 85-90% on Serine, 10-15% on Threonine and only 1-3% on Tyrosine. Therefore, specific enrichment strategies for phosphotyrosine have been developed.

**Aim** To establish a robust workflow to enrich and detect phosphotyrosine(pTyr)-containing peptides from cancer cell lines, tumor tissue and from low level input from clinical biopsies.

**Approach** Guo et al in PNAS 2007 and Rikova in Cell 2007 have shown that pTyr enrichment using the mouse pTyr-100 antibody from Cell Signaling Technologies followed by LC-MS/MS allows identification and quantification of hundreds of pTyr peptides. However, input levels of the enrichment protocol are 20 mg protein, which is very high compared to clinically available amounts. We have assessed performance of the enrichment protocol at the level of 10 mg and 1 mg protein obtained from cancer cell lysates. Additionally, to improve the performance at low level, the enrichment was performed +/- the mass spectrometry-compatible detergent N-octyl glucoside.

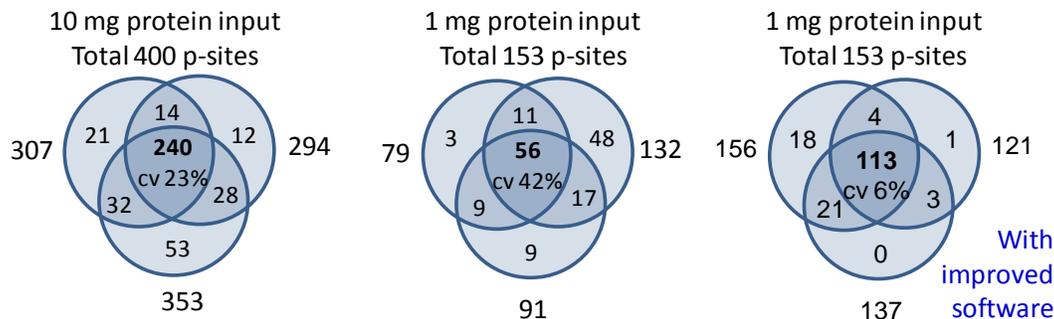


Figure. Numbers of identified pTyr containing phosphopeptides in replicate analyses using 10 mg and 1 mg of protein input from cancer cell line samples.

**Results** Using 10 mg of protein input of U87 lysate 400 phosphosites were detected with 248 sites detected in 3/3 replicates. Of the phosphopeptides >85% have a tyrosine as the site of phosphorylation. At 1 mg input 1% N-octyl glucoside improved reproducibility of detection presumably by decreasing a-specific binding of non-phosphorylated peptides. In this experiment, 56 phosphopeptides were detected in 3/3 replicates with a total of 153 sites detected. The overlap improved with improvements in the software (propagation of peptide identities across runs, see also page 81). Key in pTyr phosphopeptide enrichment at low level, is careful manipulation of low volume (4-5  $\mu$ l) pTyr-100 agarose beads and release of pTyr peptides from the beads using 0.15% TFA. Detected tyrosine-phosphorylated protein kinases include EGFR, SRC, FAK and ERK and many others (~ 80 different protein kinases).

**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.

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

## Global profiling of endogenous phosphorylation events by titanium dioxide based phosphoproteomics for signaling pathway and drug perturbation studies in cancer

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<sup>1</sup>OncoProteomics Laboratory, Dept. Medical Oncology, VU University medical center, Amsterdam, The Netherlands.

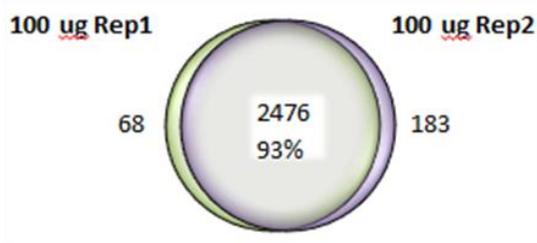
**Background:** Protein phosphorylation, one of the key post translational modifications, is crucial for the propagation of chemical, mechanical<sup>1</sup> and electrical<sup>11</sup> signals that are implicated in a variety of vital biological functions as well as in many diseases. Kinases propel phosphorylation events on serine, threonine and tyrosine amino acid residues of a protein in an ATP-driven fashion and many kinases are self-phosphorylated as a means of controlling their activity. Global profiling of phosphorylation events by mass spectrometry based proteomics is a powerful technique to understand these signaling mechanisms and to find novel drug targets for diseases. However, phosphoproteins are less abundant and hence tryptic peptides should be specifically enriched for phospho peptides from a greater pool of non-phospho peptides before sequencing. One of the common methods to distinctly enrich phospho peptides is titanium dioxide (TiO<sub>2</sub>) chromatography. TiO<sub>2</sub> has high affinity for phosphopeptides and its selectivity can be greatly improved by adding enhancers during the enrichment process.

**Aims:** (1): Establish and optimize a reproducible workflow for unbiased and highly distinct phosphopeptide enrichment; (2): assess scalability and (3): comparison and/or combination of this workflow with magnetic TiO<sub>2</sub> and polyMAC methods. Here we focus on aim 1.

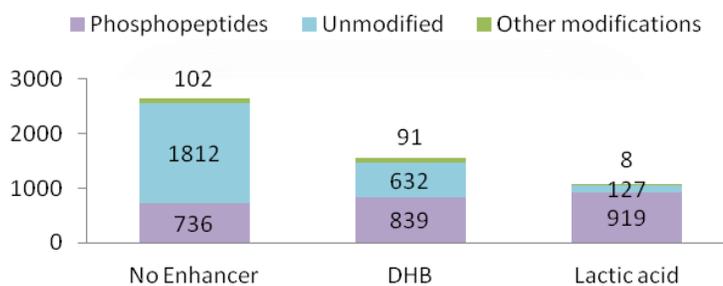
**Approaches:** First, different combinations of parameters like protein amount, TiO<sub>2</sub> bed height and enhancers were tested and optimized to obtain greater number of phosphopeptides with high selectivity. Samples were measured in duplicates to assess the reproducibility of the workflow. Second, scalability of this workflow will be tested on various levels. Third, this workflow will be compared and/or combined with magnetic TiO<sub>2</sub> and polyMAC methods to improve coverage.

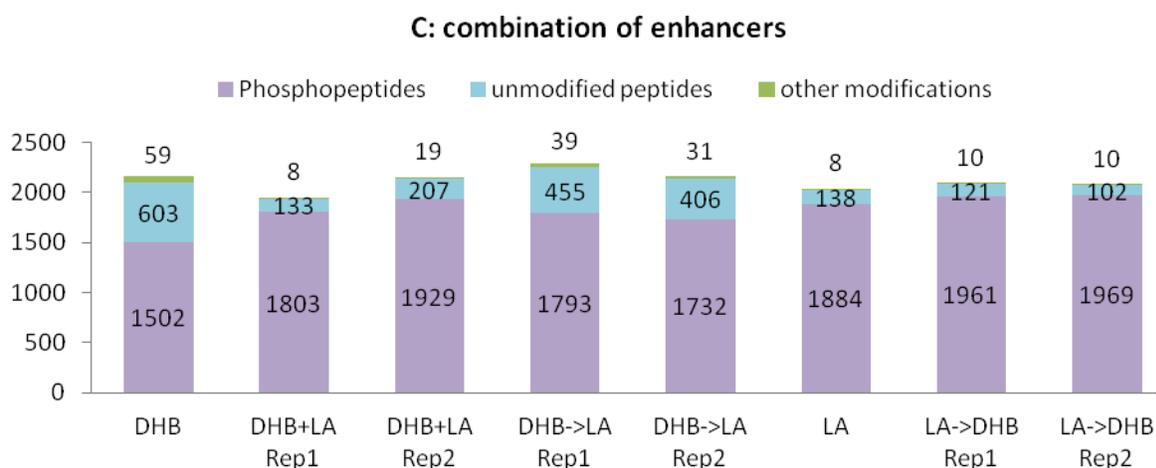
**Results:** Affinity purification using titanium oxide without additives allowed for profiling up to 1000 phosphopeptides from relatively low protein input levels (100-200 µg). Usage of enhancers like di-hydroxy benzoic acid (DHB) and lactic acid (LA) during phosphopeptide capture step greatly improved the selectivity of phosphopeptides captured. Without enhancers, many non-phosphopeptides were captured and selectivity was only 28%. But addition of enhancers like DHB and Lactic acid significantly reduced the amount of non-phosphopeptides and improved selectivity to 54% and 87% respectively (see figure panel B). Next, parameters like protein amount, TiO<sub>2</sub> bed-height and database search pipeline were optimized which further improved the coverage and selectivity of the phosphoproteome. In the recent measurements, ~2600 unique phosphopeptides representing ~1400 genes with ~94% enrichment for phosphopeptides and ~93% overlap among replicates were obtained including many protein kinases. Further optimization, scaling and comparison and/or combination of this workflow with other methods are in progress.

### A: reproducibility of analysis



### B: selectivity of enhancers





**Figure 1: (A):** Reproducibility of experiment at 100ug level. **(B):** Selectivity of Enhancers. **(C):** Combination of enhancers

**Conclusions and outlook:** The workflow for titanium dioxide affinity-based enrichment methods was successfully established and at present we could sequence over 2000 phosphopeptides in a single nano-LC MS run from only 100  $\mu$ g of protein input. Further optimization of the workflow, for example usage of different elution buffer to release tightly bound phosphopeptides, could improve the phosphoproteome coverage. This workflow is expected to give novel insights into the signaling pathways and drug perturbation studies in cancer.

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

## Deciphering global kinase signaling patterns in a panel of colorectal cancer cell lines in relation to Aurora kinase A by phosphoproteomics

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<sup>1</sup>Oncoproteomics Laboratory, Dept. Medical Oncology, <sup>2</sup>Angiogenesis laboratory, Dept. Medical Oncology; <sup>3</sup>Tumor Profiling Unit, Dept. Pathology, VU University Medical Center, Amsterdam, The Netherlands.

**Background:** Two mechanisms for genomic instability in colorectal cancer (CRC), chromosomal instability (CIN) and microsatellite instability (MSI), account for 85% and 15% of CRC, respectively. It is hypothesized that CIN and MSI colon tumors have differential kinase signaling patterns. Previous work identified a number of amplified genes that map to chromosome 20q, a region frequently amplified in a subset of CIN CRC and that is associated with poor prognosis. One gene that drives 20q gain is Aurora kinase A (AURKA), a key mitotic serine/threonine kinase regulated by auto phosphorylation at Thr<sup>288</sup> residue in the activation loop. It has a variety of functions including formation of mitotic spindles, separation of centrosomes, and organization and alignment of chromosomes. AURKA could serve as a potential drug target in CRC therapy and understanding the AURKA signaling network is an important step towards this goal.

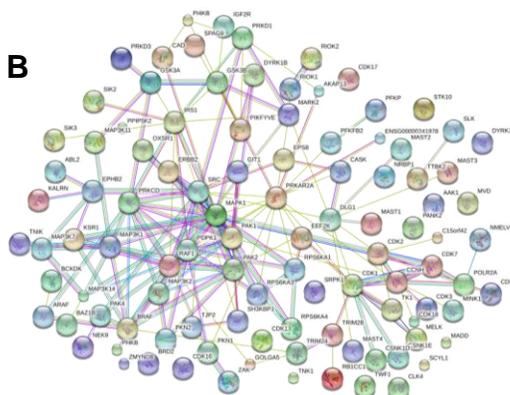
**Aims:** (1): To identify aberrant hyperactive signaling pathways in a panel of CRC cell lines in relation to AURKA protein expression levels (2): To assess the effect of an AURKA inhibitor in the cells with the highest and lowest AURKA expression to directly uncover the kinase network associated with AURKA signaling.

**Approaches:** (1): Global TiO<sub>2</sub> based-phosphoproteomics of a panel of eight CRC cell lines (5 CIN and 3 MSI). (2): Analysis of the phosphoproteome following perturbation with the AURKA inhibitor MLN8237. To this end, cells were synchronized in G2-M phase of the cell cycle with nocodazole. A phosphoproteomic screen of these samples would reveal both the kinase network and substrates of AURKA.

**Results:** Western blot analysis showed differential AURKA protein expression among eight CRC cell lines of which SW480 and CaCo2 had highest AURKA expression. Based on this data, 2 cell lines with highest and 2 cell lines with relatively lower AURKA expression levels shall be used to perturb the kinase network in G2-M phase and G1-S phase of the cell cycle. A preliminary screen was performed to this end and phospho-AURKA was detectable by western blot after nocodazole treatment for 17 hours of Caco2 cells at G2-M phase of the cell cycle.



Fig. 1A. Western blot of AURKA protein levels in a panel of CRC cell lines. Black: CIN; Red: MSI. 1B. protein-protein interaction network of the identified 111 Kinases (out of 1444 phosphoproteins) identified in CaCo2 including 89 protein



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**Conclusions and outlook:** Phosphoproteomics is a powerful approach to study kinase and phosphorylation signaling events. The Caco2 phosphoproteome contained over 100 kinases including 89 protein kinases by the improved titanium dioxide-based phosphoproteomics workflow. This workflow will be used to decipher global kinase signaling events in CIN and MSI CRC cell lines and to uncover the kinase network of AURKA following perturbation.

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

## Phosphoproteomics of glioblastoma cells: $\Delta$ EGFR targets and erlotinib effects

J.C. van der Mijn<sup>1\*</sup>, R. Piersma<sup>2\*</sup>, Thang V. Pham<sup>3</sup>, Tom Wurdinger<sup>3</sup>, Henk Verheul<sup>1\*</sup>, and Connie R. Jimenez<sup>2\*</sup>

<sup>1</sup>Angiogenesis Laboratory, <sup>2</sup>OncoProteomics Laboratory, Department of Medical Oncology, <sup>3</sup>Dept. Neurosurgery, VU University Medical Center, Amsterdam, the Netherlands. \*Equal contribution.

**Background** An in-frame deletion mutation in Epidermal Growth Receptor (EGFR),  $\Delta$ EGFR is a common and potent oncogene in glioblastoma (GBM), promoting growth and survival of cancer cells. This mutated receptor is ligand independent and constitutively active. Downstream targets of  $\Delta$ EGFR play a significant role in malignancy.

**Aim and approach** Identification of tyrosine phosphorylation events in U87 and U87dEGFR GBM cells and assess the effect of erlotinib treatment of mutant GBM cells. To this end we employed antibody-based phosphotyrosine peptide capture followed by nanoLC-MS/MS analysis.

### Results

Phosphoproteomics of U87 and U87dEGFR GBM cells revealed 362 unique phosphoproteins with 583 unique phosphosites, the largest dataset reported to date. In addition, the dataset contained 49 phosphorylated protein kinases and 36 kinases with phosphorylation in the activation loop. A subset of 82 phosphosites was > 2 fold upregulated in the  $\Delta$ EGFR mutant GBM cells in 2 independent experiments, of which 23 phosphoproteins were down-regulated following erlotinib treatment (see figure).

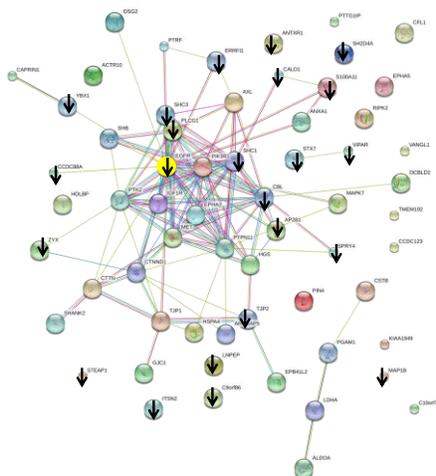


Figure Network of phosphoproteins reproducibly >2-fold upregulated in  $\Delta$ EGFR mutant GBM cells. Arrows indicate phosphoproteins that are down-regulated after erlotinib treatment. Yellow circle denotes EGFR.

**Conclusions and outlook** The phosphoproteomics results demonstrate that multiple hyperactivated protein kinases are not inhibited by erlotinib and as such may provide GBM cells an escape mechanism. Some of these have been implicated in therapy resistance in other tumor types. The data warrant follow-up and when validated will provide a rationale for combination therapy in GBM of erlotinib with eg., sunitinib.

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

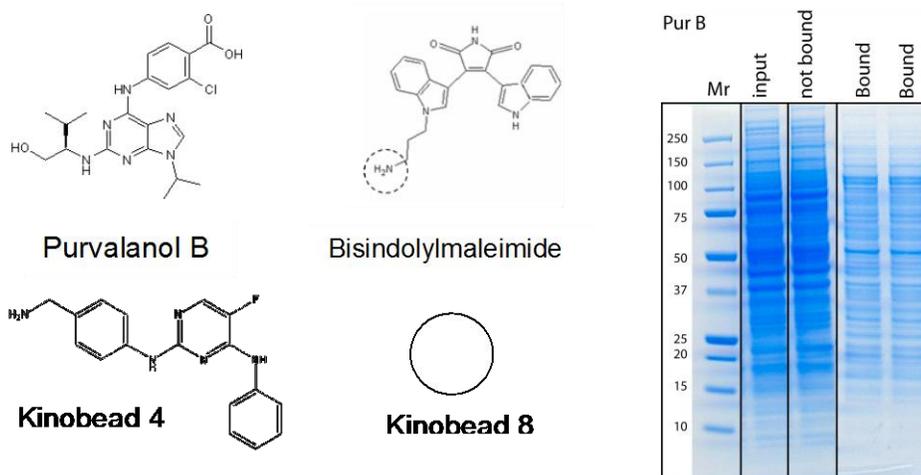
## Development of kinobeads for comprehensive kinome proteomics

Sander R. Piersma<sup>1</sup>, Inge de reus<sup>1</sup>, Jimmy Weterings<sup>2</sup>, Iwan de Esch<sup>2</sup>, Henk MW. Verheul<sup>1</sup> and Connie R. Jiménez<sup>1</sup>

<sup>1</sup>OncoProteomics Laboratory, Dept. Medical Oncology, VU University Medical Center; <sup>2</sup>FEW-VU, Amsterdam

**BACKGROUND** The human kinome represents a family of 518 protein kinases. Protein kinases, enzymes that catalyze protein phosphorylation, are key regulators of signal transduction and are often implicated in the development of cancer. Moreover, many small-molecule inhibitors have been developed against protein kinases, several of which are currently used in the clinic such as sunitinib (Sutent) and sorafenib. However, because of the low cellular abundance, kinases are hard to detect comprehensively by LC-MS/MS in cell/tissue lysate, therefore, enrichment strategies for kinases are needed.

**AIM** Development of specific protein kinase capturing methods, for enrichment of kinases from complex protein mixtures, prior to identification with mass spectrometry.



**APPROACH** We use immobilized broad selectivity kinase inhibitors coupled to beads (kinobeads), directed to the ATP-binding site of protein kinases, to capture kinases from cell lysate or tissue lysate. Captured proteins are identified using a GeLC-MS/MS workflow. We coupled Purvalanol B and Bisindolyl maleimide (commercially available ATP-competitive inhibitors) to sepharose resin via a primary amine or hydroxyl group. In addition to these two ligands two additional ATP-competitive inhibitors were synthesized and coupled to the resin: kinobead 4 (Cellzome patent) and kinobead 8, a novel broad-specificity ligand (structure to be disclosed elsewhere).

**RESULTS** Four different kinobeads have been synthesized. Mass spectrometry analysis of captured proteins by immobilized purvalanol B beads resulted in the (reproducible) identification of 80 protein kinases, compared to 20 protein kinases in cell lysate (HCT 116 colon cancer cell line). With the addition of 3 new ligands a more comprehensive protein kinase capture was obtained.

**OUTLOOK** Kinobeads allow for comprehensive profiling and analysis of protein kinases by LC-MS/MS. Using these tools, cell lines can be interrogated with kinase inhibitors for drug-target and off-target discovery. In addition to target discovery, kinase profiles of patient tissue may be used for patient stratification and may personalize targeted therapeutic intervention.

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

## Colorectal cancer proteomics

**General aim:** To identify novel protein biomarkers that can be used for development of a stool-based or blood-based tests for early diagnosis of CRC and for patient stratification.

**Embedding:** National consortium Decrease Colorectal Cancer Deaths (DeCoDe). Within the framework of the Center for Translational Molecular Medicine (CTMM) a consortium of CRC researchers, spanning the field from early detection (aiming at population based screening) to molecular testing for personalised therapies. Topics covered are molecular stool and blood testing, MR based molecular imaging, image guided surgery and PET scanning using new molecular markers.

Collaborators involved in the colorectal proteomics effort with an emphasis on screening/ early detection are: Dr. Remond Fijneman and Prof. Gerrit Meijer and other members of the Tumor Profiling Unit of the VUmc Dept. Pathology. In the context of colorectal cancer stem cells and metastasis, we collaborate with Dr. Onno Kranenburg of the UMCU Dept. of Surgical Oncology. In the context of targeted therapy we collaborate within the VUmc Dept. of Medical Oncology with Prof. Henk Verheul. Therefore, we cover the whole spectrum of clinical needs in colorectal cancer management and we aim for a systems biology insight into colorectal cancer development and progression (see figures below).

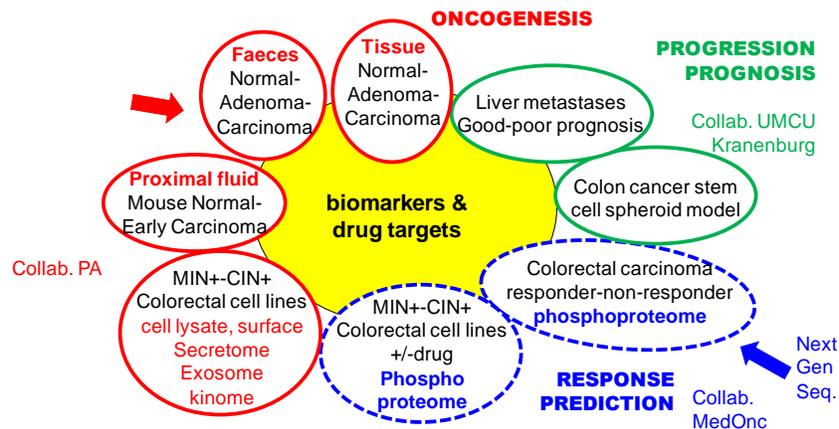


Figure. Colorectal proteomics. Data integration facilitated by the CTMM TraIT project and network-based analyses will enable better a molecular understanding of colorectal cancer oncogenesis and progression as well as biomarker selection and development. Dotted lines indicate projects in starting-up phase.

### References to reviews

- Jimenez CR, Knol JC, Meijer GA and Fijneman RJ. Proteomics of colorectal cancer: overview of discovery studies and identification of commonly identified cancer-associated proteins and candidate CRC serum markers. *J. Proteomics*. 73: 1873-1895 (2010) Review.
- Bosch LJW., Carvalho B., Fijneman RJA., Jimenez CR., Pinedo HM., van Engeland M., and Meijer GA. Molecular tests for colorectal cancer screening. *Clin Colorectal Cancer*. 2011 Mar 1;10(1):8-23. Review.

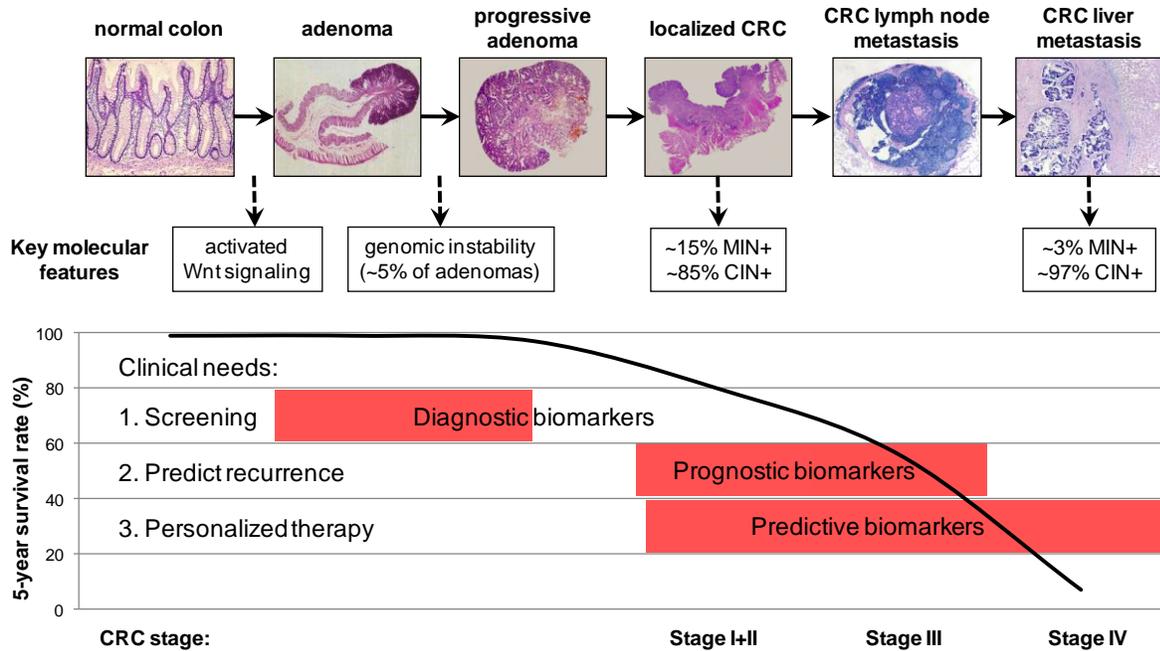


Figure. Clinical needs for biomarker discovery in relation to colon carcinogenesis. The top panel shows hematoxylin and eosin stained examples of different stages of colon tumor development. Some of the key molecular features with clinical implications are given underneath, as well as a graph that illustrates the steep decline in 5-year survival rate when CRC is diagnosed in its later stages. The clinical need for biomarkers is depicted within the graph, with shaded colors indicating the stages from which colon tumor samples should be selected for biomarker discovery. Colorectal cancer develops from normal colon through a benign precursor lesion, the colon adenoma. Adenomas have undergone several molecular alterations compared to normal colon tissue, of which activation of the Wnt signaling pathway is most prevalent. The far majority of approximately 95% of adenomas will not progress into carcinomas. Therefore, from a clinical point of view, diagnostic biomarkers that discriminate non-progressive adenomas from progressive adenomas and CRC are of more interest for cancer screening than biomarkers that discriminate benign adenomas from normal colon. Genomic instability is a main characteristic of CRC. MIN+ tumors have a better prognosis than CIN+ tumors due to their lower frequency of metastases. Prognostic biomarkers are needed that predict disease recurrence, to determine who would benefit from adjuvant chemotherapy. The 5-year survival rate for stage IV CRC is less than 10%. Novel targeted therapies are being developed, which require predictive biomarkers that indicate who will benefit from what therapy. (from review Jimenez et al., J. Proteomics 2010)

## Proximal fluid proteome profiling of mouse colon tumors reveals biomarkers for early diagnosis of human colorectal cancer

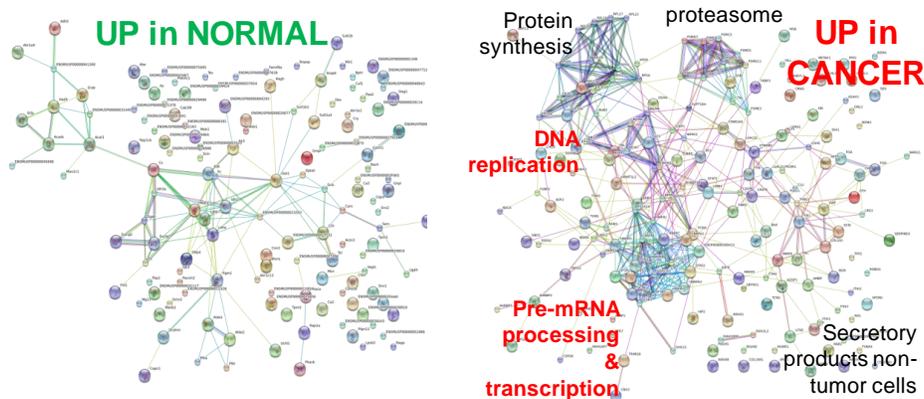
Remond J.A. Fijneman<sup>1</sup>, Meike de Wit<sup>2</sup>, Maral Pourghasian<sup>1,2</sup>, Sander Piersma<sup>2</sup>, Marc O Warmoes<sup>2</sup>, Mehrdad Lavaei<sup>2</sup>, Pien M. van Diemen<sup>1</sup>, Chloe Piso<sup>1</sup>, Els C. Robanus-Maandag<sup>3</sup>, Ron Smits<sup>4</sup>, Riccardo Fodde<sup>4</sup>, Victor W.M. van Hinsbergh<sup>2,5</sup>, Gerrit A. Meijer<sup>1</sup> and Connie R. Jimenez<sup>2</sup>

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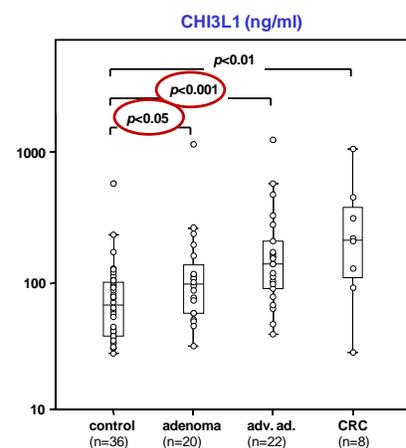
**Background:** Colorectal cancer (CRC) is the second leading cause of cancer death in the Western world. Detection of CRC at an early stage of disease is associated with a much better prognosis for the patient, and is a realistic approach to reduce CRC mortality rates. Several randomised trials have shown that FOBT screening, ie detection of blood-derived haem in feces, reduces CRC mortality by ~16%. Nevertheless, the FOBT test performance is relatively poor, and it is commonly recognized that sensitivity and specificity of non-invasive CRC screening tests need to be improved, for which novel biomarkers are urgently needed.

**Aim:** The aim of this study is to identify identify novel protein biomarkers for the early diagnosis of CRC.

**Approach:** Proximal fluids are a rich source of candidate biomarkers as they contain high concentrations of tissue-derived proteins. The Fabp1Cre;Apc(15lox/+) mouse model represents early-stage development of human sporadic CRC. Proximal fluids were collected from normal colon and colon tumors and subjected to in-depth proteome profiling by tandem mass spectrometry. Carcinoembryonic antigen (CEA) and CHI3L1 human serum protein levels were determined by ELISA.



**Results:** Of the 2,172 proteins identified, quantitative comparison revealed 192 proteins that were significantly ( $P < 0.05$ ) and abundantly (>5-fold) more excreted by tumors than by controls. Further selection for biomarkers with highest specificity and sensitivity yielded 52 candidates, including S100A9, MCM4, and four other proteins that have been proposed as candidate biomarkers for human CRC screening or surveillance, supporting the validity of our approach. For CHI3L1, we verified that protein levels were significantly increased in sera from patients with adenomas and advanced adenomas compared with control individuals, in contrast to the CRC biomarker CEA.

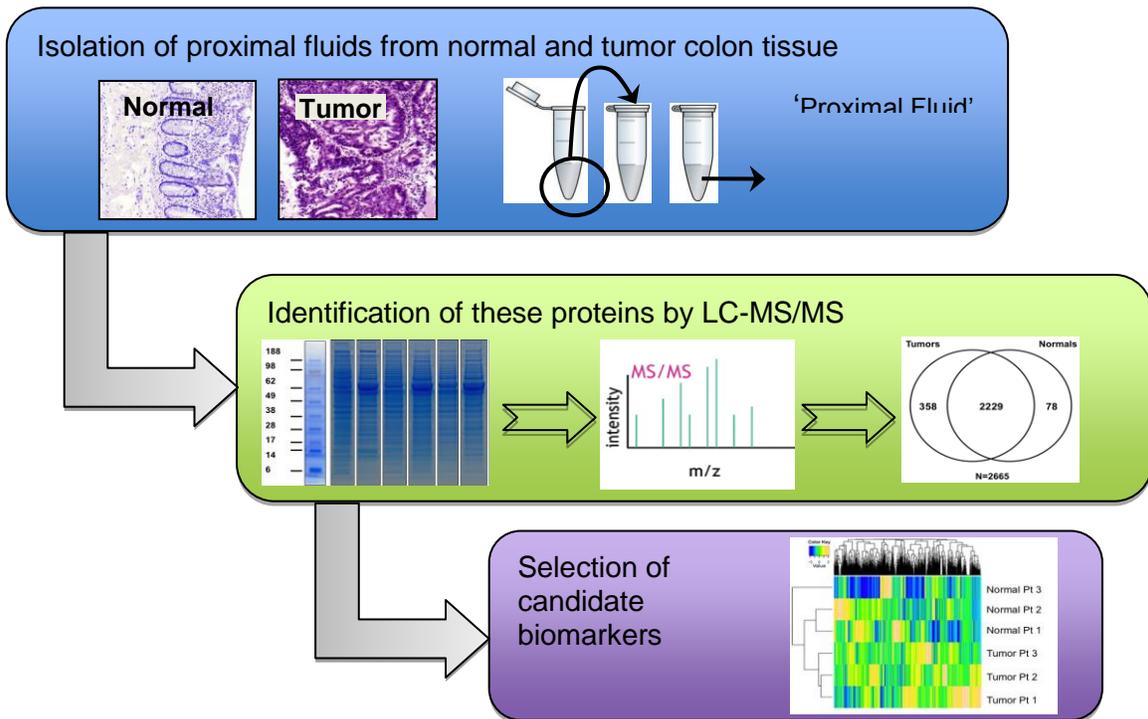


**Outlook** These data show that proximal fluid proteome profiling with a mouse tumor model is a powerful approach to identify candidate biomarkers for early diagnosis of human cancer, exemplified by increased CHI3L1 protein levels in sera from patients with CRC precursor lesions.

**Reference**

Fijneman RJ, de Wit M, Pourghasian 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 and Jimenez CR. Proximal fluid proteome profiling of mouse colon tumors reveals biomarkers for early diagnosis of human colorectal cancer. Clin Cancer Res 2012; 18(9); 2613-24.

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



## Proteome Profiling of Proximal Fluids from Human Colon Cancer Tissue Reveals Novel Candidate Biomarkers for Early Diagnosis

Meike de Wit<sup>1,2</sup>, Huub Kant<sup>2</sup>, Sander Piersma<sup>2</sup>, Thang Pham<sup>2</sup>, Sandra Mongera<sup>1</sup>, Maaike PA van Berkel<sup>2</sup>, Epi Boven<sup>2</sup>, Gerrit A Meijer<sup>1</sup>, Remond JA Fijneman<sup>1\*\*\*</sup> and Connie R Jimenez<sup>2\*\*\*\*</sup> *Shared corresponding authors*

<sup>1</sup>Department of Pathology (Tumor Profiling Unit), <sup>2</sup>Department of Medical Oncology, VU University Medical Center, Amsterdam, the Netherlands. \*Shared last author.

**Background:** Colorectal cancer (CRC) is the second leading cause of cancer death in the Western world. Detection of CRC at an early stage of disease is a realistic approach to reduce CRC mortality rates. Currently available non-invasive CRC screening tests leave room for improvement, for which novel biomarkers are urgently needed. The aim of this study is to identify novel protein biomarkers that can be used for development of a blood-based or stool-based screening test for early diagnosis of CRC.

**Approach:** Tumor “proximal fluids” are a rich source of tumor-derived proteins, comprising proteins that are either secreted, shed by membrane vesicles (exosomes), or externalized due to cell death. Fresh human colon carcinoma tissue and matched normal colon tissue samples were obtained from three patients, tissues were briefly rinsed and incubated in PBS at 37°C for one hour. These proximal fluids of normal and colon carcinoma tissues 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  $p < 0.05$ .

**Results:** A total of 2665 proteins were identified from proximal fluids of three human colon carcinoma and patient-matched normal colon control tissues. Of these, 367 proteins were significantly more secreted by colon carcinoma samples than by controls. The biological processes associated with proteins more present in the tumor proximal fluids included translation, RNA splicing, DNA replication and repair, gene expression and extracellular structure. The most promising candidate biomarkers were selected based on several stringent criteria, e.g., 5-fold more present in tumor proximal fluids than normal proximal fluids and present in a CRC cell line proteomic dataset, leading to 68 candidate biomarkers. These include several proteins that have been proposed in literature as markers for (stool-based) CRC screening.

**Conclusion:** We conclude that proximal fluid proteome profiling of human colon carcinoma tissue is a powerful strategy to discover novel candidate biomarkers for CRC screening. We identified 68 candidate biomarkers that could potentially be used for development of a blood-based or stool-based screening test for early diagnosis of CRC. Further validation studies are required to investigate whether these candidates are robust biomarkers for CRC screening.

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

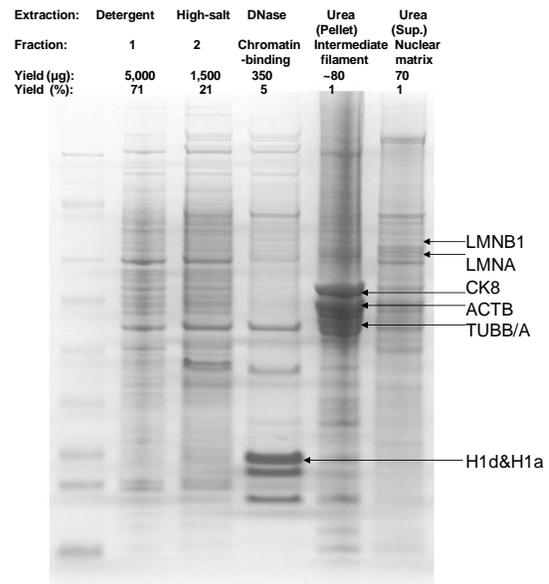
## Sub-nuclear proteomics in colorectal cancer: Identification of proteins enriched in the nuclear matrix fraction and regulation in adenoma to carcinoma progression

Jakob Albrethsen<sup>1</sup>, Jaco C Knol<sup>1</sup>, Sander R. Piersma<sup>1</sup>, Thang V. Pham<sup>1</sup>, Meike de Wit<sup>2</sup>, Sandra Mongera<sup>2</sup>, Beatriz Carvalho<sup>2</sup>, Henk MW Verheul<sup>1</sup>, Remond JA Fijneman<sup>2</sup>, Gerrit A Meijer<sup>2</sup>, and Connie R Jimenez<sup>1</sup>

<sup>1</sup>OncoProteomics Laboratory, Dept. Medical Oncology, <sup>2</sup>Dept. Pathology (Tumor Profiling Unit), VU University Medical Center, Amsterdam. The Netherlands

**Background** Abnormalities in nuclear phenotype and chromosome structure are key features of cancer cells. Investigation of the protein determinants of nuclear sub-fractions in cancer may yield molecular insights into aberrant chromosome function and chromatin organization and in addition may yield biomarkers for early cancer detection.

**Aims** 1. To evaluate a proteomics workflow for profiling protein constituents in sub-nuclear domains in colorectal cancer tissues. 2. To apply this workflow to a comparative analysis of the nuclear matrix fraction in colorectal adenoma and carcinoma tissue samples.



**Results** First we established the reproducibility of the entire workflow. In a reproducibility analysis of three nuclear matrix fractions independently isolated from the same colon tumor homogenate, 889 of 1,047 proteins (85%) were reproducibly identified at high confidence (minimally 2 peptides per protein, at 99% CI at the protein level), with an average CV for the number of normalized spectral counts per protein of 27%. This indicates a good reproducibility of the entire workflow from biochemical isolation to nanoLC-MS/MS analysis. Second, using spectral counting combined with statistics, we have identified proteins that are significantly enriched in the NM fraction relative to two earlier fractions (the chromatin-binding and intermediate filament fractions) isolated from 6 colorectal tissue samples. The total dataset contained 2,059 non-redundant proteins. Gene ontology mining and protein network analysis of NM enriched proteins revealed enrichment for proteins implicated in “RNA processing” and “mRNA metabolic process”. Finally, an explorative comparison of the nuclear matrix proteome in colorectal adenoma and carcinoma tissues revealed many proteins previously implicated in oncogenesis as well as new candidates. A subset of these differentially expressed proteins also exhibited a corresponding change at the mRNA level.

**Conclusions** Together, the results show that sub-nuclear proteomics of tumor tissue is feasible and a promising avenue for exploring oncogenesis.

**Reference.** Albrethsen J, Knol JC, Piersma S, Pham TV, de Wit M, Mongera S, Carvalho B, Verheul HM, Fijneman RJ, Meijer GA, Jimenez CR. Sub-nuclear proteomics in colorectal cancer: Identification of proteins enriched in the nuclear matrix fraction and regulation in adenoma to carcinoma progression. *Mol Cell Proteomics*. 2010, 9(5):988-1005.

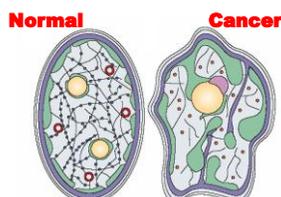
*This research is supported by the VUmc-Cancer Center Amsterdam.*

## Chromatin-associated proteins in colorectal adenoma and carcinoma tissues

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**Background** The hallmarks of cancer cells include genetic instability and altered nuclear morphology. Research into protein correlates of these aberrations could provide novel insights into cancer pathogenesis and may constitute a valuable source of protein markers.



**Method** We biochemically extract the so-called chromatin-binding (CB) tissue fraction from colorectal cancer (CRC) progression stages and compare the relative proteomic differences by label-free LC-MS/MS-based spectral counting.

**Results** The CB fraction is enriched for nuclear proteins including histons and is depleted of major structural proteins, as well as extra-vascular Serum Albumin. Biochemical fractionation and label-free LC-MS/MS identifies over 1,856 proteins. We confirmed increased levels of several of the (pre)clinical markers for CRC, as well as several previously reported candidate markers for CRC and, in addition, we identify many proteins that not previously have shown to be increased in CRC. A subset of these differentially expressed proteins also exhibited a corresponding change at the mRNA level in previously in-house generated transcriptomics datasets. The up-regulated nuclear proteins are among others associated with DNA recombination, replication and repair.

**Conclusions** Comparative proteomics of the CB fraction of cancer and precursor stages has potential for the identification of candidate biomarkers and for advancing clinical proteomics towards implementation in a pathological framework. Here, we present several novel candidate protein markers for early stage CRC for further validation.

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

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

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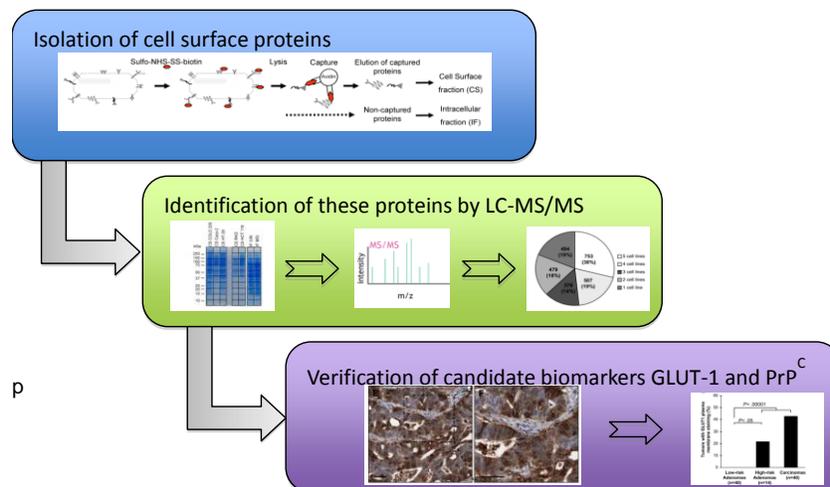
VU University Medical Center, Department of <sup>1</sup>Pathology (Tumor Profiling Unit) and <sup>2</sup>Medical Oncology (OncoProteomics Laboratory), De Boelelaan 1117, 1081HV Amsterdam, The Netherlands. <sup>3</sup>Uppsala University, The Rudbeck Laboratory, Department of Genetics and Pathology, SE-751 85 Uppsala, Sweden. <sup>4</sup>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<sup>C</sup>; gene symbol *PRNP*;  $P < .005$ ).

**Conclusion:** This study revealed GLUT1, PrP<sup>C</sup>, 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, <sup>19</sup>F-MRI and PET.



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. 2011 Sep 2.

## Stool proteomics reveals novel candidate biomarkers for colorectal cancer screening

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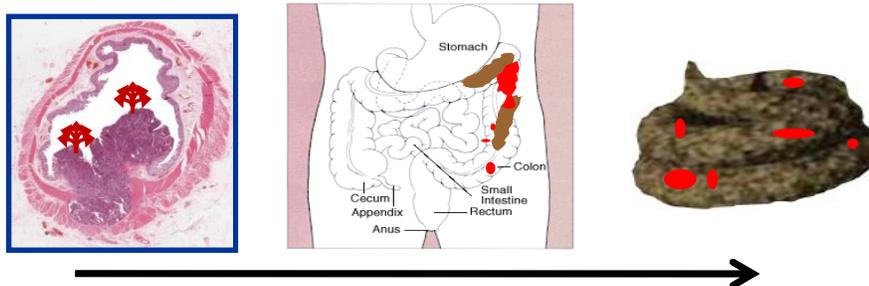
### Background

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. 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-binomial test.



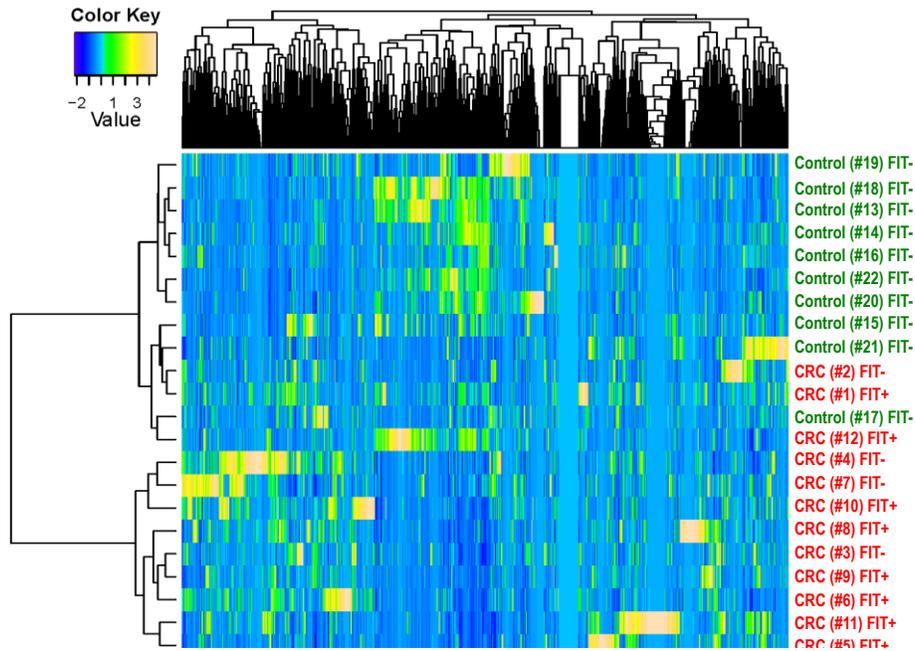
Stool as direct link to the colon

### Results

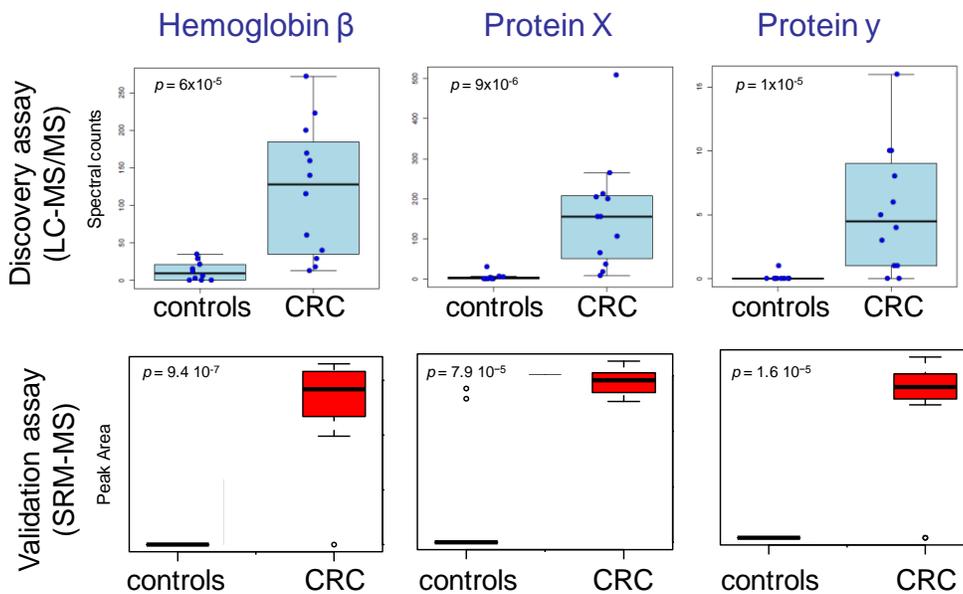
In total 830 human proteins were identified and levels for 221 of these differed significantly between stool samples from CRC patients and control subjects. Of these, 134 proteins were significantly enriched in CRC. Unsupervised hierarchical cluster analysis using all 830 proteins as input, revealed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

### Conclusions and outlook

Proteome profiling on stool revealed 134 proteins significantly enriched in CRC compared to control stool samples. This set contains several proteins, like complement component 4B, with discriminative potential higher than or complementary to hemoglobin in stool tests for CRC screening. Promising candidate protein markers are currently being selected to be followed up in a validation series of 240 stool samples by Selected Reaction Monitoring mass spectrometry (SRM-MS).



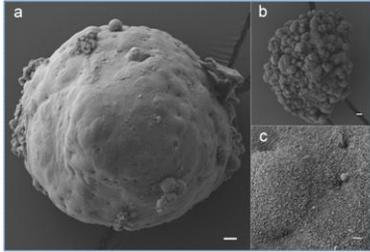
**Figure 1. Proteome analysis of human stool samples.** **A.** Stool samples were obtained from 12 CRC patients and 10 control individuals, as determined by colonoscopy. Proteins were isolated and subsequently analyzed by in-depth proteomics using gel electrophoresis, peptide extraction and nano Liquid Chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS). Spectral counts mapped to known peptides were used to quantify presence of human proteins. **B.** Unsupervised cluster analysis based on all 830 human proteins detected in stool samples from CRC patients and control patients, showing two clusters. One cluster contains CRC patients only; the other cluster contains all control patients plus three CRC patients. FIT results on the same stool samples are indicated by FIT+ (positive test results) or FIT- (negative test result).



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### Proteomics of cancer stem cell-enriched colon cancer spheroids

Colorectal tumors contain a small subpopulation of cells that have the unique capacity to self-renew, to initiate tumor formation, and to generate non-tumorigenic differentiated offspring. These cancer stem cells (CSC; or tumor-initiating cells) are thought to originate from normal tissue stem cells. Normal tissue stem cells possess a variety of resistance mechanisms that allow them to endure a lifetime of genotoxic insults. By inheriting these traits, cancer stem cells are thought to be endowed with an intrinsic resistance to chemotherapy. Proteomics of tumor spheroids may yield insight into the relationship between chemoresistance and cancer stemness.



The OncoProteomics Laboratory is involved in several cancer stem cell projects with two institutes: 1 with Benjamin Emmink and Dr. Onno Kranenburg of the research group Surgical Oncology of the University Medical Center Utrecht (UMCU) and 2. With Dr. Uros Rajcevic and Dr. Simone Niclou of the NorLux Oncology Laboratory in Luxemburg. The two projects on colorectal cancer spheroids are described in the sub-projects.

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

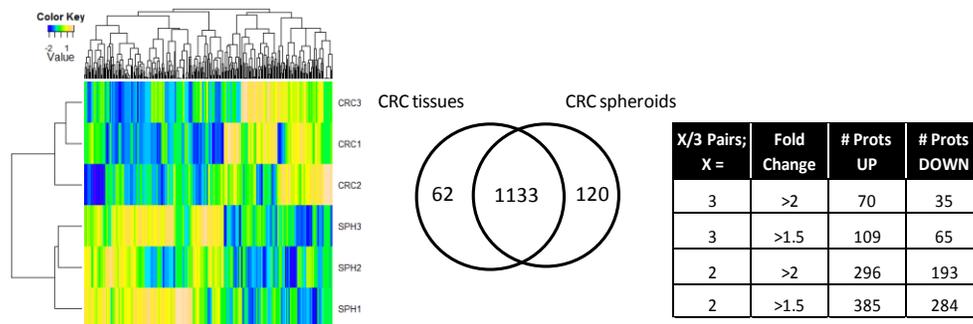
Uros Rajcevic<sup>1</sup>, Jaco C. Knol<sup>2</sup>, Sander Piersma<sup>2</sup>, Sébastien Bougnaud<sup>1</sup>, Thang V. Pham<sup>2</sup>, Eirik Sundlisaeter<sup>3</sup>, PerØyvind Enger<sup>3</sup>, Rolf Bjerkvig<sup>1,3</sup>, Simone P. Niclou<sup>1</sup> and Connie R. Jimenez<sup>2</sup>

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**Background.** Preclinical cancer research has thrived on in vitro culturing systems. However, it has become increasingly clear that monolayer cultures used to study molecular and biological aspects of tumor cells, as well as their response to therapeutic agents, have lost a great many features exhibited by in vivo tumors. In cell monolayers, the architecture of a tumor tissue and the complex interactions and connections of its constituent cell types and matrix molecules are largely lost, intratumoral differences in nutrition, oxygenation, and drug penetration are abrogated, and growth on a plastic substratum can induce significant changes in cellular genotype and phenotype. In contrast, three-dimensional cell culture systems have biological characteristics which are more closely related to those of in vivo tissue. Spheroids can not only be generated from (dissociated) cultured cells, but also directly from tissue biopsy fragments in an organ culture type of fashion (Bjerkvig (1990)). Importantly, these "tumor tissue spheroids" preserve tissue characteristics of the original tumor in situ that are not only lost in monolayer cultures of cell lines, but also in spheroids derived from dissociated tissue cells. These tissue spheroids still preserve tumor-initiating potential as demonstrated by xenografting experiments in animal models and therefore may provide a suitable model to test therapeutic agents in vitro in a more relevant setting.

**Aim and approach:** To assess what is happening at the protein level following CRC spheroid culture, and 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.** Using GeLC-MS/MS, 1315 unique proteins were identified across the sample pairs of the three patients. 103 proteins were more than 2-fold regulated in all 3 pairs. Spheroid enriched proteins were associated with (negative regulation of the) cytoskeleton, chromatin remodeling, nucleic acid metabolic process, RNA metabolism and translation. In addition the spheroids were enriched ALDHA1, a protein previously reported as a colon cancer stem cell marker.



**Conclusions** Colon cancer spheroids express an overlapping but distinct protein profile as compared to their corresponding resection material. The dataset may harbor potential new marker candidates for colon cancer stem cells. These proteins may have prognostic value and be involved in drug resistance. Further studies are warranted.

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

## Comparative proteomics of colon cancer stem cells and differentiated tumor cells identifies BIRC6 as a potential therapeutic target.

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**Background** Patients with liver metastases from colon carcinoma show highly variable responses to chemotherapy and tumor recurrence is frequently observed. Therapy-resistant cancer stem cells have been implicated in drug resistance and tumor recurrence. However, the factors determining therapy resistance and tumor recurrence are poorly understood. Novel tissue culture protocols allow the isolation and propagation of tumor-derived 3D-spheroid cultures that are enriched in CSC's (Figure). Spheroid cultures are highly tumorigenic when transplanted into immunodeficient mice, and generate tumors that closely resemble the original patient tumor, at least when analyzed by histological methods.

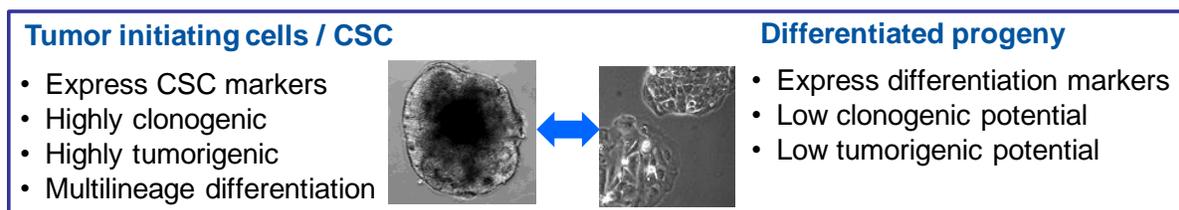


Figure. Model used for comparative proteomics

**Aim and approach** The aim of this study was to gain insight into tumorigenic mechanisms by comparing the proteomes of patient-derived cancer stem cell cultures and their differentiated isogenic offspring. We established colonosphere cultures derived from resection specimens of liver metastases in patients with colon cancer. These colonospheres, enriched for colon cancer stem cells, were used to establish isogenic cultures of stably differentiated nontumorigenic progeny.

**Results** Proteomics based on one-dimensional gel electrophoresis coupled to nano liquid chromatography tandem MS was used to identify proteome differences between three paired cultures of adherent cells and spheroids. The resulting data were analyzed using Ingenuity Pathway Software. Out of a total data set of 3048 identified proteins, 32 proteins were at least twofold up-regulated in the colon cancer stem cells when compared with the differentiated cells. Pathway analysis showed that "cell death" regulation is strikingly different between the two cell types. Interestingly, one of the top-up-regulated proteins was BIRC6, which belongs to the class of Inhibitor of Apoptosis Proteins. Knockdown of BIRC6 sensitized colon cancer stem cells against the chemotherapeutic drugs oxaliplatin and cisplatin.

**Conclusions and outlook** This study reveals that differentiation of colon cancer stem cells is accompanied by altered regulation of cell death pathways. We identified BIRC6 as an important mediator of cancer stem cell resistance against cisplatin and oxaliplatin. Targeting BIRC6, or other Inhibitors of Apoptosis Proteins, may help eradicating colon cancer stem cells.

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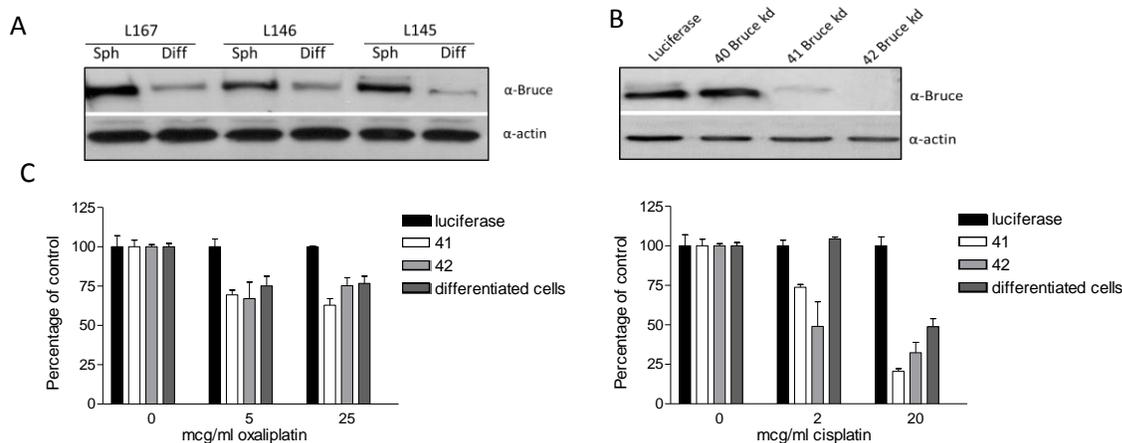


Figure. A BIRC6 is enriched in spheroids and the tumorigenic Aldefluorhigh fraction of colonosphere cells (data not shown). B-C. BIRC6 confers resistance against cisplatin and oxaliplatin

### Colon cancer proteomics subproject 7c

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

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**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.

Proteomics of colorectal metastase tissue: high expression of serpinB5 (Maspin) is associated with poor survival in stage III and IV colorectal cancer

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\* equal contribution to study

**Background:** Little is known about the factors driving metastasis formation in colorectal cancer (CRC). Here we set out to identify genes and proteins in liver metastases that correlate with poor disease free survival (DFS) in stage IV patients. Such factors may predict a propensity for metastasis in earlier stages of CRC.

**Methods:** Mass spectrometry-based proteomics was used to identify differentially expressed proteins in patients with DFS shorter than 6 months (n=5) and longer than 24 months (n=5). IN parallel, Micro-array analysis was used to identify differentially expressed genes in liver metastases from stage IV CRC patients with disease-free survival (DFS) shorter and longer than 12 months. Immunohistochemistry on tissue microarrays containing tumors from 243 stage II and 179 stage III patients was subsequently performed to assess correlations with stage-specific outcome.

**Results:** Both gene expression profiling and proteomics on stage IV tumors identified the tumor suppressor serpinB5/maspin to be most strongly and significantly correlated with short disease free survival. High maspin expression is also an independent prognostic factor for poor disease-free, overall and disease-specific survival in patients with stage III, but not in patients with stage II tumors. Furthermore high maspin expression correlates with poor differentiation, mucinous differentiation and microsatellite instability.

**Conclusions:** High maspin expression correlates with poor outcome in colorectal cancer after spread to the local lymph nodes. Therefore, maspin may have a stage-specific function possibly related to tumor cell dissemination and/or metastatic outgrowth.

# Brain cancer proteomics

## *Brain cancer proteomics, sub-project 1*

### Proteomics of meningiomas in correlation with growth rate

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**Introduction** The underlying mechanism for the variability in growth rate within the group of meningiomas that are classified as WHO I is unknown. Possibly the genome is selectively repressed, an adaptive property that is governed by the regulation of gene expression, mostly at the level of transcription i.e., the production of messenger RNA from the DNA of the gene in question. These alterations may lead to altered levels of the corresponding proteins. Therefore, large-scale identification of proteins, proteomics, may offer insight into the molecular mechanisms in different WHO I phenotypes as well as yield novel candidate biomarkers that provide information for both classification and prognostication of individual tumors.

**Aim** To identify different protein patterns between meningiomas histological type WHO I (no clinical growth), WHO I (clinical significant growth) and WHO II.

**Methods** Proteomic analysis was performed on 6 samples (2 in each of the above mentioned groups). Total tissue lysates were made by homogenizing 20 mg tissue directly in 500 µl SDS sample buffer. The tissue lysates were subsequently subjected to SDS-PAGE (40 µg per sample) followed by slicing of the whole gel lane, in-gel protein digestion using trypsin and nanoLC-MS/MS (GeLC-MS/MS) on a LTQ-FTMS instrument. Proteins were identified using database searching of the IPI database with the SEQUEST search engine. For visualization and comparison of the results, the proteins were loaded in the tool Scaffold.

**Results** Using nanoLC-MS/MS, a total of 1734 different protein were identified (based on at least two peptides identified at > 95% confidence). Importantly, unique patterns of protein expression were identified in the different groups. These proteins represent different functional classes that are differentially enriched. For example using gene ontology mining we found that a substantial part of the 52 proteins unique for the WHO I-no growth group were involved in the cell cycle whereas the 23 proteins uniquely present in WHO I-growth and WHO II were enriched in the gene ontologies 'response to external stimulus, defense response' 'stress response' adhesion and cell proliferation. With respect to molecular function, the group WHO I-no growth was enriched for proteins with annotation 'transcription factor activity' in the molecular function class whereas the term 'integrin binding was enriched in the WHO I- growth and WHO II samples. In the cellular component ontology, 'extracellular matrix part was enriched as well as the KEGG pathway ECM-receptor interaction in the WHO I-growth and WHO II samples. Finally, 20 proteins were unique for WHOII samples, including several mitochondrial proteins and enzymes.

**Conclusions** This pilot proteomic analysis of meningiomas yielded the largest protein dataset to date and identified differential proteins related to WHO I meningiomas with different growth patterns and WHO II meningiomas. These proteins will be validated using immunohistochemistry of an independent series and may provide candidate biomarkers for both classification and prognostication of individual tumors.

**Book chapter:** Saskia Peerdeman, Okay Saydam , Connie R. Jimenez. In 'Tumors of the nervous system'. Meningiomas: clinical needs and molecular insights. Springer. 2011, in press.

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

## Proteomics reveals minichromosome maintenance proteins as novel tumor markers for meningiomas

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<sup>1</sup>Department of Neurology, Neurogenetic Unit, Massachusetts General Hospital, Harvard Medical School,

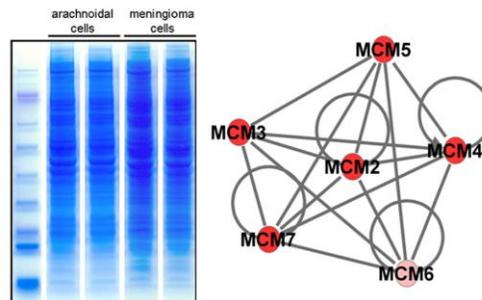
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**Introduction** Meningioma is one of the most common central nervous system tumors accounting for 32.1% of all reported brain tumors. Recent studies on protein profiling in tumors provided crucial information about the pathogenesis of several cancers at the molecular level.

One of these studies, proteomics is the process that allows to screening a broad protein profile that permits a direct analysis of proteins differentially or uniquely expressed by a cell or a tissue type. To date, to our knowledge there is no report on proteomics based protein profiling in meningiomas compared to arachnoidal tissues, the origin of this tumor.

**Aims** 1. To identify novel protein tumor markers for diagnostic and/or prognostic purposes for meningiomas. 2. To discover previously unknown proteins and/or signaling pathways involved in tumorigenesis of meningiomas.

**Methods** Proteomics studies were performed by NanoLC-LTQ-FT MS. We first compared protein profiling of meningioma cells to primary arachnoidal cells. In validation studies, qRTPCR and western blots were performed in human meningioma tumor samples (WHO grade I: 14 samples, WHO grade II: 7 samples, WHO grade III: 4 samples) compared to arachnoidal tissue controls (3 samples).



**Results** Protein profiling of meningiomas revealed changes in the expression levels of 92 proteins associated with various biological functions such as DNA replication, recombination, cell cycle, and apoptosis. Seventy-three proteins were found to be detected and up-regulated only in meningioma cells, whereas only nineteen proteins were up-regulated in arachnoidal cells and non-detected in meningiomas. Among those several interesting candidates, we focused on the most up-regulated proteins in meningiomas, minichromosome maintenance (MCM) proteins and found that MCMs are up-regulated in meningiomas compared to arachnoidal tissue controls.

**Conclusion** Our data suggested that MCMs are up-regulated in meningiomas and might serve as diagnostic markers for this tumor.

**Reference:** Saydam O, Senol O, Schaaij-Visser TB, Pham TV, Piersma SR, Stemmer-Rachamimov AO, Wurdinger T, Peerdeman SM, Jimenez CR. Comparative protein profiling reveals minichromosome maintenance (MCM) proteins as novel potential tumor markers for meningiomas. *J Proteome Res.* 2010 Jan;9(1):485-94.

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

## miR-200a inhibits meningioma cell migration and tumor growth by directly targeting non-muscle heavy chain IIb

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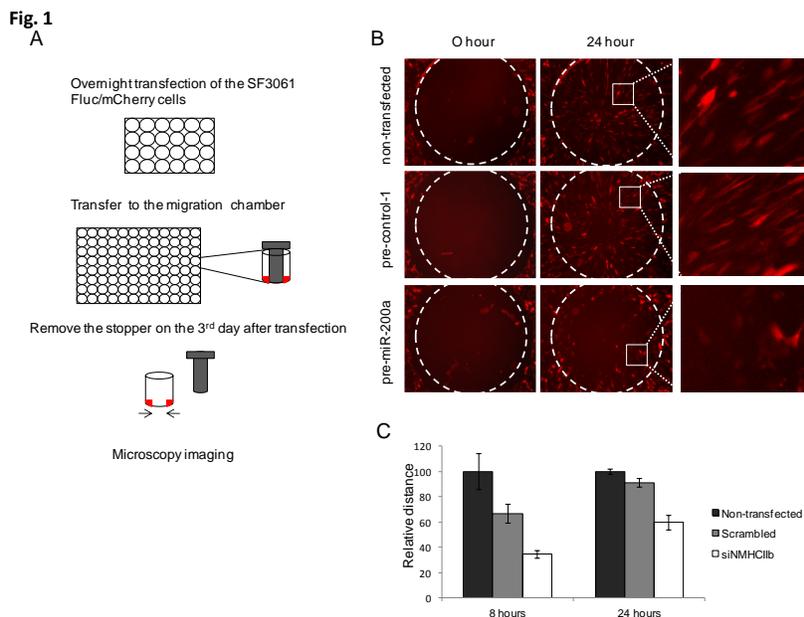
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**Background** miR-200a has been shown to be implicated in the pathogenesis of meningiomas, one of the most common central nervous system tumors in humans.

**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 over 2000 proteins, 130 proteins were dysregulated in response to miRNA manipulation. Among those interesting proteins, we focused on non-muscle heavy chain IIb (NMHCIIb) and found that NMHCIIb was significantly decreased in the miR-200a overexpressing SF4433 cells. Using qRT-PCR and western blot analysis, we validated that the upregulation of miR-200a decreased the NMHCIIb expression in both mRNA and protein levels. We also showed that miR-200a directly targets NMHCIIb by targeting the binding sites in 3'UTR of the mRNA. NMHCIIb is one of three isoforms of non-muscle myosin II heavy chains and has been shown to play a key role in the biological processes such as cell division and cell migration. To assess whether miR-200a is involved in the regulation of these processes through targeting NMHCIIb gene, we overexpressed miR-200a in the malignant meningioma cell line, SF3061 and performed a migration assay. We observed a significant decrease in the migration rate and attenuation in the migration phenotype of miR-200a overexpressing cells in comparison to controls (Fig. 1). Besides, siRNA ablation of NMHCIIb resulted in the similar migration phenotype in these cells as well as a significant decrease in cell growth.

**Conclusions** These results suggest that NMHCIIb targeting might be one of the regulatory mechanisms driven by miR-200a in meningiomas.



## Marker identification on brain tumor stem cells from malignant glioma

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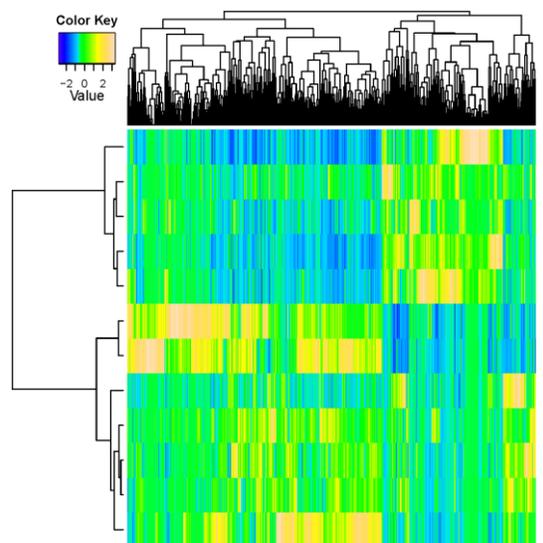
**Background:** Glioblastoma multiforme (GBM) is one of the most lethal forms of human cancer. Median survival is only 12 to 15 months for glioblastoma patients. Genetic and signaling pathways involved in the development of glioblastoma have been relatively well characterized but the cellular origin of these tumors is unknown. A new approach to glioblastoma proposes targeting cancer stem-like cells which have been shown to be crucial for the initiation and maintenance of malignant disease including glioblastoma. No reliable markers exist up-to-date, that would be able to differentiate the tumor stem cells from the normal stem cells.

**Aims:** **1.** To reveal the proteome of GBM biopsy spheroids established from human glioblastomas and compare it with the proteomes of normal neurospheres and glioma stem cells grown in neurospheres by means of label-free GeLC-MS/MS. **2.** To use immunohistochemistry on glioma tissue microarrays as a mode of marker validation on clinical material.

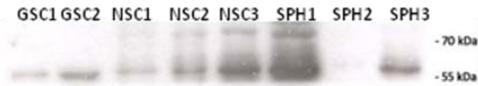
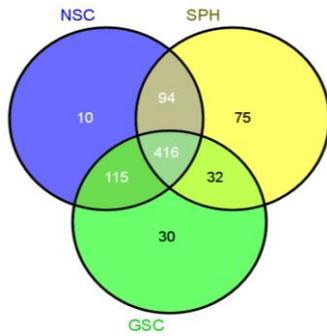
**Patients and Methods:** Primary cell cultures were used. Glioma spheroids (5 samples; I-M in the graph) were created from samples obtained from surgery, normal neurospheres were grown from subventricular parts of normal brain tissues (5 samples) and glioma stem cell neurospheres were grown from cell lines (2 cell lines; G, F). The proteins resulting from these cells were subjected to a label-free proteomics discovery workflow consisting of GeLC-MS/MS using the LTQ-FTMS and database searching using SEQUEST. A large set of tissue microarrays (TMA) was created from glioma (grades I-IV) tissue samples from more than 300 glioma patients. Antibodies against selected markers were used in a WB on an independent set of primary cell cultures and to stain the TMA and of different grade of tumors.

**Results:** We identified 1982 proteins with at least two peptides sequenced – of which 747 were differentially expressed in three-way comparison (ANOVA  $p < 0,05$ ). Samples of NSC (A-E) were well distinguished from glioma spheroids (I-M) and GSC (F,G).

Most proteins (416) were expressed in all three types of cells, while 30 were uniquely expressed in glioma stem cells 10 in neural stem cells and 75 were only expressed in tumor spheroids.

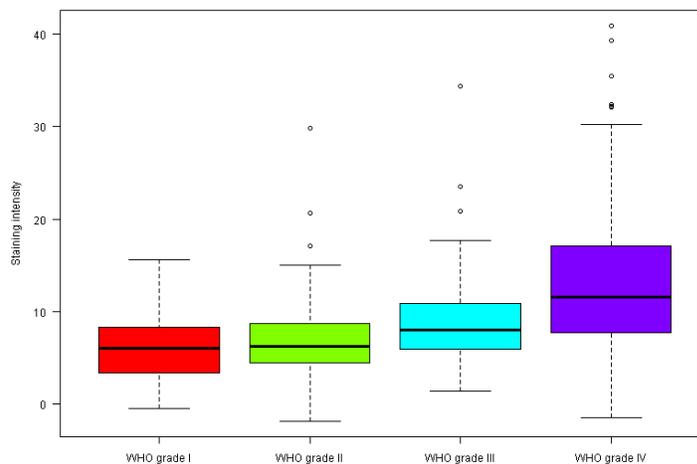


*'This project is supported by the Fonds National de la Recherche (FNR) of Luxembourg.'*



Using different bioinformatic and statistical tools we selected a set of biomarker candidates to be validated by alternative means. The first batch of these markers was differentially expressed and came from the intersection of the cell type. The expression of marker X was confirmed by WB. It's expression in the context of tissue was verified by TMA analysis of gliomas grades I-IV. The expression difference was shown significant between grades I/II (benign) and I/IV (benign/malignant).

Our favorite marker, marker y, a transcription factor, was shown to be most significantly pointing at the differences between tumor grades at molecular level as its expression differs between all the grades highly significantly except I/II (see figure below).



**Conclusions:** By using a combination of MS/MS-based proteomics and immunohistochemistry we are proposing a set of markers to distinguish between glioma grades at molecular level. The expression of these markers, when correlated to each other and to patient survival may have an important impact in glioma survival prediction.

**Outlook:** Confident, specific NSC and GSC markers could facilitate their proper differentiation which is crucial for the diagnostics. Novel NSC markers could make an alternative to existing NSC markers in FACS analyses of NSCs, while novel GSC markers could be interesting as drug targets in glioma or as diagnostic tools.

*This research was supported by FNR Luxembourg (grant PDR-08-007 to UR) and CRP-Santé Luxembourg.*

# Breast Cancer Proteomics

## Breast cancer proteomics, sub-project 1

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

Marc.O. Warmoes<sup>1</sup>, Janneke Jaspers<sup>2</sup>, Thang V. Pham<sup>1</sup>, Sander Piersma<sup>1</sup>, Gideon Oudgenoeg<sup>1</sup>, Maarten P.G. Massink<sup>3</sup>, Hanne Meijers-Heijboer<sup>3</sup>, Quinten Waisfisz<sup>3</sup>, S. Rottenberg<sup>2</sup>, Epie Boven<sup>1</sup>, Jos Jonkers<sup>2</sup>, Connie R. Jimenez<sup>1</sup>

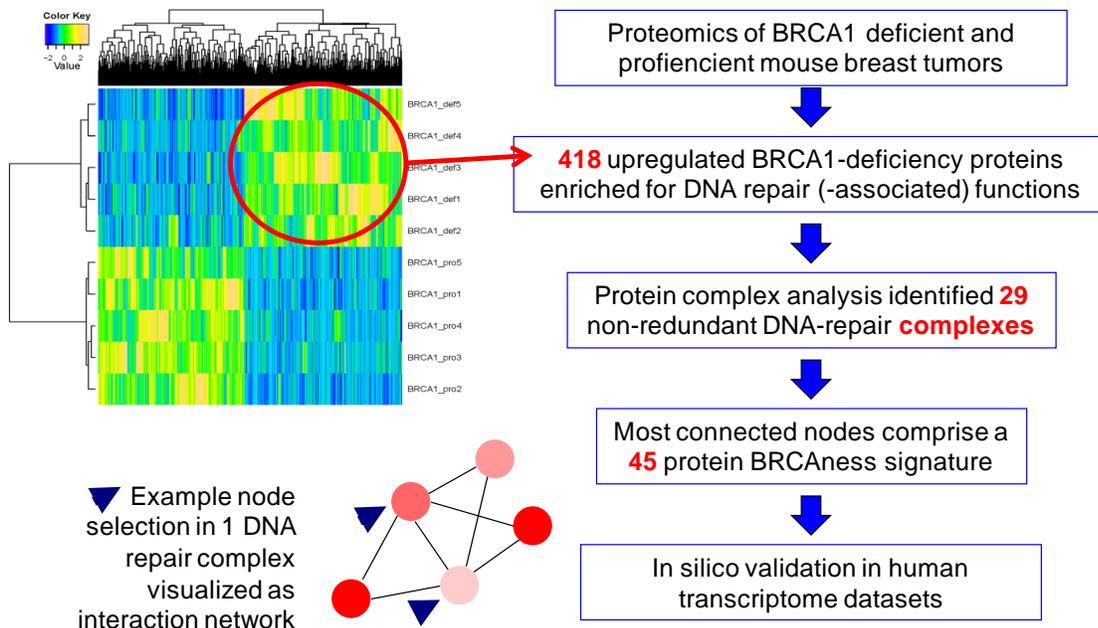
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**Background:** Breast cancer is the most common malignancy in women in the Western world. The outcome of breast cancer treatment is strongly improved if patients can be diagnosed and treated early. This especially holds for patients with BRCA1 hereditary breast cancer, a type of cancer with mostly defects in the homology based DNA-repair pathway.

**Aim** To identify proteins that are associated with BRCA1 deficient hereditary breast cancer. These proteins could have potential use as screening, 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.

**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 prognostic power was assessed by the use of publicly available gene expression human breast cancer data sets.



**Results** We identified a total of 3614 proteins, of which 804 were significantly regulated between the genomic instable BRCA1-deficient and the genomic stable BRCA1-proficient breast tumors. Pathway and protein complex analysis identified DNA-repair and associated functions like chromatin modeling and RNA-processing as the major functions associated with the proteins upregulated in the BRCA1 deficient tumors. A signature of 45 proteins comprising the most connected upregulated nodes of each (DNA repair) complex was shown to have diagnostic and prognostic power.

**Conclusions and outlook** Proteomics of genetic mouse models for genetic breast cancer is a powerful strategy to discover novel candidate BRCA1 deficiency markers with human relevance. Further validation studies are required to investigate whether the BRCAness signature is predictive for sensitivity to PARP inhibition in BRCA deficient as well as tumors with deficiency in homology repair.

*This research was supported by the GenE/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, in press.

#### *Breast cancer proteomics, sub-project 4*

### Immunohistochemical validation in human breast cancer of selected protein members of the mouse 45 protein BRCA1 deficiency signature

Petra van der Groep<sup>1,2</sup>, Marc Warmoes<sup>3</sup>, Joey Lam<sup>3</sup>, Yvonne Smolders<sup>2</sup>, Epie Boven<sup>3</sup>, Sven Rottenburg<sup>4</sup>, Jos Jonkers<sup>4</sup>, Paul van Diest<sup>2</sup>, Elsken van der Wall<sup>1</sup>, Connie Jimenez<sup>3</sup>

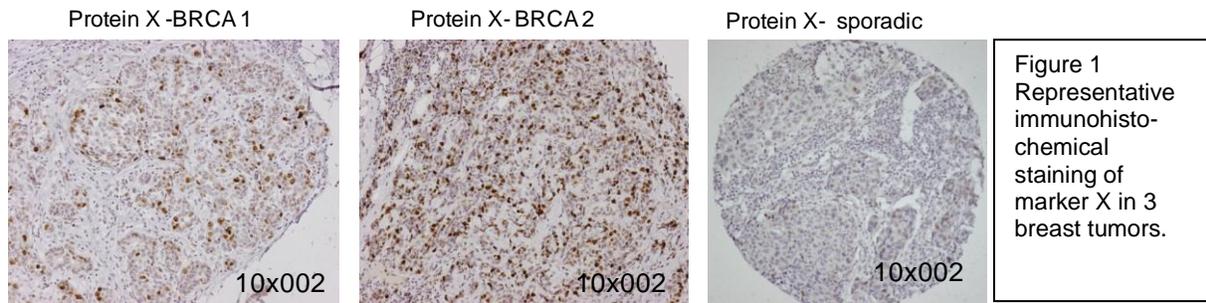
<sup>1</sup>Division of Internal Medicine and Dermatology and <sup>2</sup>Dept. of Pathology, University Medical Center Utrecht, Utrecht, <sup>3</sup>Oncoproteomics Laboratory, Dept. of Medical Oncology, VU University Medical Center, Amsterdam, <sup>4</sup>Division of Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

**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. In a mouse model for *BRCA1* deficient breast cancer, we previously identified a signature comprised of 45 proteins involved in a range of DNA repair and chromatin remodeling functions that was able to classify human *BRCA1* and *BRCA2* deficient breast tumors *in silico* using transcriptomics datasets.

**Aim and approach** To validate the mouse *BRCA1* deficiency signature at the protein level in human *BRCA* mutation related breast cancers using immunohistochemistry.

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. Tissue microarrays were constructed and stained for marker X, Y and Z (markers were selected out of the top ten of the 45 protein BRCA1 deficiency signature) by immunohistochemistry. 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



Most of the tumors were of ductal type and high grade (Table 1). 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 1) 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 ( $p=0.000$ ) (table 1).

		Sporadic	BRCA 1	BRCA 2	p value
N		99	38	23	
Type	Ductal	87	30	20	
	Lobular	8	0	3	
	Medullary	0	2	0	
	Other	3	0	0	
	Missing	1	6	0	
Age	Mean	57	42	47	
Grade	1	9	0	2	
	2	30	5	5	
	3	54	23	7	
	Missing	6	10	9	
X	Neg	69	3	3	0.000
	Pos	30	28	17	
	Missing	0	7	3	
Y	Neg	33	21	7	0.136
	Pos	52	16	13	
	Missing	14	1	3	
Z	Neg	65	7	6	0.000
	Pos	16	27	12	
	Missing	18	4	5	

Table 1. Expression patterns of marker X, Y and Z in invasive breast cancer of non-*BRCA*, *BRCA1* and *BRCA2* mutation carriers.

**Conclusions and outlook** The expression of marker X and Z is increased in *BRCA1* and *BRCA2* deficient breast tumors in comparison to the expression of these markers in sporadic breast tumors. These proteins, X and Z, warrant further studies to explore their predictive potential in retro- and prospective trial using DNA damaging agents and their value for early detection in biofluids.

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

Marc Warmoes<sup>1\*</sup>, Janneke Jaspers<sup>2\*</sup>, Sven Rottenberg<sup>2</sup>, Barath Kumar<sup>2</sup>, Sander Piersma<sup>1</sup>, Jos Jonkers<sup>2\*</sup>, Connie R. Jimenez<sup>1\*</sup>

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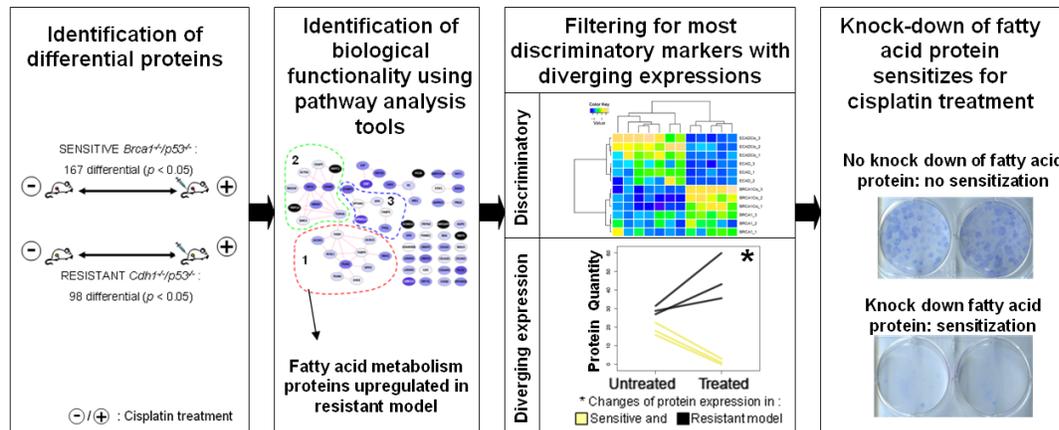
<sup>2</sup>Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

**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*<sup>-/-</sup>; *p53*<sup>-/-</sup> mammary tumors of our mouse model for hereditary breast cancer (*K14cre;Brca1*<sup>flox/flox</sup>; *p53*<sup>flox/flox</sup>) are very sensitive to the maximum tolerable dose of cisplatin, and do not acquire resistance to this drug (Rottenberg et al., PNAS 104, pp. 12117-12122, 2007). In contrast, several spontaneously developing *Ecad*<sup>-/-</sup>; *p53*<sup>-/-</sup> mammary tumors of our mouse model for lobular breast cancer (*WAPcre;Ecad*<sup>flox/flox</sup>; *p53*<sup>flox/flox</sup>) 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 treated 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 the GenE/Van Lanschot and the VUmc Cancer Center Amsterdam

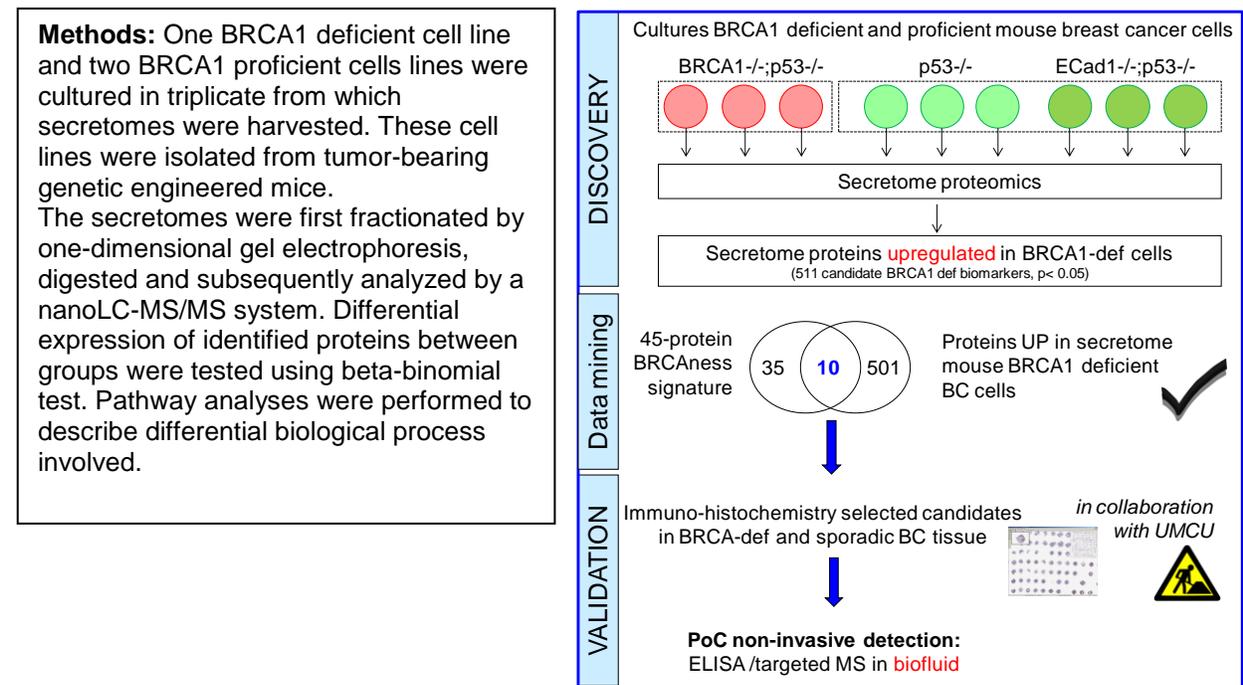
## Proteomics of mouse BRCA1-deficient and proficient mammary cell line secretomes identifies non-invasive protein biomarkers for BRCA1 deficient breast cancer

Marc Warmoes<sup>1\*</sup>, Siu W. Lam<sup>1\*</sup>, Thang V. Pham<sup>1</sup>, Sander R. Piersma<sup>1</sup>, Sven Rottenberg<sup>2</sup>, Epie Boven<sup>1</sup>, Petra van der Groep<sup>3</sup>, Paul J. van Diest<sup>3</sup>, Petra Nederlof<sup>2</sup>, Jos Jonkers<sup>2</sup>, Connie R. Jimenez<sup>1</sup>

<sup>1</sup>Oncoproteomics Laboratory, Dept. of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands; <sup>2</sup>Division of Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands; <sup>3</sup>Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands. \*Equal contribution.

**Background:** Secreted proteins (secretomes) could provide a valuable source of non-invasive protein biomarkers.

**Aim and approach:** To identify proteins specifically related to BRCA1 status by differential protein profiling of secretomes of BRCA1-deficient and proficient breast cancer cell lines



**Results:** The proteomic analysis identified a total of 2107 secreted proteins in nine experiments. A >80% overlap of identified proteins was observed in all triplicate experiments. By using defined criteria ( $p$  value < 0.05 and fold change > 1.5), we identified 509 upregulated proteins and 403 downregulated proteins in secretomes from BRCA1-deficient breast cancer. The upregulated proteins were involved in biological process including translation, RNA splicing, angiogenesis, whereas the downregulated proteins were involved in proteolysis, actin cytoskeletal organization and nucleoside metabolism. Protein candidates were annotated for overlap with the 45 protein BRCA1 deficiency signature (Warmoes et al., MCP 2012), mRNA expression in human BRCA1/2 breast cancer, presence in human biofluid and functional relation with BRCA1 providing a list of prioritized candidates for further follow-up.

**Outlook** The clinical relevance of selected proteins is currently being explored. Potential clinical applications of a protein signature related to BRCA1 dysfunction are manifold i.e. early diagnostic tool, patient allocation or treatment selection.

## Lung cancer proteomics

*Lung cancer proteomics, subproject 1*

### Novel candidate biomarkers for cisplatin response prediction and monitoring in NSCLC

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

<sup>1</sup>OncoProteomics Laboratory, Department of Medical Oncology, <sup>2</sup>Division of Molecular Genetics and Centre for Biomedical Genetics, The Netherlands Cancer Institute, Amsterdam, The Netherlands; <sup>3</sup>Department of Otolaryngology/Head and Neck surgery, <sup>4</sup>Dept. Pathology, <sup>5</sup>Dept. Pulmonology, VU University Medical Center, Amsterdam, The Netherlands

**Project background:** This project is embedded in the multi-center AIRFORCE project of the Center for Translational Molecular Medicine (CTMM) and a collaboration between the Oncoproteomics Laboratory of the VUmc and the division of Molecular Genetics of the Netherlands Cancer Institute. The AIRFORCE project in general aims at the improvement of personalized chemo-radiation of lung and head and neck cancer.

**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. Rather than following a broad strategy by analyzing the proteome of whole cell or tissue lysates, we chose to use a more specific approach based on the assumption that the best tumor markers are shed or secreted by the tumor and can be detected in blood.

**Aim** Identification of protein biomarkers that can be used for treatment response prediction and therapy monitoring.

**Approach:** Secretome was harvested on a set of tumors from conditional mouse models for SCLC and NSCLC<sup>2,3</sup>, a set of human and mouse SCLC and NSCLC cell lines and a series of human NSCLC cell lines with a range of IC50-values for cisplatin (1.5 – 15  $\mu$ M). Secretome protein profiling is performed using our in-house proteomics workflow. Here we focus on the cell line dataset.

**Results:** In total, 4 datasets were obtained. Data analysis was performed for which the cell lines were classified either resistant (IC50>5) or sensitive (IC50<5) to cisplatin. In total, 199 proteins were found to have a higher expression in the secretomes of sensitive cell lines and 233 proteins were highly expressed in secretomes of resistant cell lines. In the lysates of the cell lines, 255 proteins were higher expressed in the sensitive cells and 333 were higher expressed in the resistant cells. Supervised clustering based on differential proteins revealed clear clustering of resistant and sensitive cell lines. For marker selection, we considered the level of regulation between sensitive and resistant cells and the correlation to the cisplatin IC50, the associated biological process and whether the candidate had also been identified in a functional RNAi screen.

**Conclusions and outlook** Included for follow-up are biomarker candidates that can predict pathway defects in the Fanconi/ BRCA pathway and that are involved in the multivesicular body recycling compartment, among others. These candidates will be analyzed in clinical cohorts by targeted methods (selected reaction monitoring mass spectrometry and antibody-based methods for the analysis of sputum and tumor tissues).

*This research was supported by the CTMM Airforce project*

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

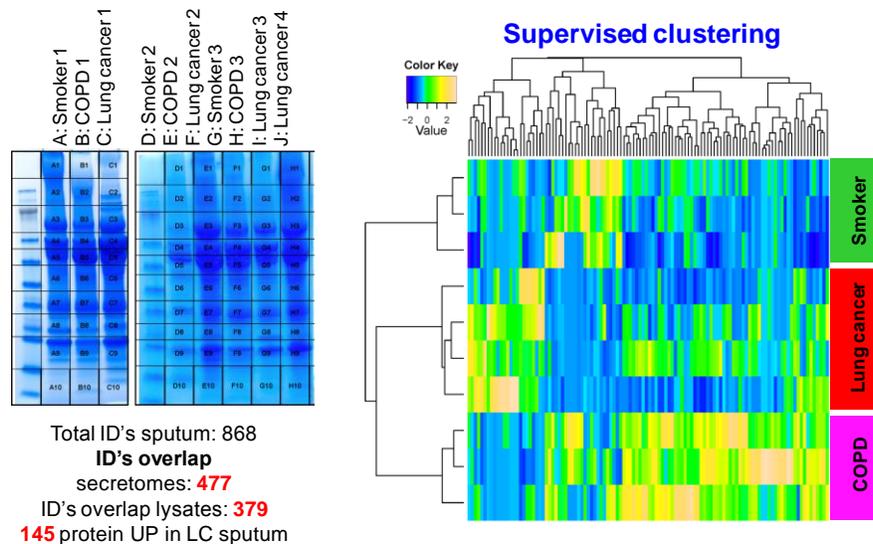
Tieneke BM Schaaij-Visser<sup>1</sup>, Burçin Colak<sup>1</sup>, Jasmijn Hubers<sup>2</sup>, Egbert F. Smit<sup>3</sup>, Erik FBJM Thunnissen<sup>2</sup>, Connie R. Jimenez<sup>1</sup>

<sup>1</sup>OncoProteomics Laboratory, Department of Medical Oncology; <sup>2</sup> Department of Pathology, <sup>3</sup>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 Carcinoembryonic 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*

## Cervix cancer proteomics

### Unravelling protein expression changes during HPV-induced transformation using a label-free proteomics approach

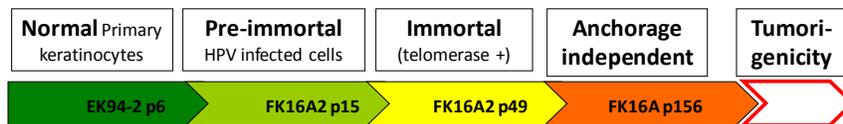
Carolien van Alphen<sup>1</sup>, Thang V. Pham<sup>1</sup>, Sander R. Piersma<sup>1</sup>, Peter J.F. Snijders<sup>2</sup>, Renske D.M. Steenbergen<sup>2\*</sup>, Connie R. Jimenez<sup>1\*</sup>

<sup>1</sup>OncoProteomics Laboratory, Dept. Medical Oncology, and <sup>2</sup>Dept. Pathology, VUmc Cancer Center Amsterdam, VU University Medical Center, Amsterdam, The Netherlands. \*Shared corresponding authors

**Background:** Cervical carcinogenesis is initiated by persistent infection with high-risk Human Papillomavirus (hrHPV), but requires additive host cell alterations for malignant transformation of a hrHPV infected cells. Unravelling these host cell events will yield novel disease markers to identify hrHPV-positive women at risk of cervical cancer and their high-grade precursor lesions.

**Aim:** To identify novel protein markers for risk assessment of hrHPV-positive women

**Approach:** Quantitative proteome analysis of a well-characterized, longitudinal *in vitro* model of cervical carcinogenesis, consisting of primary keratinocytes, HPV16-transfected keratinocytes representing consecutive stages of transformation (extended lifespan-mortal, immortality and anchorage independence) and tumorigenic cervical cancer cells (SiHa).



**Results:** The complete dataset contained 2420 distinct proteins. Unsupervised cluster analysis showed that protein expression profiles clustered according to transformation stage. Comparison of protein expression profiles at the different stages of transformation using statistics (beta-binomial model) yielded different sets of differential proteins. For the comparison 'mortal vs. immortal cells' 400 proteins were regulated (170 up in immortal cells and 230 down), for the comparison 'immortal vs. anchorage independent growth' 358 proteins were significantly regulated (158 up in anchorage-independent cells and 200 down) and finally in the multi-group comparison 560 proteins were significantly regulated. Known progression markers that were highly up-regulated across passages were identified (eg., TOP2A, Ki-67), validating our approach.

To investigate whether proteins with similar expression profiles reflect changes in specific cellular processes during transformation we performed cluster analysis of expression profiles (K-means clustering) in conjunction with gene ontology mining and pathway analysis of common expression profiles. In total 16 common protein expression profiles were obtained. Two upward profiles with most relevance for stratification of hrHPV-positive women (i.e. mortal vs immortal) were obtained. Ingenuity pathway analysis revealed association of these profiles with 'DNA Replication, Recombination, and Repair, Cell Cycle, Cellular Compromise' and 'Molecular Transport, RNA trafficking, Nervous System Development and Function'. In line with Ingenuity findings, FatiGO analysis associated the first upward regulation profile with 'Regulation DNA replication, Chromosome Organisation' and the second profile with 'Chromatin Remodelling'.

**Conclusions and outlook:** Proteins showing significantly differential expression during hrHPV-induced transformation, in particular proteins clustered in the 2 upward expression profiles discriminating pre-immortal from immortal passages, provide candidate biomarkers that may discriminate transient hrHPV infections from so-called transforming infection having an increased risk of cancer development. These candidate protein biomarkers will be followed up by immunostaining of cervical tissue specimens, representing the full spectrum of disease (normal to cancer) and cervical scrapings. .

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

## AML proteomics

### Identification of protein profiles of the bone marrow microenvironment associated with apoptosis-resistance of acute myeloid leukemia

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**Background** Both expression of intracellular apoptosis-related proteins and multiple apoptosis gene expression patterns of leukemic cells of patients at diagnosis treated at the department of Hematology were found to correlate with frequency of Minimal Residual Disease and poor prognosis. The predictive strength of the balance between pro-apoptotic and anti-apoptotic proteins appeared to be merely ascribed to anti-apoptotic members. It was found that external cellular or secreted microenvironmental factors did regulate the apoptosis-profile of leukemic cells, as supernatants of apoptosis-resistant leukemic cells were able to induce Bcl2 expression in apoptosis sensitive cells. In order to obtain insight in microenvironmentally induced apoptosis resistance, we planned to perform sensitive and quantitative identification of proteins secreted by leukemic cells. However, bone marrow plasma of AML patients is a complex protein mixture spanning a broad dynamic range of concentrations, which makes it difficult to analyse and identify low abundant proteins and peptides. In order to overcome this, emerging proteomics strategies focus on cancer cell and tissue-secreted fluid fractions to enrich for relevant proteins and peptides (Jimenez et al., 2009; Piersma et al., 2010).

**Aim** Comparative protein profiling of the secretome of apoptosis-resistant and apoptosis-sensitive primary leukemic cells in order to identify protein profiles associated with apoptosis resistance.

**Results** Proteomic analysis of secretome of apoptosis resistant (n=5) and apoptosis sensitive (n=6) primary leukemic blast was performed. In these secretomes 1492 proteins were identified: with 40 proteins significantly upregulated in secretomes of apoptosis sensitive blasts and 208 upregulated in apoptosis resistant blast (p<0,05). Several proteins involved in apoptosis were identified (eg., Pycard, Diablo, Card8, Anamorsin). Interestingly, unbiased analysis using the pathway analysis tool Ingenuity showed that the most significant protein network obtained for the proteins in the resistant secretomes were associated with 'DNA Replication, Recombination, and Repair', 'RNA Post-Transcriptional Modification' and 'Cancer'. In addition, the top pathways were all associated with splicing functions (Exon junction complex, Spliceosome complex, C complex spliceosome). Indeed, aberrant RNA splicing have been documented in cancer cells, leading to aberrant mRNAs and their encoded proteins. What is completely novel here is the unexpected release of entire splicing complexes by resistant AML blasts, possibly via exosomes (unpublished data crj). There is increasing evidence showing that abnormal RNA splicing has functional consequences for cancer cells, involved in therapy resistance. For example, 2 splicing factors that are upregulated in resistant AML secretomes are known to be involved in alternative splicing of Bcl-x and may yield anti-apoptotic Bcl forms.

**Future studies** We will investigate the functional consequences of increased splicing enzyme excretion in conditioned media of resistant AML cells. Furthermore, we will investigate whether splicing proteins are secreted via exosomes and whether exosomes from resistant AML cells can confer resistance to sensitive AML cells. Subsequently, the identified apoptosis resistance-associated proteins will be searched for in bone marrow plasma of AML patients to determine whether the presence of these proteins is indeed associated with an apoptosis resistant profile of the leukemic cells at diagnosis. The impact of these factors on clinical outcome will be established in a large set of biobanked samples of bone marrow cells at diagnosis.

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

## High-throughput body fluid peptide profiling by MALDI mass spectrometry for patient stratification by pattern diagnostics

Mass spectrometry can be used to generate diagnostic peptide peak profiles "signatures" of serum samples. Peak profiles can be used to compare different sera and correlate samples (i.e., patient groups) with clinical data to assist in diagnosis, monitoring, and/or prediction. Indeed, in recent studies, we and other researchers have successfully combined serum peptide profiling by mass spectrometry (MS) with bioinformatics and have established distinctive serum polypeptide MS patterns that correlate with cancer types and clinically relevant outcomes (eg., Voortman, Pham, Jimenez et al., Proteome Sci, 2009). Moreover, emerging evidence shows a direct link between the peptide marker profiles of cancer and differential protease activity which suggests that the patterns may have clinical utility as surrogate markers for detection and classification of cancer.

**Book chapter:** Knol, JC. and Jimenez, CR. MALDI-TOF Serum Profiling Using Semi-Automated Serum Peptide Capture With Magnetic Reversed Phase (C18) Beads. In: 'Nanoproteomics: Methods and Protocols'. Humana Press. Methods Mol Biol. 2011;790:3-16. 2011.

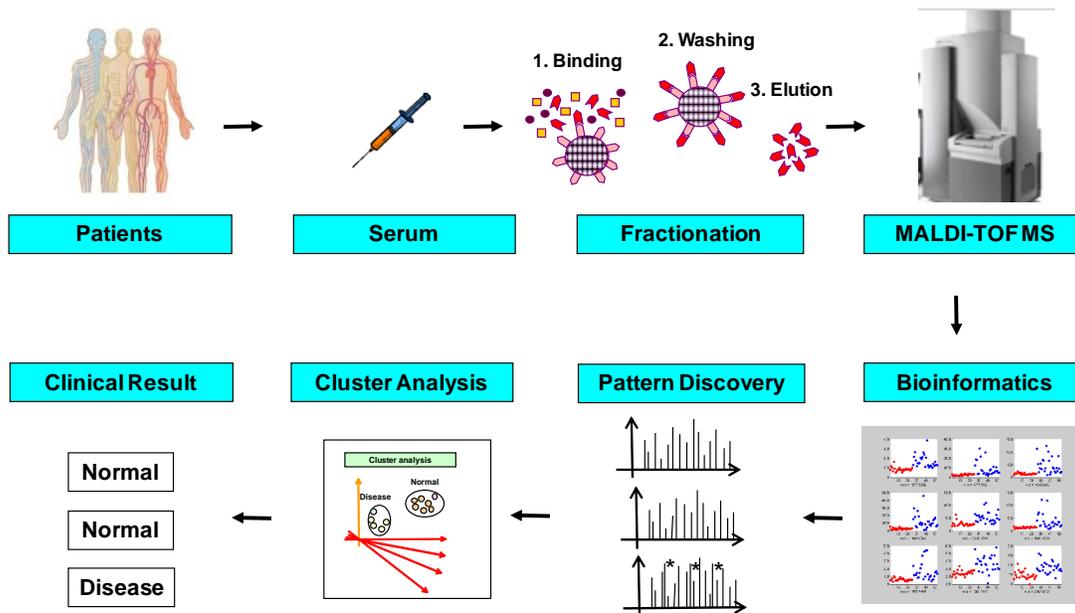


Figure High-throughput serum peptide profiling by MALDI-TO-MS for pattern analysis.

## Mass spectrometric serum peptide profiling for drug response prediction: signature validation

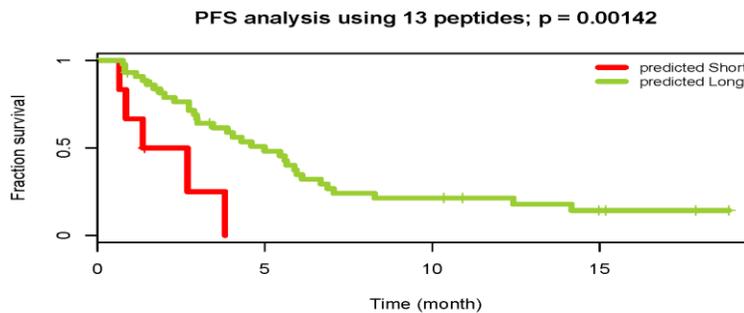
T.V. Pham<sup>1</sup>, J.C. Knol<sup>1</sup>, J.S.W. Lind<sup>2</sup>, H.M.W. Verheul<sup>1</sup>, E.F. Smit<sup>2\*</sup>, C.R. Jimenez<sup>1\*</sup>

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**Introduction** Serum peptide mass profiles of 27 non-small cell lung cancer (NSCLC) patients treated with cisplatin-gemcitabine chemotherapy and bortezomib were analyzed with a support vector machine algorithm to predict progression-free survival (PFS). Based on differential pre-treatment peptide profiles and dynamic changes in peptide abundance during treatment, two classifiers were trained, one using a 6-peptide signature and the other using a 13-peptide signature. The leave-one-out cross validation accuracies were 82% and 86% respectively on the training set (Voortman et al., Proteome Science, 2009). The aim of the current study is to evaluate the performance of the two classifiers on a completely independent dataset.

**Methods** Pre-treatment sera of 50 patients treated with erlotinib and sorafenib for advanced NSCLC (See also below) were collected and analyzed using magnetic bead-assisted serum peptide capture coupled to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Each sample was classified into “short PFS” and “long PFS” group according to the two classifiers. Subsequently survival analysis was performed. In addition, the classification result is tested for correlation with the EGFR and KRAS mutation status.

**Results** The result shows significant prognosis power for progression free survival and overall survival on the independent test set (see the figure for the result of the 13-peptide classifier). The classification based on serum peptide profiling is not significantly correlated with either EGFR or KRAS mutation status.



**Conclusions** This study shows that high throughput MALDI-TOF-MS serum peptide profiling is reproducible and able to classify for drug response in a clinical setting.

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

## Serum peptide profiling in non-small cell lung cancer patients treated with sorafenib and erlotinib: signature discovery

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Departments of <sup>1</sup>Medical Oncology and <sup>2</sup>Pulmonary Diseases VU University Medical Center Amsterdam, <sup>3</sup>Maastricht University Medical Center and <sup>4</sup>University Medical Center Groningen \**Shared corresponding authors*

**Background** The activity of vascular endothelial growth factor (VEGF) and the epidermal growth factor receptor (EGFR) inhibitors has been established in advanced non-small cell lung cancer (NSCLC). However, only a minority of patients with advanced non-small cell lung cancer (NSCLC) benefit from these new anti-cancer drugs. Serum peptide profiling by mass spectrometry is an emerging approach for disease diagnosis and biomarker discovery (Gilloly et al., 2007). Previously, we have established an automated magnetic bead-based method for off-line serum peptide capture coupled to MALDI-TOF mass spectrometry (Jimenez et al., 2007). Here we apply this method to the analysis of sera collected in a phase II study from NSCLC patients treated with sorafenib and erlotinib.

**Aim** Serum peptide profiling of patients treated with sorafenib (a VEGFR inhibitor) and erlotinib (an EGFR inhibitor) to discover peptide patterns associated with treatment outcome.

**Approach** Using magnetic bead-assisted serum peptide capture coupled to matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF/TOF-MS), serum peptide mass profiles (spectra) of 50 NSCLC patients will be obtained (pretreatment, after 7 days and after 21 days of treatment). Algorithms will be established to classify for tumor response, progression-free survival and overall survival.

**Results** Serum samples have successfully been obtained from 50 patients and measured by MALDI-TOF/TOF mass spectrometry. Support vector machine learning is currently applied for the discovery of predictive/ prognostic signatures. A 13-peptide signature leads to classification with 82% leave-one-out cross validation accuracy in a subset of 28 pre-treatment samples. The addition of differential time course peptides does not improve classification accuracy on this dataset. The classification of the 22 pre-treatment samples not used in the training procedure shows significant prognostic power in overall survival analysis.

**Outlook** We hope to assess whether (time-course) serum peptidome profiling using MALDI-TOF-MS coupled to pattern diagnostics may aid in the prediction of treatment outcome of NSCLC patients treated with VEGF and EGFR inhibitors, potentially enabling patient selection.

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

## Mining high dimensional proteomics data

*Data mining, sub-project 1*

### **On the beta binomial model for comparative analysis of spectral count data in label-free tandem mass spectrometry-based proteomics**

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**BACKGROUND** Spectral count data generated from label-free tandem mass spectrometry-based proteomic experiments can be used to quantify protein's abundances. Comparing spectral count data from different sample groups such as control and disease is an essential step in statistical analysis for the determination of altered protein level and biomarker discovery. However, tools for statistical analysis of this type of data are still immature. Our initial experiments in two cancer studies show that the current methods are unable to declare at 95% confidence level a number of protein markers that have been judged to be differential on the basis of the biology of the disease and the spectral count numbers.

**AIM** Preliminary investigation indicates that the current approaches do not take into account within-sample and between-sample variations together. Hence, our aim is to improve upon existing techniques by incorporating both the within-sample and between-sample variations into a single statistical model.

**APPROACH** We propose to use the beta-binomial distribution to model spectral count data. The variability is modeled in two directions. One is the variability within single sample with a binomial distribution, similar to the assumption employed in the *G*-test of independence. The other is the group variability in which the parameter of the binomial distribution is modeled by a beta distribution. Finally, the likelihood ratio test is employed for hypothesis testing as in the case of the *G*-test.

**RESULTS** The beta-binomial test can be applied for experiments with one or more replicates, and for multiple condition comparisons. Experimental results show that it performs favorably in comparison with other methods on several datasets in terms of both true detection rate and false positive rate. The figure below shows the results of the proposed test and four other tests on a standard test set. Six proteins were spiked in with two fold differences. The beta-binomial test has the lowest false positive rate in four cases.

**OUTLOOK** A consolidated version of the software for the beta-binomial test is available for download (<http://www.oncoproteomics.nl/index.php/resources#software>) so that other researchers can easily use the tool for comparative analysis of spectral count data.

#### **Reference**

Pham TV, Piersma SR, Warmoes M and Jimenez CR. On the beta-binomial model for analysis of spectral count data in label-free tandem mass spectrometry-based proteomics. *Bioinformatics*. **26**: 363-369 (2010).

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

## Strategies for analyzing spectral count data in label-free tandem mass spectrometry-based proteomics

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**Background** Label-free strategies for quantitative proteomics provide a versatile and economical alternative to labeling-based proteomics strategies. We have shown for different types of biological samples obtained via relatively simple and more complex workflows using analysis of technical and biological replicates (cancer cell secretome, Piersma et al., *Journal of Proteome Research* 2010; subnuclear fractions isolated from clinical material, Albrethsen et al., *Molecular and Cellular Proteomics* 2010; depleted cerebrospinal fluid, Fratantoni et al., *Proteomics Clinical Applications* 2010) that spectral counting-based label-free quantitation is a promising avenue for biomarker discovery. Analyzing spectral count data generated from these studies is however not straightforward as commonly used techniques for genomics data analysis are not suitable.

**Methods** We employ the beta-binomial distribution for significance analysis of independent samples, which integrates both within-sample variation and between-sample variation into a single statistical model (Pham et al., *Bioinformatics* 2010). For cluster analysis we devise a novel distance measure between samples based on the Jeffrey divergence. This measure prevents highly abundant proteins from dominating others in contribution to the total sample difference. Finally, a new test also based on the beta-binomial distribution has been developed for significance analysis of paired samples (manuscript in preparation).

**Results & Conclusions** We showed that the beta-binomial test performs favorably in comparison with other methods on several datasets in terms of both true detection rate and false positive rate. In addition, it can be applied for experiments with one or more replicates, and for multiple condition comparisons. We showed the quality of the new distance measure for clustering. Finally, we demonstrated the usefulness of our strategies in the identification of several potential biomarkers in the secretomes of mouse embryonic fibroblasts, the enrichment of brain derived proteins in the depleted cerebrospinal fluid fraction, and the identification of proteins enriched in the nuclear matrix fraction and regulation in adenoma to carcinoma progression in colorectal cancer.

**References:** Pham TV and Jimenez CR. Strategies for analyzing spectral count data in label-free tandem mass spectrometry-based proteomics. *Bioinformatics*. 2012, accepted manuscript.

**Book chapter:** Pham TV, Piersma SR, Warmoes M, Jimenez CR. Statistical analysis of spectral count data in label-free tandem mass spectrometry-based proteomics. *Springer Protocols. Neuroproteomics* issue (2011).

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

## Network-based data analysis for marker prioritization for targeted mass spectrometry validation

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**Background** Much progress has been made in several biomarker studies associating genes to a wide range of diseases. However, there have been few successes in new therapeutic strategies mostly because the mechanisms underlying the diseases are not understood to a large extent. Vidal et al. (Cell, 2011) have convincingly argued that the so-called “one-gene/one-enzyme/one-function” concept is insufficient to explain the complexity of the biological system. Most cellular components exert their functions through interactions with other cellular components. Complex diseases like cancer reflect perturbations in multiple intracellular and intercellular networks that link cells and tissues into organ systems. Thus, one needs to take on the challenge of analyzing the molecular network taking place within cells for a better understanding.

**Aim** This project aims to establish a robust pipeline with principled bioinformatics and statistical methods for network-based cancer data analysis.

**Approach** We will adopt existing tools for enrichment analysis using established databases like GO and KEGG. Network visualization and overlaying of quantitative genomics data will be facilitated by Cytoscape and related software plugins. Subsequently, we will develop advanced analysis methods in a stepwise approach with an increasing order of difficulty. First, we perform a global analysis incorporating existing network knowledge to detect the most interesting pathways exhibiting differential expression patterns between two experimental condition like normal-cancer, or untreated-treated. Secondly, we assess whether there is any differential interaction in the pathway. We identify gene pairs differentially correlated between clinical subgroups, while incorporating the expression of other genes in the pathway as confounders into analysis. Finally, we model the regulatory network in each clinical group explicitly and compare the networks. Such comparison should pinpoint hub genes and sub-networks discriminating the clinical groups. We will experiment with model datasets based on genetic mouse models of BRCA1 deficient breast cancer, an APC mutant colon cancer model, a HPV progression model. For all models proteomics and transcriptomics data are available as well as other OMICS data. In addition we will apply the tool to the analysis of clinical material (including OMICS analyses on-going on the context of the CTMM DeCoDe and Airforce projects).

**Results** We have implemented a simple algorithm based on a network clustering algorithm available in Cytoscape. We analyzed a colon cancer dataset reported in (Albrethsen et al. 2010). Out of 200 sub-networks, 15 are significantly different between colon adenomas and carcinomas. Initial manual examination reveals interesting candidates in the context of both colon cancer and treatment drug.

**Outlook** The result of the network-based analysis will help identifying important protein candidates (regulators) as well as important relationships relating to different input conditions for further targeted validation.

*This research was supported by Avanti.*

## Computational analysis of phosphoproteomics data

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**Background** Phosphoproteomics profiling is a method to identify aberrant signaling pathways in cancer cells and tissues. Phosphoproteomics can be used to identify candidate drivers of cancer progression. 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 <sup>1)</sup> the low intensity of phosphorylated peptides detected by LC-MS/MS as compared to unmodified peptides and the lability of phosphopeptides, because <sup>2)</sup> phosphorylation site localization algorithms are not perfect and because of <sup>3)</sup> the complicated dynamic biochemical process of phosphorylation and dephosphorylation and its feedback 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 phosphopeptide 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 phosphoproteomics); 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 phosphopeptide 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.

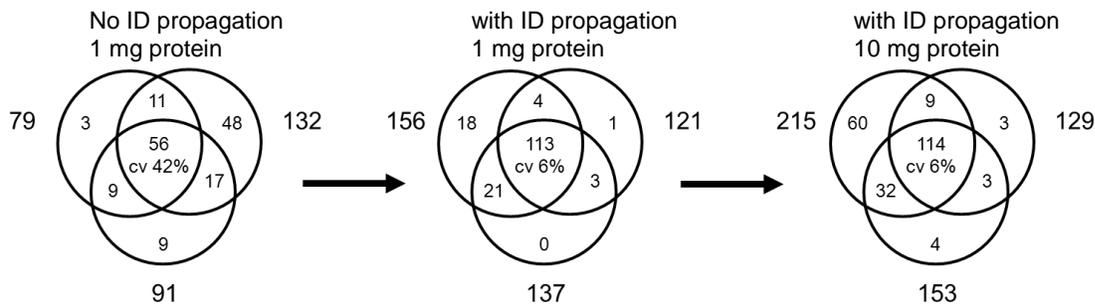
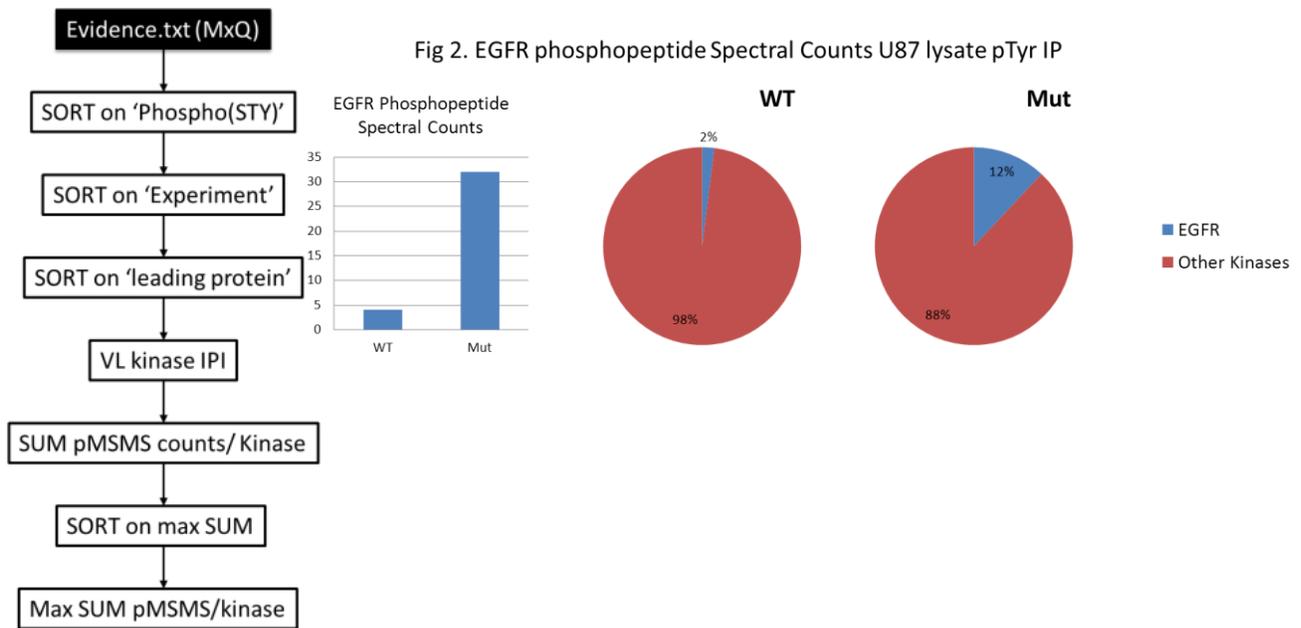


Fig. 1. Effect of ID-propagation on low level pTyr phosphopeptide identification and quantification. Triplicate analysis of phosphopeptide IDs from 1 mg protein of a cancer cell line lysate shows that the overlap of identified peptides increases when the peptide identities are propagated from runs with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

### Phosphopeptide MS/MS spectral counting to identify candidate driver kinases

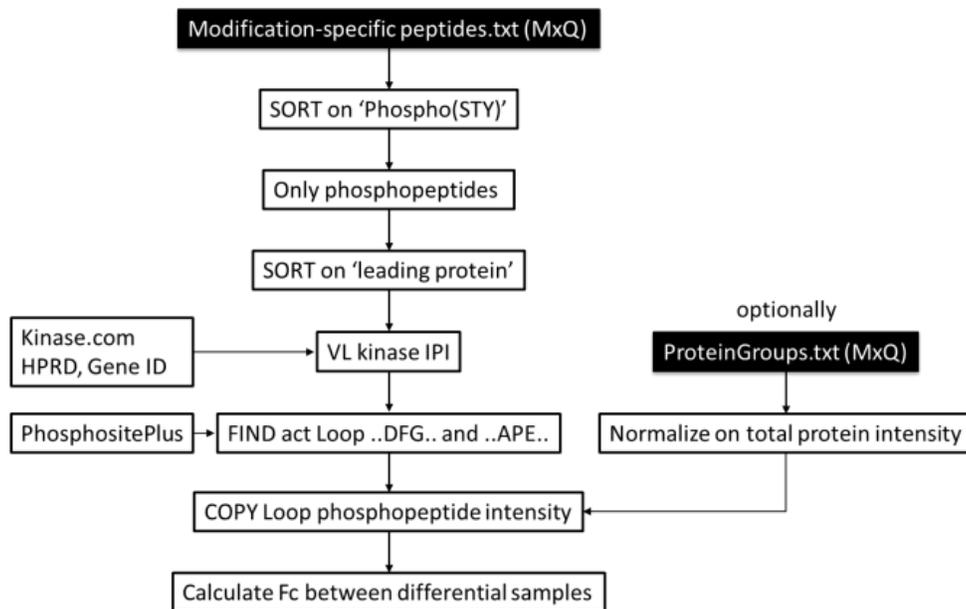
In Figure 2 the workflow for extraction of phosphopeptide MS/MS counts from MaxQuant Evidence.txt output is shown. Several SORT, COPY and SUM steps are required to extract the relevant intensity values from the Evidence.txt file as well as an ID mapping step to extract protein kinases from the file. 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. A pie chart and bar graph representation is convenient for identification of hyperphosphorylated protein kinases: see example phosphopeptide spectral counts for EGFR WT vs  $\Delta$ VIII 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.txt 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.

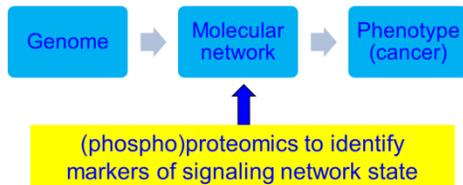
Fig 3. Data analysis workflow for Protein kinase activation loop phosphorylation analysis



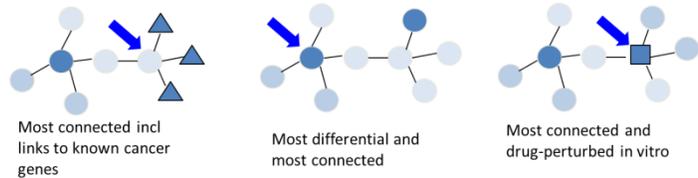
### Signaling network analysis to prioritize candidate driver/activated kinases

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.

Conceptual framework  
integrated cancer genomics and proteomics



SCENARIOS



### 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.

*This research was supported by Avanti*

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## **Other core/ collaborator abstracts**

# Neuroproteomics

## Introduction

Neurodegenerative diseases such as Alzheimer's and Parkinson's disease may represent one of the most important issues for public health in the coming years, due to the increase in life expectancy of general population. Both of these diseases are characterized by a long pathogenetic course in which the neurodegenerative processes start several years before the appearance of the first symptoms. The treatment options for these disease are still few and not effective in advanced stages due to the irreversibility of neuronal damage. Considering this, the focus should be in the early detection of these diseases to ensure that the treatment options may be administered before the neuronal damage is too large to be rescued.

To this purpose, cerebrospinal fluid (CSF) represents a suitable sample for proteomic biomarker discovery in neurodegenerative diseases, reflecting pathological changes occurring in the central nervous system structures.

The Oncoproteomics laboratory has been active in the last 2 years in three main fields related to the application of proteomics for early diagnosis of neurodegenerative diseases:

1. CSF proteomics for early detection of Alzheimer's disease (clinical NEUro PROteomics for neurodegenerative diseases, cNeuPRO project, 6<sup>th</sup> European Framework funded)
2. CSF proteomics for biomarker discovery in MENdelian Forms of Parkinson's disease (MEFOPA, project, 7<sup>th</sup> European Framework funded)
3. Proteomic analysis of CSF-derived microvesicles (MEFOPA, project, 7<sup>th</sup> European Framework funded)

Besides these 3 projects, the OPL participates in several collaborative neuroproteomics projects with the VUmc departments of Clinical Chemistry, Neurology and Anatomy and Neurosciences as well as the AMC. See the neuroproteomics abstracts for further details on running projects.

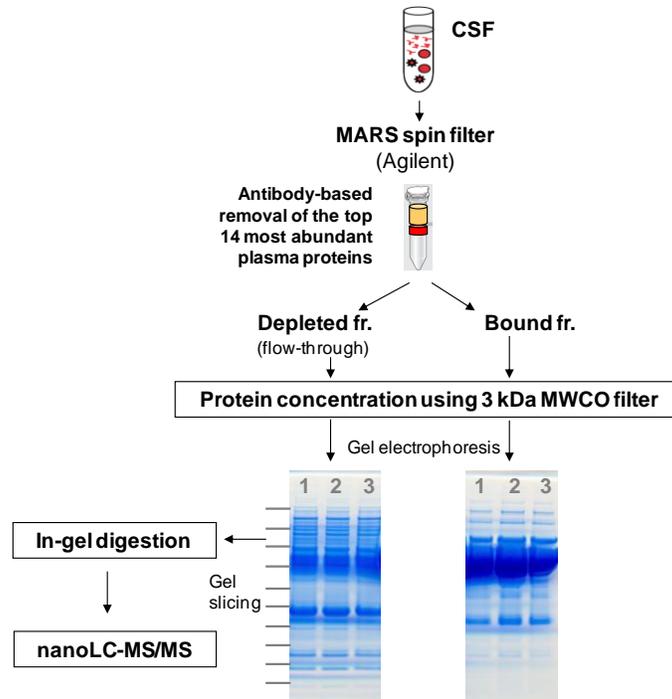


Figure. Abundant protein depletion sample pretreatment method for in-depth analysis of the cerebrospinal fluid proteome and biomarker discovery (Figure from: Fratantoni and Jimenez, Springer Protocols. Neuroproteomics issue. 2010)

## In-depth analysis of the cerebrospinal fluid proteome for biomarker discovery: abundant protein depletion sample pretreatment method

Silvina A. Fratantoni, Sander R. Piersma and Connie R. Jimenez

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**Background:** Cerebrospinal fluid (CSF) contains peptides and proteins important for brain physiology and potentially also relevant to brain pathology. Therefore, CSF provides an attractive source for biomarker discovery in brain cancer and neurological diseases. CSF proteomics provides an analytical challenge as approximately 80% of proteins originate from serum, and removal of these major proteins is necessary to study brain-derived proteins and disease biomarkers that are present at low concentrations.

**Aim:** To compare CSF sample pretreatment methods to allow for in-depth and comparative analysis of the CSF proteome.

**Approach:** Evaluation of 2 protocols for batch-mode abundant protein depletion, ie., the Multiple Affinity Removal System MARS cartridge (Agilent) and Seppro-IgY Supermix

**Results and conclusions:** In this study, we compared the performance of two multi-affinity depletion methods in spin filter format: MARS Human 14 and Seppro-IgY Supermix. MARS and IgY spin filters yielded comparable reproducibility of protein identification (71%-74%) and quantification (17%-18%) but a significant difference in the total number of identified CSF proteins (767 and 703 proteins, respectively). Therefore, the MARS spin filter provides a significantly better and more sensitive analysis.

**Outlook:** For biomarker discovery in cerebrospinal fluid, removal of major serum proteins is necessary. Abundant protein depletion using the MARS spin filter allows for detection of brain-derived proteins and provides the most sensitive approach. Future studies will apply this method to biomarker discovery in neurological disease and brain cancer.

### References

Fratantoni, SA., Piersma, SR., **Jimenez, CR.** Comparison of the performance of two affinity depletion spin filters for quantitative proteomics of cerebrospinal fluid: Evaluation of sensitivity and reproducibility of CSF analysis using GeLC-MS/MS and spectral counting. *Prot. Clin. Applic.* 2010, 4, 613-617.

Fratantoni, S. and **Jimenez, CR.** Abundant protein depletion sample pretreatment method for in-depth analysis of the cerebrospinal fluid proteome and biomarker discovery. *Springer Protocols. Neuroproteomics issue.* 2010.

*This research was supported by the EU cNeuPRO project*



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

## CSF proteomics reveals novel biomarker BRI2 that is increased in amyloid plaques in Alzheimer's disease

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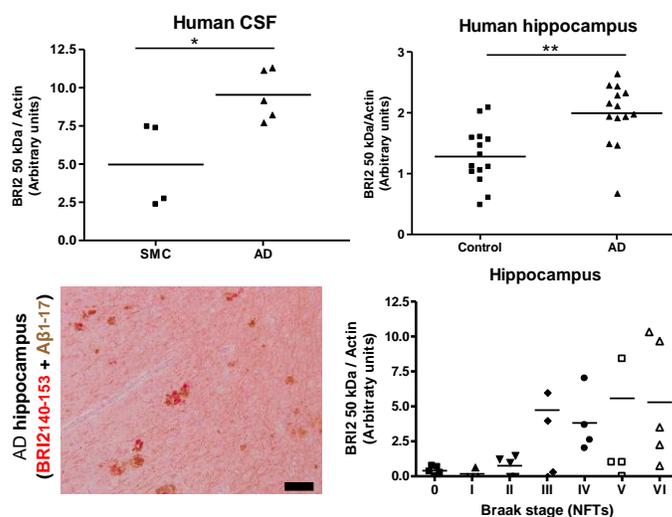
Departments of Clinical Chemistry and Neurological Laboratory<sup>1</sup>, Alzheimer Center<sup>2</sup>, Pathology<sup>3</sup>, Medical Oncology and OncoProteomics Laboratory<sup>4</sup>, VU University Medical Center, Amsterdam, The Netherlands. Department of Neuropathology<sup>5</sup>, Heinrich Heine University Medical School, Düsseldorf, Germany.

**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. These data suggest that BRI2 is a promising biomarker for early diagnosis and that it could be modified in early stages of AD. As far as we know, the relationship of BRI2 within human AD pathology is not known.

The aim of this study was therefore, to analyze 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** A significant increase of extracellular BRI2 deposition was observed in AD hippocampus human tissue compared to controls. Moreover, extracellular BRI2 staining was co-localized with approximately 50% of all the amyloid fibrillar plaques but not with diffuse or dense-core plaques. Extracellular BRI2 deposition starts already in at Braak stage 3. Western blot analysis also showed a significant increase of a BRI2-specific band at 50 kDa.



**Conclusions** Our data show that BRI2 levels are increased in AD patients, supporting the potential utility of BRI2 as an AD biomarker. Furthermore, BRI2 partly co-localized with fibrillar plaques, suggesting an important involvement of BRI2 in AD pathology. Confirmation of this hypothesis may open new insights in AD aetiology which would allow the development of new preventive and disease-modifying therapies

## CSF biomarkers for Parkinson's disease patients with LRRK2 mutations

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**Background** Parkinson's disease (PD), one of the most common neurodegenerative disorders, is characterized by neuronal degeneration of substantia nigra-pars compacta with motor and cognitive symptoms. Studying sporadic PD prior to the onset of clinical symptoms is impractical, making early intervention challenging. Autosomal dominant mutations in the leucine-rich repeat kinase (LRRK2) gene, the most common known genetic cause of parkinsonism, result in a clinical phenotype similar to sporadic PD. LRRK2 mutation carriers have been extensively characterized by PET studies, demonstrating neurochemical changes similar to sporadic PD cases, as well as detectable dopaminergic dysfunction in asymptomatic carriers. Thus, subjects with LRRK2 mutations constitute an excellent cohort for studying preclinical and early PD.

**Aim** The aim of this project is to identify novel early detection markers for PD patients with LRRK2 mutations and to obtain insight into the pathological role of LRRK2.

**Approach** Comparative CSF proteomics of asymptomatic and symptomatic PD patients carrying mutations in LRRK2. As an extra reference CSF of sporadic PD and controls will be included as well.

**Results** During 2011, high quality CSF samples for proteomic analysis have been collected in two European centres, Tübingen University Hospital and Oslo University Hospital. We have currently available CSF from 7 symptomatic patients carrying LRRK2 G2019S mutation, 3 CSF samples from asymptomatic patients, 20 CSF from idiopathic PD patients with no mutation on LRRK2, and 17 healthy controls. The four groups will be compared using our in-depth proteomic approach for biofluid biomarker discovery, which includes depletion of high abundant proteins, SDS-PAGE, protein identification on a Q-Exactive mass spectrometer and label-free protein quantification.

**Acknowledgements:** This research was supported by the European FP7 project: MEFOPA

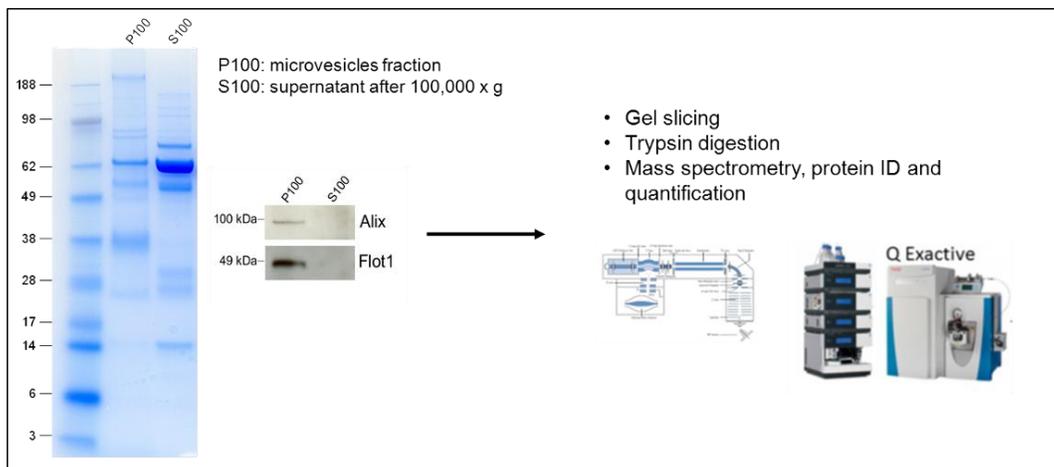
## Proteomic analysis of CSF-derived microvesicles

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**Background:** Extracellular microvesicles (eMVs) are a class of membrane bound organelles secreted by various cell types. eMVs include (i) exosomes: 40-100 nm diameter membraneous vesicles of endocytic origin (ii) ectosomes (also referred to as shedding microvesicles, SMVs): large membranous vesicles (50-1000 nm diameter) that are shed directly from the plasma membrane (PM) and (iii) apoptotic blebs (50-5000 nm diameter): released by dying cells. Recently eMVs have been investigated as a new tool to discover biomarkers for different diseases, including neurodegenerative disorders.

**Aim:** The aim of this project was develop an optimal protocol for isolation of microvesicles from cerebrospinal fluid (CSF) to verify the presence of these organelles and assess if they can be used as a new biomarker discovery tool.



**Approach:** Differential centrifugation was used to obtain a preparation of microvesicles from pooled samples of CSF (6-12 mL). Proteins extracted from 100,000 x g (P100) centrifugation pellet (P100) which correspond to enriched microvesicles, were separated with SDS-PAGE and compared with those of total CSF (S100) and pellet coming from 20,000 x g (P20) centrifugation to analyse the quantitative enrichment of proteins in the P100 fraction.

**Results:** Mass spectrometry identified around ~ 2000 proteins in the three fractions, evidencing enrichment in several exosomal markers in our microvesicle preparation. Principal biological function of the proteins enriched in the microvesicles preparation were related to response to wounding, axonogenesis, glycosaminoglycan metabolic process, response to calcium, while cellular localization analysis evidenced an enrichment in extracellular proteins and cytoplasmic membrane-bound vesicle terms.

**Conclusions and outlook:** marker profiles and biological function analysis showed that our preparation is enriched in microvesicles. These results demonstrate how the proteome of microvesicles in CSF can be analysed and used as new instrument for neurodegenerative diseases biomarker discovery.

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

## Proteomics discovery of novel CSF biomarkers for Multiple Sclerosis

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**Background** Clinical disease progression is the cause of major disability in MS and is irreversible. Disease progression in MS cannot be prevented or cured yet and is difficult to predict. The biological correlate of disease progression is axonal damage. Body fluid biomarkers are potentially valuable for predicting disease progression and for monitoring of therapy responsiveness in MS patients. CSF proteomics provides a promising opportunity for in-depth analysis of the CSF proteome and to obtain protein biomarkers.

**Aim** The aim of the current study is to identify novel candidate biomarkers for disease progression of MS, using the recently optimised in-depth nano-LC-MS/MS proteomics method.

**Results** We analyzed well-defined and age and gender stratified CSF samples including CSF of 5 relapse-onset MS patients that had radiologically confirmed severe disease progression at follow-up, 5 relapse-onset MS patients with radiologically confirmed stable disease at follow-up, 5 non-inflammatory controls and 5 inflammatory controls. After abundant protein depletion and proteomics analysis by gel-nanoLC-MS/MS analysis a dataset of 924 CSF proteins was obtained. Supervised cluster analysis of the data revealed 24 proteins that could discriminate OND patients from the MS patients. Among these proteins were inflammatory and cell adhesion proteins. 16 proteins were differentially regulated between RRMS and SPMS, including HLA-1 and neuron-specific enzymes.

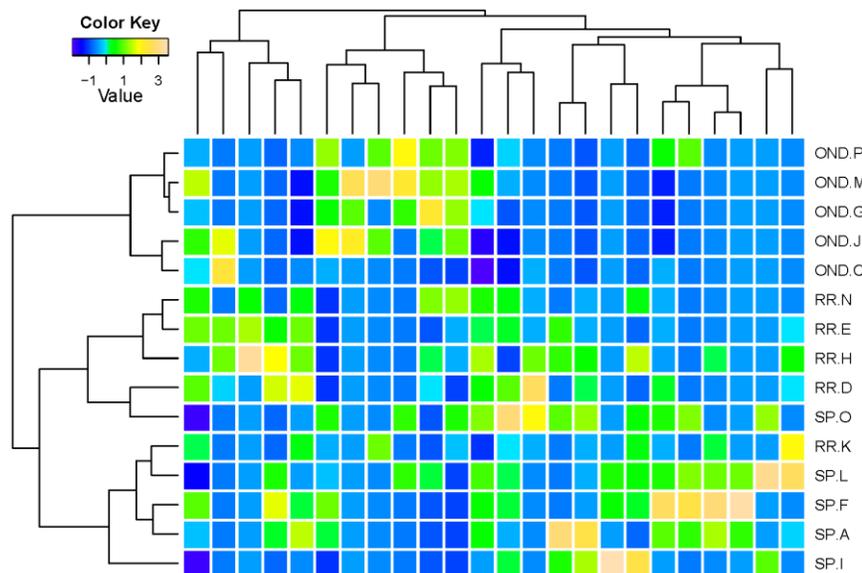


Figure Supervised cluster analysis. Heat map view of differential CSF proteins (3-group comparison: 24 proteins). Differential proteins are largely clustered according to group label

**Conclusions and outlook** We identified CSF proteins that can discriminate different MS disease groups. A multiplex targeted mass spectrometry assay is currently being developed in the laboratory of F. Berven to enable validation in a large cohort.

## Proteomic analysis of the locus ceruleus in Parkinson's disease

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**BACKGROUND** The locus ceruleus is among the earliest affected brain regions in Parkinson's disease (PD) showing Lewy body pathology and neuronal loss.

**AIM and APPROACH** To improve our understanding of the pathogenesis of PD, we performed a proteomic analysis of post-mortem locus ceruleus tissue of six pathologically confirmed PD patients, and six age- and gender-matched non-neurological controls. Post-mortem locus ceruleus tissue was excised and homogenized in sample buffer and proteins were fractionated using 1D-gel-electrophoresis followed by in-gel tryptic digestion and nanoLC-FTMS. Databases were used for peptide and protein identification. Locus ceruleus proteomes of PD patients were compared to controls by the number of spectral counts using a beta-binomial model.

**RESULTS** In total 2495 proteins were identified, of which 87 proteins were differentially expressed in the locus ceruleus of PD patients compared to controls. The majority of these differentially expressed proteins are known to be involved in processes that have been implicated in the pathogenesis of PD previously, including mitochondrial dysfunction, oxidative stress, protein misfolding, cytoskeleton dysregulation and inflammation. Several individual proteins were identified that have hitherto not been associated with PD, such as regucalcin, which plays a role in maintaining intracellular calcium homeostasis, and isoform 1 of kinectin, which is involved in transport of cellular components along microtubules. In addition, pathway analysis suggests a pathogenetic role for aminoacyl-tRNA-biosynthesis.

**CONCLUSIONS and OUTLOOK** These findings indicate that the proteome of the locus ceruleus of PD patients and non-neurological controls provides data that are relevant to the pathogenesis of PD, reflecting both known and potentially novel pathogenetic pathways. Further evaluation of the biological mechanisms underlying the observed alterations in protein expression levels and how they relate to protein aggregation and neuronal death may ultimately lead to new therapeutic targets and/or novel diagnostic biomarkers for PD.

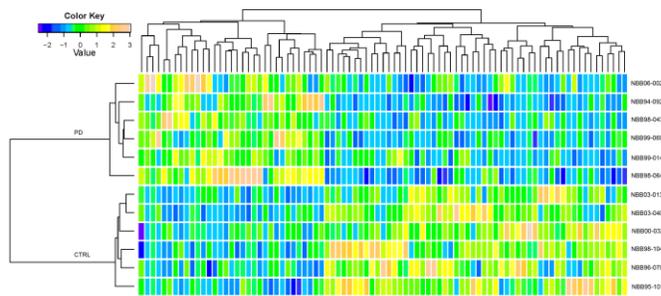


Figure: Heat map view of supervised cluster analysis using the differentially expressed proteins. Supervised clustering of 87 proteins (horizontal axis) that had significantly different expression levels between PD patients (PD) and controls (CTRL; vertical axis), with 33 upregulated and 54 downregulated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

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## Identification of new proteins associated with Cerebral Capillary Amyloid Angiopathy Relevance for amyloid clearance in Alzheimer's disease

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**Background:** Alzheimer's disease (AD) is the most common form of dementia and is characterised by the accumulation of the amyloid  $\beta$  (A $\beta$ ) protein in the cortical areas of the brain, due to a disturbance in the clearance of A $\beta$ . The blood-brain-barrier (BBB) plays an essential role in the transport of A $\beta$  from the brain to the periphery. A disturbance in A $\beta$  clearance across the BBB is observed in AD cases that show deposition of A $\beta$  in and around cortical capillaries, also referred to as capillary cerebral amyloid angiopathy (capCAA). capCAA is observed in 40% of AD cases and is associated the clinical progression of AD. Little is known about the molecular mechanism leading to capCAA in AD pathogenesis.

**Aim** The aim of this study is to identify novel differentially expressed proteins in cases with only capCAA pathology and to obtain new insight into the underlying mechanisms that cause a disturbed clearance of A $\beta$  across the BBB.

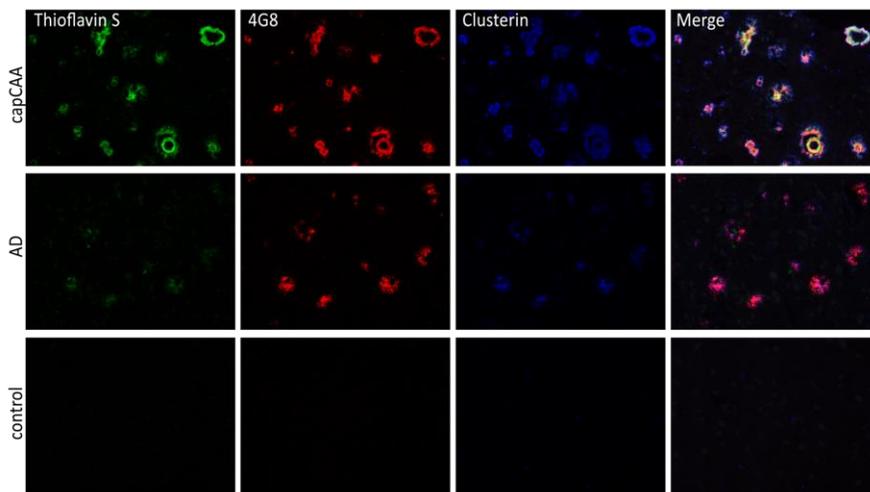
**Approach** We have recently identified clinical AD cases that show extensive capCAA pathology without the common AD hallmarks like A $\beta$  deposits in the parenchyma and neuronal accumulation of hyperphosphorylated tau (neurofibrillary tangles). A proteomics analysis was performed using post-mortem 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 A $\beta$  aggregates. For each condition 2 cases were selected (total of 6). 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** 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- $\beta$  peptides and is involved in the clearance of A $\beta$  peptides and fibrils is localized in A $\beta$ -laden capillaries, (Figure 1A). Clearance of A $\beta$  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.

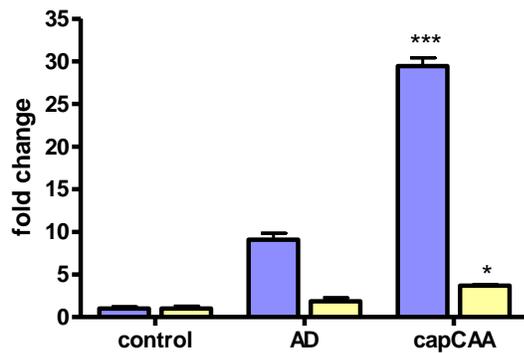
**Outlook** The availability of cases with capCAA only, i.e. without AD-related changes, enables us to identify novel proteins that may play a role in the clearance of A $\beta$  from the brain. These proteins will be further analysed using cell-based bioassays and animal models, to assess their value as either biomarkers or as targets for therapeutic intervention.

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

Figure 1



A. 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.



B Quantification of clusterin

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

## Proteomics analysis of kainic acid-induced changes in the mouse hippocampus reveals microRNAs as regulators of neural stem cell apoptosis

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**Background:** Adult hippocampal neurogenesis (AHN), the birth of new neurons in the adult hippocampus, is a process tightly regulated both by cell proliferation and apoptosis. Alterations in AHN are associated with a variety of neurological disorders including epilepsy. The sharp increase in neurogenic cell numbers observed in young human epilepsy patients can be mimicked in mice by systemic administration of kainic acid (KA). This KA-induced status epilepticus model shows an increase in immature neurons 3 days after treatment.

**Aim and approach:** We hypothesize that the regulation of apoptosis of newborn neurons may be affected during status epilepticus

By means of proteomics, transcriptomics and microRNAomics we investigate differential gene expression after KA-induced status epilepticus and its relationship to induction of AHN.

**Results:** We found several apoptosis related targets and microRNAs to be differentially expressed in the mouse dentate gyrus as a result of KA treatment. Interestingly, while protein levels of some members of the BCL family were decreased, mRNA levels remained unchanged, suggesting a possible role for microRNA regulation. This role was confirmed by a dose-dependent decline in levels of an endogenous BCL target protein upon introducing exogenous microRNA in neural stem cells *in vitro*. Also, this interaction takes place through binding of microRNAs to the 3'UTR of the BCL protein mRNA.

**Conclusion and outlook:** our results suggest that apoptosis of newborn neurons is reduced after KA-induced status epilepticus. This reduction in apoptosis may be induced in part by alterations in the interactions of microRNAs with their mRNA targets. Current research is focused on the functional characterization of these interactions and their contribution to hippocampal neural stem cell apoptosis.

## Identification of biomarkers for diagnosis and progression of multiple sclerosis by MALDI-TOF/mass spectrometry

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<sup>a</sup> NUBIN, Department of Clinical Chemistry and Department of Molecular Cell Biology and Immunology, <sup>b</sup> OncoProteomics Laboratory, Department of Medical Oncology, <sup>c</sup> Department of Neurology d Department of Radiology and Department of Physics and Medical Technology, <sup>e</sup>. Department of Molecular Cell Biology and Immunology, VU University Medical Center Amsterdam; <sup>f</sup>. Department of Neurology, Medical University of Graz, Graz, Austria; <sup>g</sup>. Section of Biochemistry and Clinical Biochemistry, Department of Biochemistry and Molecular Biology, University of Ferrara, via Luigi Borsari 46, Ferrara 44121, Italy

**Background:** Body fluid biomarkers for clinical subtyping, predicting and monitoring of disease progression are of considerable interest in multiple sclerosis (MS). Proteomics tools are optimal for the unbiased simultaneous detection of large series of peptides and proteins.

**Objectives:** To identify novel candidate biomarkers discriminating MS patients from patients with other neurological diseases, and relapsing-remitting (RR) from secondary progressive (SP) MS patients using a high-throughput MALDI-TOF-based mass spectrometry method.

**Methods:** Paired CSF and serum samples of 41 RR, 30 SP, 13 primary progressive (PP) well documented MS patients and 25 patients with non-inflammatory other neurological diseases (OND) were analysed.

**Results:** Out of a total of 100 detected peptides in CSF and 200 peptides in serum, eleven peptides were differentially regulated in serum or CSF comparing MS patients and the OND control group. In addition, eight peptides were differentially regulated in serum or CSF comparing RRMS and SPMS patients. Specific peaks regulated in MS were tentatively identified as fragments of secretogranin III and complement C3. The peak intensity of the CSF peak with m/z value 8607.7 correlated to atrophy ( $r=-0.27$ ,  $P<0.005$ ), black hole volumes ( $r=0.31$ ,  $P<0.008$ ) and total lesion load ( $r=0.34$ ,  $P<0.003$ ). A serum peptide with m/z value of 872.4 that was elevated in SPMS correlated to EDSS ( $r=0.341$ ,  $p<0.005$ ) and atrophy ( $r=-0.286$ ,  $P<0.028$ ).

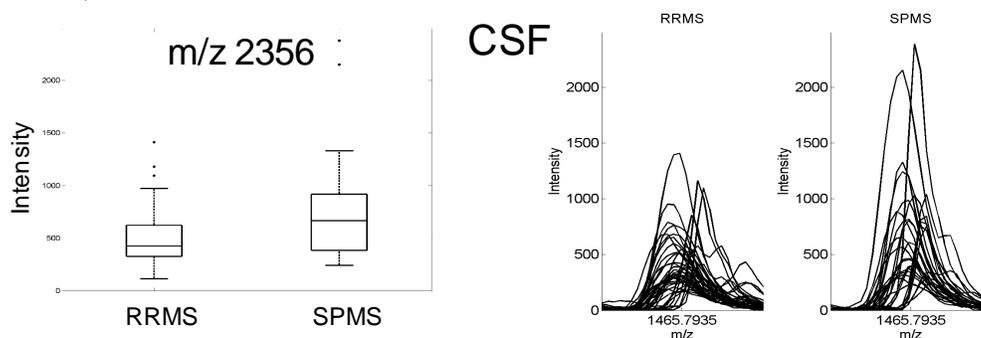


Figure. Example of a peptide with differential levels in the clinical groups

**Conclusions:** Using a high-throughput body fluid profiling by MALDI-TOF Mass spectrometry, small proteins and peptides were identified as promising candidate biomarkers for diagnosis and disease progression of MS.

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## Diagnosis of Alzheimer's disease by mass spectrometric detection of specific amyloid-beta peptide fragments in CSF in combination with pattern analysis.

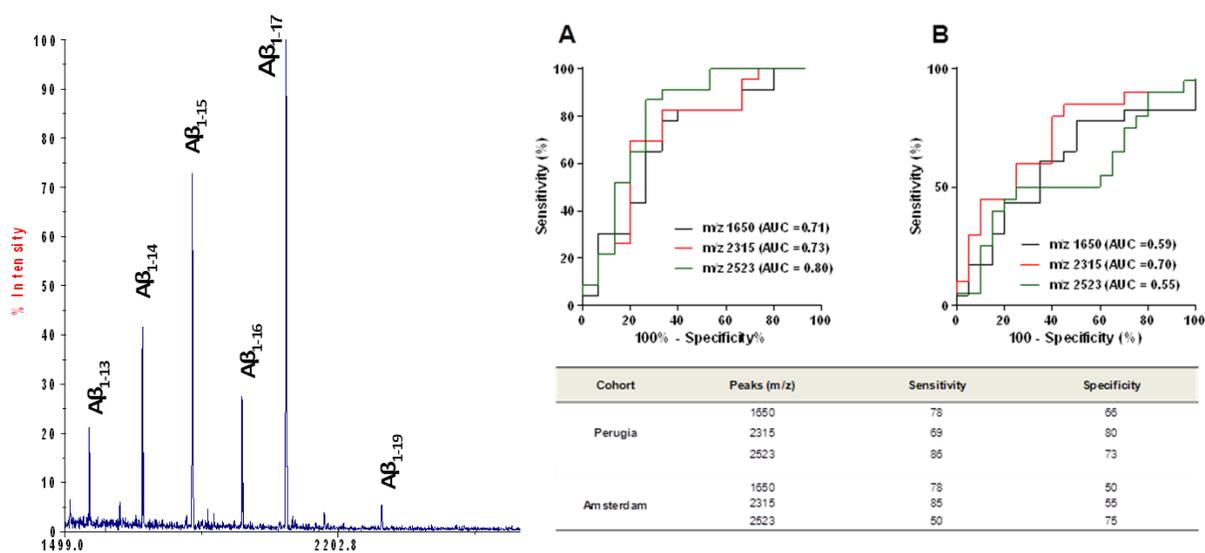
Davide Chasserini<sup>1,2</sup>, Thang V Pham<sup>1</sup>, Jaco C Knol<sup>1</sup>, Lucilla Parnetti<sup>2</sup>, Wiesje M. van der Flier<sup>3</sup>, Philip Scheltens<sup>3</sup>, Charlotte Teunissen<sup>3</sup>, and Connie R. Jimenez<sup>1</sup>

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**Background:** Alzheimer's disease (AD) is the world's most common neurodegenerative disease. A striking pathological feature of AD is the deposition of extracellular plaques, consisting mainly of amyloid beta peptides. One of the most abundant peptides in the plaques is the 42 aminoacid-form of A $\beta$  (A $\beta$ <sub>1-42</sub>). The A $\beta$ <sub>1-42</sub> fragment is generated by sequential cleavage steps of the amyloid precursor protein (APP) by the action of different proteases. A $\beta$ <sub>1-42</sub> in CSF is further degraded by a range of endogenous proteases. However, proteolytic degradation fragments of A $\beta$ <sub>1-42</sub> are difficult to detect using standard methods such as ELISA for the lack of specific antibodies. Mass spectrometry is an emerging technique for the detection and identification of peptide profiles in body fluids and has potential for diagnostic approaches. Some recent studies have shown that a number of A $\beta$  fragments are present in higher concentrations in CSF of AD patients. In particular the short isoform A $\beta$ <sub>1-16</sub> has been reported as increased in AD patients.

**Aim:** In this project we tested the hypothesis that specific combinations of A $\beta$  fragments and other peptides present in CSF can be used to detect early AD and the progression to AD from the mild cognitive impairment phase.

**Approach:** we used two different approaches: i) a targeted peptidomic analysis of immunoprecipitated amyloid fragments to specifically detect and quantify A $\beta$  peptides (< 42 amino acids) which was in-house developed, ii) a more comprehensive approach, using C18 beads to fractionate CSF from patients with AD and mild cognitive impairment and possibly find a discriminative pattern of peptide break-down products to predict AD progression. The two techniques were used in two different cohorts one coming from Perugia University, Italy and one from the Alzheimer's centre VUmc Amsterdam. The total number of patients was 181. Peptides were detected and quantified with MALDI-TOF mass spectrometry.



**Results:** In the Perugia cohort  $A\beta_{1-15}$  and  $A\beta_{1-16}$  were quantitatively increased in AD patients when compared to controls (figure 3), and, comparing only these two groups, the difference was significant ( $p=0.032$  for  $A\beta_{1-15}$  and  $p=0.014$  for  $A\beta_{1-16}$  Mann-Whitney U test). No significant changes were noticed for the MCI groups. This finding was not confirmed in Amsterdam cohort, where the levels of  $A\beta_{1-15}$  and  $A\beta_{1-16}$  did not change significantly among the diagnostic groups. C18 beads fractionation yielded in Perugia cohort 15 differentially expressed peaks among the four groups, while in the Amsterdam cohort 16 peaks were differential. Only three differential peaks overlapped between the two cohorts, these had m/z of 1650, 2315, and 2523. In Perugia cohort the best predictor for AD was the peak with m/z 2523, with a sensitivity of 86% and a specificity of 73% to detect AD (AUC =0.80). In the Amsterdam patients the best predictor for AD was the peak with m/z 2315 (AUC= 0.70, sensitivity 85%, specificity 55%).

**Conclusions and outlook:** To our knowledge, this is the first large-scale CSF peptide mass profiling analysis using two independent patient cohorts. Further studies will be aimed to: i) identify the overlapping differential peptides in CSF and possibly development of an antibody-based test, ii) build a statistical model to combine differential peaks (either from immunoprecipitation or C18 beads fractionation) and calculate the performance of these putative biomarkers in distinguishing AD from controls but also, and more importantly, in detecting early AD in MCI patients, iii) combine the differential peaks also with CSF classical AD biomarkers ( $A\beta_{1-42}$ , total tau and phosphorylated tau) to see if this combination might improve the performance of the current tests for AD diagnostics.

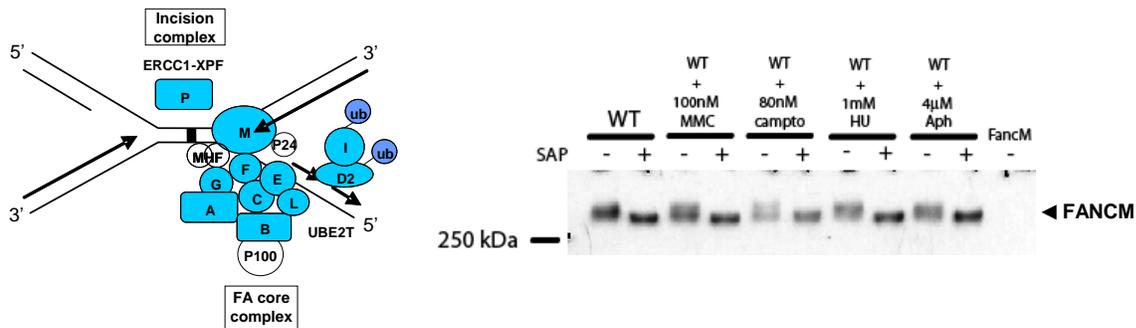
**Acknowledgements:** This research is supported by the Hersenstichting

## FANCM phosphorylation in response to topoisomerase I inhibitor camptothecin

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**BACKGROUND** Fanconi anemia (FA) is a rare, genetic instability syndrome characterized by congenital abnormalities, progressive bone marrow failure and a high predisposition to cancer especially AML and squamous cell carcinoma. The genes affected in FA patients encode proteins that assemble in a nuclear complex essential for the maintenance of genetic stability. FANCM is one of the components of this complex and seems essential for the recruitment of the complex to DNA damaged by crosslinking agents like mitomycin C and cisplatin (Fig 1). In contrast to other FA proteins, FANCM appears to have a broader role in genome maintenance at the stalled DNA replication fork, as shown by the specific sensitivity of FANCM-deficient cells to the topoisomerase I inhibitor camptothecin. The regulation of FANCM activity is currently unclear, but DNA damaging agents including camptothecin induce a strong phosphorylation of FANCM, which might be involved in this regulation (Fig 1).



**Figure 1** FANCM is part of the FA core complex and phosphorylated upon DNA damage

**AIM** Identification of FANCM phosphorylation sites upon treatment with camptothecin, to learn more about its regulation.

**APPROACH** FANCM was purified from camptothecin treated HeLa cells or 293 cells stably transfected with flag-tagged FANCM. Immunoprecipates were separated on a 3–8% Tris acetate SDS-PAGE gel. Coomassie-stained bands corresponding to FANCM were cut from the gel and processed for in-gel trypsin digestion, prior to mass spectrometry. To identify phosphorylated amino acids, selective phosphopeptide enrichment was carried out using titanium dioxide chromatography and these peptides were analysed by liquid-chromatography followed by tandem mass spectrometry (LC-MS-MS). Peptides were analyzed with Sequest and MaxQuant

**RESULTS** Purification of endogenous FANCM from HeLa cells identified a single FANCM specific phosphopeptide. The site S661 was positively identified as being phosphorylated only in cells treated with 5nM camptothecin. Prediction programs showed that this amino acid is a possible target of PKA, PKC and PKG. In addition to the large scale preparation of endogenous FANCM from HeLa cells, we purified exogenous wild type FANCM from treated and untreated HEK293 cells. In this experiment several sites were found to be phosphorylated, but only the phosphorylation of S1437 and S1448 was induced by camptothecin treatment.

**OUTLOOK** It seems that we have been able to identify amino acids in FANCM that are phosphorylated upon treatment with DNA damaging agents. The significance of these findings need to be further investigated by functional studies.

## Fishing for new Fanconi genes by proteomics of protein complexes

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**BACKGROUND** Genes that participate in the Fanconi anemia (FA)/BRCA DNA damage response pathway have been recognized as important cancer-related genes, involved in a variety of both hereditary and sporadic cancers. Cells with defects in this pathway display an abnormally high sensitivity to chemotherapeutic agents such as cisplatin, a feature that is highly relevant for the choice of treatment in the clinic. With currently at least 13 FA genes known to be involved, the FA pathway may be considered complex (see Figure 1). Nevertheless, there is strong evidence that new players - especially in the important 'downstream branch' - remain to be identified.

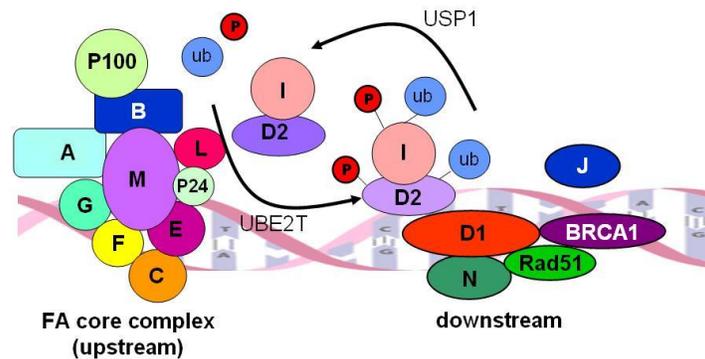


Figure 1. Current model of the FA/BRCA pathway, showing the 13 known FA genes and p24, p100, and BRCA1. The latter 3 proteins formally do not qualify as genuine FA genes, since so far no FA patients have been identified with mutations in these genes. Proteins p24 and p100 are essential components of the core complex, while BRCA1 binds to BRIP1/FANCI and FANCD1/BRCA2. Key reaction in the pathway is the monoubiquitination of FANCD2 and FANCI into FANCD2-ub and FANCI-ub, which requires a functional core complex (upstream part of the pathway). FANCL provides the E3 ligase activity for this reaction. UBE2T acts as the ubiquitin conjugating enzyme, while the deubiquitinating enzyme USP1 recycles these proteins. FANCD1 (BRCA2), FANCN (PALB2) and FANCI (BRIP1) act downstream of this step, as does BRCA1. Females carrying monoallelic mutations in any of these 4 'downstream' genes have an increased risk to develop breast cancer.

**AIM** We primarily focus on finding interactors for the two FA proteins that were most recently identified in our laboratory: FANCI and PALB2.

**APPROACH** Our collaboration with a.o. Dr. David Livingston (Harvard Medical School, Boston, USA) has demonstrated that protein complex purification is a valuable tool for identifying new players in the FA/BRCA pathway. FANCI and PALB2 protein complexes were isolated from human HeLa cells via coimmunoprecipitation (co-IP) with 3 rabbit polyclonal antibodies against FANCI and 2 against PALB2. Co-IPs with two unrelated antibodies of the same type served as negative controls. Proteins present in the precipitates were identified by mass-spectrometry.

**RESULTS** In the size range of ~75 kDa - ~300 kDa, a total of ~51 hits were specifically found for one or more of the 3 FANCI co-IPs and ~68 hits for the PALB2 co-IPs. The previous executed pilot study for FANCI could be confirmed. Among the specific hits for PALB2, a known interactor of PALB2, BRCA2, was detected. The interactions found with mass-spectrometry could be validated for all tested candidates for both FANCI and PALB2. Thus far, one of the putative interacting factors for PALB2 could also be verified by reverse co-IP, although weak.

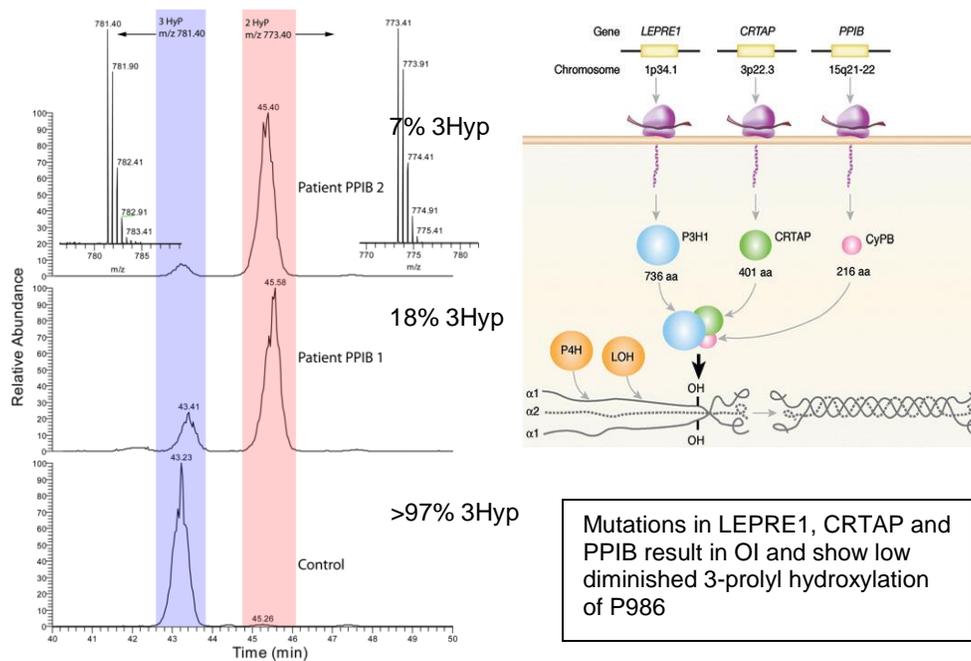
**CONCLUSION** The experiments indicate that this approach can uncover novel proteins binding to FA proteins. Whether these are associated with new FA complementation groups remains to be elucidated.

## PPIB Mutations Cause Severe Osteogenesis Imperfecta

Fleur S. van Dijk, Isabel M. Nesbitt, Eline H. Zwikstra, Peter G.J. Nikkels, Sander R. Piersma, Silvina A. Fratantoni, Connie R. Jimenez, Margriet Huizer, Alice C. Morsman, Jan M. Cobben, Mirjam H.H. van Roij, Mariet W. Elting, Jonathan I.M.L. Verbeke, Liliane C.D. Wijnaendts, Nick J. Shaw, Wolfgang Högler, Carole McKeown, Erik A. Sidermans, Ann Dalton, Hanne Meijers-Heijboer, and Gerard Pals

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Deficiency of cartilage-associated protein (CRTAP) or prolyl 3-hydroxylase 1 (P3H1) has been reported in autosomal-recessive lethal or severe osteogenesis imperfecta (OI). CRTAP, P3H1, and cyclophilin B (CyPB) form an intracellular collagen-modifying complex that 3-hydroxylates proline at position 986 (P986) in the  $\alpha 1$  chains of collagen type I. This 3-prolyl hydroxylation is decreased in patients with CRTAP and P3H1 deficiency. It was suspected that mutations in the PPIB gene encoding CyPB would also cause OI with decreased collagen 3-prolyl hydroxylation. To our knowledge we present the first two families with recessive OI caused by PPIB gene mutations.



The clinical phenotype is compatible with OI Sillence type II-B/III as seen with COL1A1/2, CRTAP, and LEPRE1 mutations. The percentage of 3-hydroxylated P986 residues in patients with PPIB mutations is decreased in comparison to normal, but it is higher than in patients with CRTAP and LEPRE1 mutations. This result and the fact that CyPB is demonstrable independent of CRTAP and P3H1, along with reported decreased 3-prolyl hydroxylation due to deficiency of CRTAP lacking the catalytic hydroxylation domain and the known function of CyPB as a cis-trans isomerase, suggest that recessive OI is caused by a dysfunctional P3H1/CRTAP/CyPB complex rather than by the lack of 3-prolyl hydroxylation of a single proline residue in the  $\alpha 1$  chains of collagen type I.

### Reference

van Dijk FS, Nesbitt IM, Zwikstra EH, Nikkels PG, Piersma SR, Fratantoni SA, Jimenez CR, Huizer M, Morsman AC, Cobben JM, van Roij MH, Elting MW, Verbeke JI, Wijnaendts LC, Shaw NJ, Högler W, McKeown C, Sidermans EA, Dalton A, Meijers-Heijboer H, Pals G. PPIB mutations cause severe osteogenesis imperfecta. *Am J Hum Genet.* 2009 Oct;85(4):521-7. Epub 2009 Sep 24.

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

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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

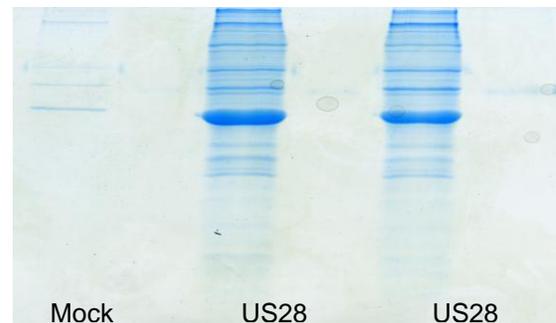
Immunoprecipitation of the HA-tagged US28 in mammalian cells transfected with US28 to isolate US28-complexes for proteomics analysis

### 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<sup>129</sup>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.** Typical result showing the different immunoprecipitates obtained from mammalian cells.

## Cancerous inhibitor of PP2A, CIP2A, as target for Fusicoccin and 14-3-3 proteins

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**Background** Protein-protein interactions form the basis of many regulatory processes in virtually every biological process. 14-3-3 proteins have emerged as key regulators of a wide range of such processes in animal and plant cells. They regulate and coordinate processes like cell cycle progression, apoptosis, signal transduction, protein synthesis, folding and degradation, cellular trafficking and ion homeostasis. Not surprisingly, changes in the expression and activity of 14-3-3 proteins (and 14-3-3 protein interactions) underlie a range of human diseases; e.g. cancer, Alzheimer's disease, the neurological Miller-Dieker and spinocerebellar ataxia type 1 diseases and bovine spongiform encephalopathy (BSE).

The discovery of small ligands that modulate these 14-3-3/target protein interactions offer an attractive opportunity for the development of new therapeutic treatments. To date, one small molecule is known to affect the interaction between 14-3-3 and a target protein. This molecule is called Fusicoccin (FC) (Fig. 1) and is produced by a fungus that infects plants. In nature plants, fungi and even animals produce other molecules that have the characteristic 5-8-5 ring core structure of Fusicoccin; together these compounds are called Fusicoccanes. We have recently found that FC is an effective anti-cancer molecule as it induces apoptosis in a number of tumor cell lines derived, from ovarian, lung and colon tumors.

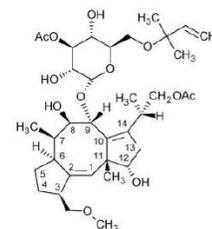


Fig. 1. Structure of Fusicoccin

**Aim and approach** The aim of our project is to identify the protein(s) to which Fusicoccin binds in combination with 14-3-3 proteins. Our approach is to use Fusicoccin affinity chromatography and identify by mass spectrometric analysis which proteins bind in a specific way to Fusicoccin coated magnetic beads.

**Results** Fusicoccin was coupled covalently to magnetic beads and functionally tested with the plant H<sup>+</sup>-ATPase peptide and 14-3-3 proteins. Next, cell lysate was prepared from OVCAR3 cells, FC-beads were added to the extract and proteins interacting specifically with the beads were eluted with the 14-3-3 blocking peptide R18. The protein fraction eluted with R18 were subjected to mass spectrometry analysis by the group of Dr. Jimenez. One of the identified proteins fulfilled an important prerequisite as potential Fusicoccin target, namely a C-terminal tip ending with -TV, with the Thr reported to be phosphorylated. As the name indicates, CIP2A acts as inhibitor of phosphatase 2A and has an important function in tumor cell growth and progression. In a number of tumors CIP2A is strongly up-regulated. Using fluorescent anisotropy we confirmed that Fusicoccin strongly enhances the affinity between the CIP2A C-terminal peptide (16AA) and 14-3-3 proteins (Fig. 2). In collaboration with Dr. C. Ottmann (MPI, Dortmund) we also succeeded in the co-crystallisation of the trimeric complex (FC/CIP2A/14-3-3) and we determined its structure at high resolution.

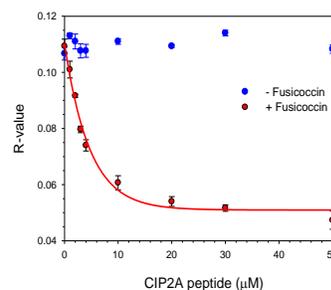


Fig. 2. Fluorescence anisotropy assay. The affinity between the CIP2A phospho-peptide and 14-3-3 $\zeta$  is strongly enhanced by Fusicoccin (De Boer, unpubl.

**Conclusions and outlook.** The combination of FC-affinity chromatography and mass spectrometry resulted in the identification of a protein that has the potential to be targeted by the small molecule stabilizer Fusicoccin. Currently several lines of research are in progress: i) *in vivo* interaction between CIP2A and 14-3-3s, ii) identification of the kinase that phosphorylated S<sup>904</sup>, iii) how is CIP2A functionally affected by the interaction with 14-3-3 and Fusicoccin? and iv) molecular modelling of the wide range of naturally available Fusicoccin analogues (Fusicoccanes; [6]) into the CIP2A binding pocket

## Identification of novel molecular interactions of the cardiac sodium channel carboxyl-terminal domain

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**Background:** The voltage-gated cardiac sodium (Na) channel is a critical mediator of normal cardiac conduction. Loss of Na channel function (decreased inward Na current during the action potential upstroke) has been associated with an increased risk for cardiac arrhythmias in primary electrical disease as well as in acquired cardiac lesions, such as acute myocardial ischemia. In recent years, it has become apparent that ion channels are part of large, multi-protein complexes. Mutations affecting these interacting proteins or sodium channel mutations that disrupt interaction with these proteins can impair the sodium current resulting in a clinical phenotype. The 243-residue intracellular carboxyl-terminal (CT) region is involved in inactivation gating, contains multiple sequence motifs forming consensus protein-protein interaction domains and is the site of multiple mutations associated with primary arrhythmias. The C-terminus therefore represents a very relevant and exciting field of research to further our understanding of this complex membrane protein.

**Aim:** In this study, we undertook a tandem affinity purification (TAP) tag approach for identification of novel partners interacting with the Na<sub>v</sub>1.5 C-terminal domain.

**Approach:** We engineered a protein A tag and a calmodulin binding peptide tag in tandem, separated by a TEV-protease cleavage site fused to the hNa<sub>v</sub>1.5 CT. Tandem tagged Nav1.5 CT constructs and “tags-only” (as control) were transiently expressed in an H10 (rat-derived cardiomyocyte) cell line and protein complexes were purified from cytosolic cell lysates. After purification on an IgG column, one round of TEV enzyme cleavage and subsequent purification on a calmodulin column, eluates were subjected to SDS-PAGE. Gel lanes of the Nav1.5-CT and the control elute were then processed by in-gel protein digestion and analyzed by liquid chromatography followed by tandem mass spectrometry (nanoLCMS/MS).

**Results:** A total of 1310 proteins were identified in the Nav1.5 CT and control sample, of which 42 were found exclusively in the Nav1.5 CT sample. Three candidate proteins were selected for validation by co-immunoprecipitation (Co-IP) based on fold change (spectral counts CT / spectral counts empty) and literature search. One of these proteins was validated by co-IP and further functional studies are ongoing.

**Outlook:** Using an unbiased TAP-tag approach, promising novel molecular components of the sodium channel macromolecular complex in heart will be identified, which may yield important insights into the molecular basis of arrhythmias and may suggest novel therapeutic approaches to treatment of these life-threatening conditions.

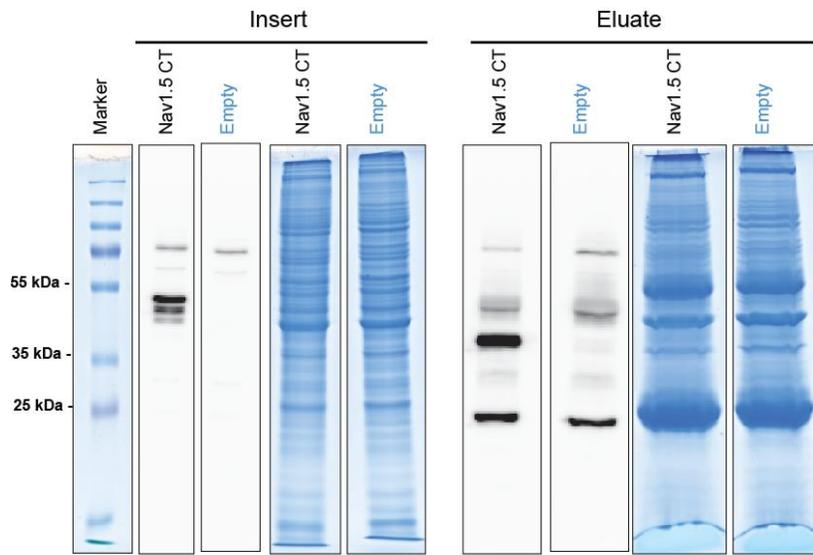
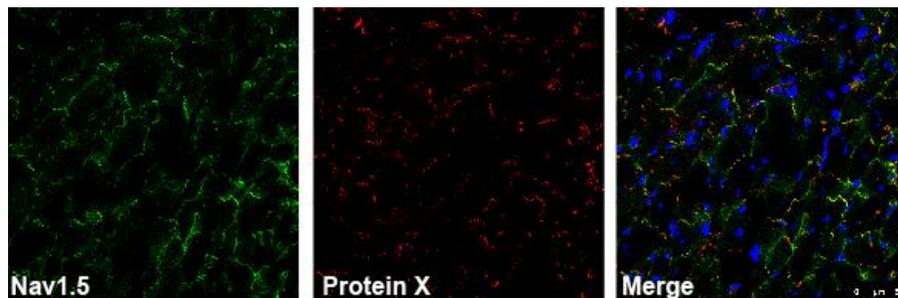
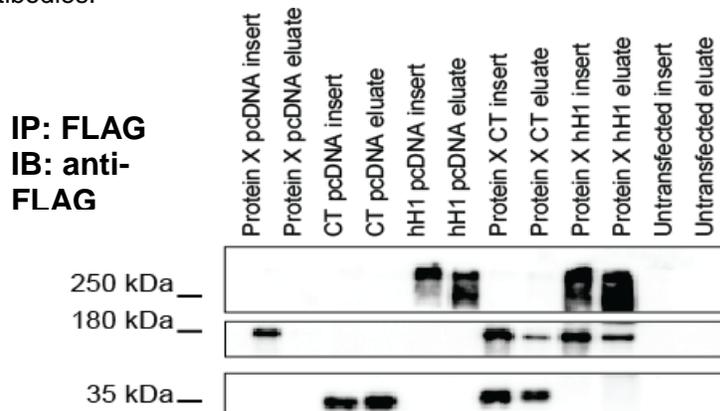


Figure 1: Purification of sodium channel macromolecular complexes. Coomassie-stained SDS-PAGE lanes and respective immunoblots using anti-Nav1.5 C-terminus antibody showing tagged-Nav1.5 CT and 'tags-only' (empty) as a control in the insert (left panel) and the eluates following one purification step (IgG and TEV cleavage) (right panel).

Figure 2: 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 immunoprecipitated with anti-FLAG beads. Immunoprecipitates (eluates) were analyzed by immunoblotting with the indicated antibodies.



2B: Endogenous Nav1.5 associates with protein X *in vivo*. Immunohistochemistry of mouse left ventricular cryosections using anti-Nav1.5 and anti-Protein X antibodies showing co-localization.

## In vitro PKC $\alpha$ -phosphorylated cardiac troponin reduces maximal force and increases Ca<sup>2+</sup>-sensitivity in failing human cardiomyocytes

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**Aims:** Protein kinase C $\alpha$  (PKC $\alpha$ ) phosphorylates several myofilament proteins which are implicated in heart failure, however, the exact consequences for contractile function are unclear. This study aimed to identify the PKC $\alpha$ -phosphorylation sites on cardiac troponin (cTn) and the impact of PKC $\alpha$ -mediated phosphorylation of cTn on myofilament function in human failing cardiomyocytes.

**Methods and results:** Two novel phosphorylation sites were identified by mass spectrometry: Ser198 on cTnI and Ser179 on cTnT. Using a targeted mass spectrometry method, multiple reaction monitoring, Ser198 on endogenous cTnI was confirmed to be a PKC $\alpha$  substrate in human failing tissue and was significantly phosphorylated along with sites Ser42, Ser44, Thr143 when recombinant cTn was treated with PKC $\alpha$ . Whole cTn exchange was applied using cardiac Tn(DD) which has Ser23/24 on cTnI mutated into aspartic acids (D) to rule out *in vitro* cross-phosphorylation of the protein kinase A sites on cTnI by PKC $\alpha$ . Exchange using cTn(DD) pretreated with PKC $\alpha$  in permeabilized cardiomyocytes from patients with end-stage idiopathic dilated cardiomyopathy increased significantly the Ca<sup>2+</sup>-sensitivity of force and reduced the maximal force (F<sub>max</sub>) compared to exchange using control (untreated) cTn(DD) complex. Furthermore, subsequent PKC $\alpha$  incubation of the exchanged failing cells restored pCa50 to baseline levels.

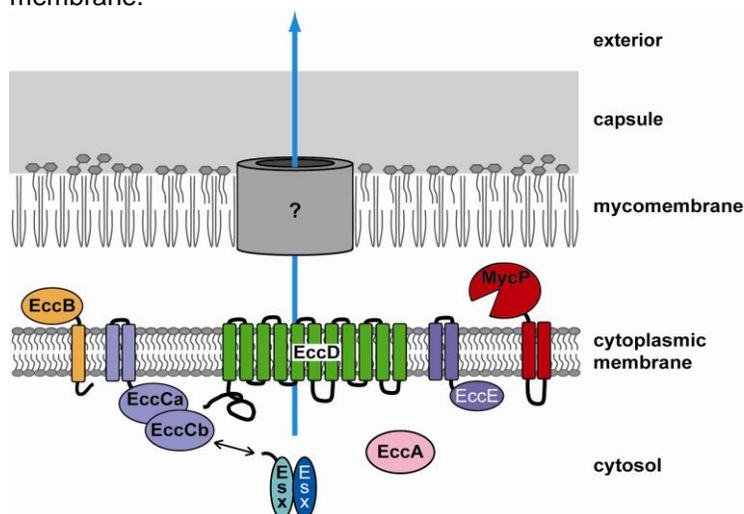
**Conclusion:** All PKC sites are significantly phosphorylated in human cTn complex treated with PKC $\alpha$ , with a high degree of selectivity for Thr143. Exchange of PKC $\alpha$ -phosphorylated recombinant human cTn in failing cardiomyocytes increased Ca<sup>2+</sup>-sensitivity and decreased F<sub>max</sub>, whereas subsequent treatment of cells with PKC $\alpha$  decreased Ca<sup>2+</sup>-sensitivity.

## Proteomics of Mycobacterial Pathogens

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**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.



**Figure 1.** Schematic representation of the mycobacterial cell envelope and the predicted subcellular localization and membrane topology of the known proteins that form a type VII secretion system.

**Aim and approach** In this project, we will identify and characterize proteins from the outer membrane, cell surface and secretome of different pathogenic mycobacteria. In addition, we will also characterize 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 will be analyzed.

**Results** LC-MS analysis confirmed the exclusive location of our OM and IM marker proteins in our newly generated outer membrane and inner membrane fraction, respectively. Furthermore, this analysis showed that in total 163 proteins (including the two outer membrane indicator proteins) were more than 90% extracted from the cell envelope using our detergent extraction procedure. This list was cleaned by removing all lipoproteins and proteins known by functional annotation as inner membrane protein, resulting in a short list of 37 putative new outer membrane proteins. A number of these proteins are now studied in more detail.

The same procedure was used to study the substrates of the SecA2 pathway in pathogenic mycobacteria. Proteomic analysis showed 16 putative substrates that were differentially fractionated between the wild-type and the *secA2* mutant strain. Subsequent analysis showed that 3 of these substrates were indeed SecA2 dependent.

Finally, the composition of the type VII secretion machinery was determined using proteomics on isolated

membrane complexes. The data showed that 4 proteins together form this machinery. The results were confirmed using specific antibodies.

**Conclusions and outlook** The *secA* mutant of pathogenic mycobacteria is an interesting vaccine candidate. However, thus far it was not known why this mutant showed reduced virulence. Two of the identified substrates could explain the reduced virulence and we are now testing which protein is responsible. These data will help to develop a new tuberculosis vaccine.

**Publications**

Sani M, Houben EN, Geurtsen J, Pierson J, de Punder K, van Zon M, Wever B, Piersma SR, Jiménez CR, Daffé M, Appelmek BJ, Bitter W, van der Wel N, Peters PJ. (2010) Direct visualization by cryo-EM of the mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. *PLoS Pathog.* 6:e1000794.

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

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<sup>1</sup>Department of Infectious Disease Immunology, Statens Serum Institut, Copenhagen, Denmark; <sup>2</sup>OncoProteomics Laboratory, Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands; <sup>3</sup>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.

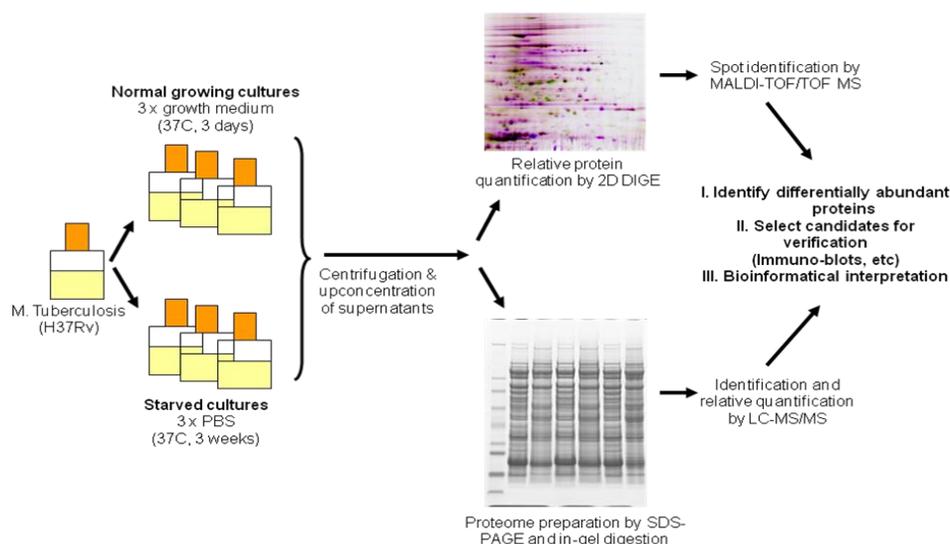
**Aim and Approach** In vitro models which mimic latent infection are valuable tools to describe the changes in metabolism which occur when the bacterium exists in a non-growing form. We used two complementary proteomic approaches, label-free LC-MS/MS analysis and two-dimensional DIGE, to determine the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

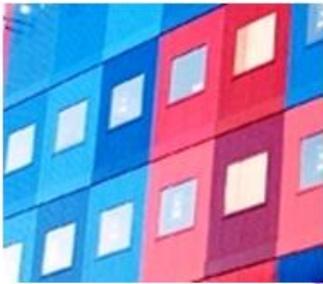
**Results** By label-free LC-MS/MS analysis of fractionated samples, 947 proteins were identified from culture filtrates of log phase and nutrient starved cultures, and the protein levels of 239 proteins were increased in nutrient starved culture filtrates, while 204 were decreased. Notably, members 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

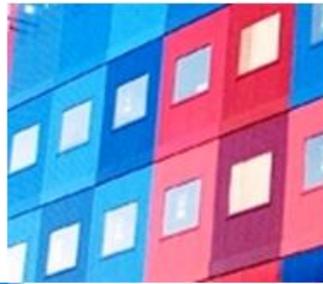
Submitted to *Molecular and Cellular Proteomics*

### Workflow:



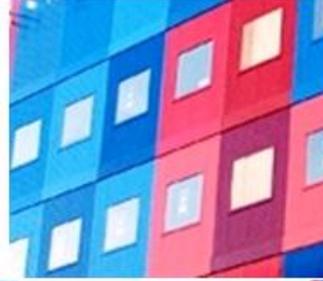


**Biomarkers**

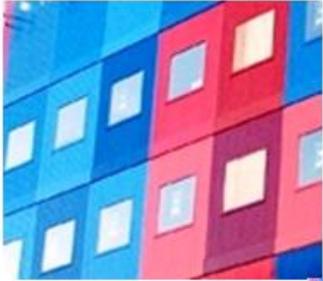


**Proteomics**

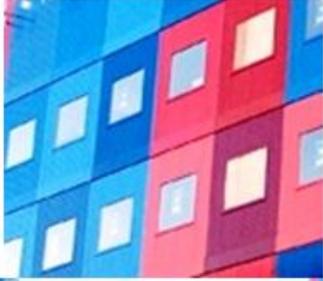
**Mass Spectrometry**



**Discovery**

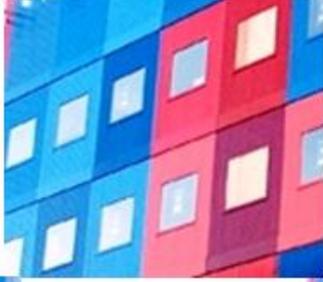


**Multi-disciplinarity**

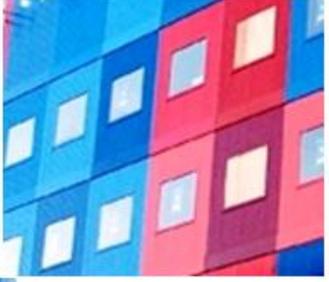


**Collaboration**

**Disease Pathways**



**Clinical Translation**



**Early Detection**



**Personalized Treatment**

**VUmc**  **Cancer Center Amsterdam**

**VU University Medical Center**  
**Department of Medical Oncology** 