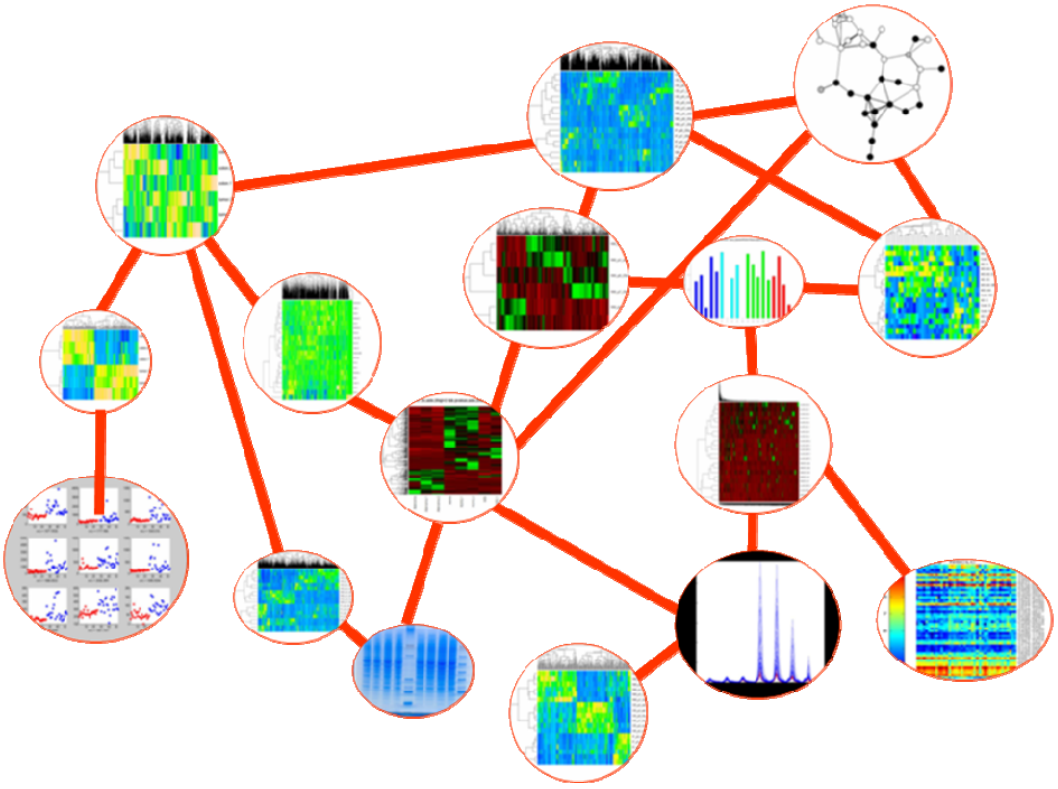
OncoProteomics Laboratory

Progress Report

2008-2009



CCA VUmc O P L
ONCO
Proteomics Laboratory

VUmc Cancer Center Amsterdam

Progress Report 2008-2009

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Foreword

This progress report 2008-2009 covers the part of the second and third year of the OncoProteomics Laboratory (OPL), that was established in April 2006 with support from the VUmc-Cancer Center Amsterdam. The 3-year anniversary we are celebrating on Sept. 30, 2009 with a mini-symposium, that will feature own and collaborative research as well as several international speakers in the field of cancer proteomics.
In this report, you can read about our mass spectrometry infrastructure and workflows, as well as about our scientific research, including 3 research highlights and summaries of running core and collaborative projects.

In the past 1,5 years, we consolidated and extended several research lines. We made the transition from studies for proteomics method exploration and validation to some successful cancer applications, both in model systems and human material and data mining became more and more important:

- New statistical approaches for the analysis of spectral count data were implemented
- The first paper using our in-depth proteomics pipeline for stimulated platelet secretome analysis was published as well as papers on statistics of proteomics data and three invited reviews on proteomics technology and sub-cellular proteomics for biomarker discovery. In addition, we were co-author on several papers of collaborators.
- Progress was made in data mining of the different subcellular proteomics datasets of colorectal cancer tissue (OPL research in collaboration with Dr. Remond FIjneman and Prof. Gerrit Meijer of the VUmc-Tumor Profiling Unit). These projects have lead to the identification of novel biomarker candidates for colorectal cancer screening (patent filed and several manuscripts). The project that identified candidate markers for imaging is described in research highlight 1.
- Proteomics of a genetic mouse model for human BRCA1 deficient breast cancer has identified new BRCA1-related proteins with potential for detection of human genetic breast cancer (work in collaboration with Jos Jonkers of the NKI, described in research highlight 2).
- Proteomics of a cancer stem cell model revealed new proteins associated with stemness (work in collaboration with Onno Kranenberg UMCU, described in research highlight 3).
- Moreover, since Sept. 2008, the OPL is part of two large-scale Center for Translational Molecular Medicine (CTMM) consortia, DeCoDe (DeCrease Colorectal cancer Death) and AIRFORCE (lung cancer). The DeCoDe project provides for funding for a PhD student, Mehrdad Lavaei, who will start in Jan. 2010, and for a new mass spectrometer for targeted, candidate-based proteomics. This instrument is optimal for HTP biomarker candidate validation, and therefore, will nicely complement the existing infrastructure that is optimal for candidate discovery. The AIRFORCE project provided funding for a post-doc, Tieneke Schaaij-Visser who started her biomarker discovery in mouse models for lung cancer, in collaboration with Prof. Anton Berns of the NKI.
- Recently we started setting up kinome capture methods and phosphoproteomics to allow for a new exciting line of research ‘patient stratification for targeted therapy’ together with the new department head of Medical Oncology, Prof. Henk Verheul.

In 2008 and 2009, several young (foreign) scientists and students joined the OPL for research projects:

- Drs. Ann Cathrine Kroksveen from the University of Bergen came for CSF proteomics
- From the Hogeschool Leiden, 2 HLO students joined the OPL in 2009 for short research projects and 4 bioinformatics students worked on tools for proteomics data analysis
- Mehrdad Lavaei and Huub Kant, both master oncology students, explored resp. cancer cell line-derived exosomes and human tumor tissue secretomes as new avenues for biomarker discovery

Finally, I hope you will enjoy reading this report on the second and third years of the OPL and this report triggers new ideas and collaborative projects.

Dr. Connie Jimenez,
Head of the OncoProteomics Laboratory, Dept. Medical Oncology

Group photo OncoProteomics Laboratory, sept. 2009. From left to right, top: Jaco C. Knol, Inge de Reus, Connie R. Jimenez, Tieneke Schaaii-Visser, Silvina Fratantoni, Huub Kant. Bottom: Sander Piersma, Marc Warmoes, Mehrdad Lavaei, Thang V. Pham.

Members of the Research Group:

Head:
Dr. Connie R Jimenez

Research associates:
Dr. Sander Piersma (OPL-core: nanoLC-FTMS)
Dr Thang V. Pham (OPL core: data analysis)

Technicians
Dr. Jaco C. Knol (OPL core: MALDI-TOF/TOF)
Dr. Silvina Fratantoni (CSF biomarkers and assistance with wet work collaborative projects)
Ing. Inge de Reus (back-up FTMS, kinobead and platelet projects)

Post-doctoral fellow:
Dr. Tieneke Schaaij-Visser (lung cancer biomarkers, CTMM-Airforce)

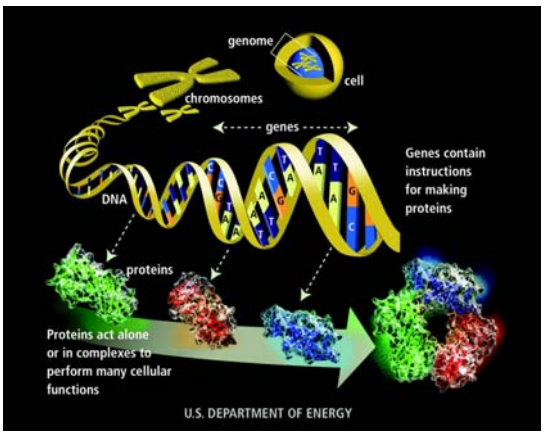
PhD student:
Marc Warmoes (breast cancer biomarkers, CenE/Van Lanschot)

Internship students
Mehrdad Lavaei (Oct 2008-Aug 2009) (will start 1-1-1010 as PhD student CTMM-DeCoDe)
Leon de Boer (april-juni 2009)
Jisca van Lavieren (april-juni 2009)
Huub Kant (feb-sept 2009)

Introduction

The OncoProteomics Laboratory (OPL) 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 CCA/V-ICI researchers.

Proteomics creates a link between genomic information and biological function through large-scale analysis of protein expression, protein modification and protein-protein interactions
Recent 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 of hundreds to 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 signatures 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 diagnostics and treatment of cancer.

To this end, we have developed and implemented robust strategies for biomarker discovery in tumor tissue and biofluids including workflows for sub-nuclear and cell surface proteomics as well as for multi-affinity depletion of biofluids. In addition, cancer cell conditioned media and tumor secretomes hold great promise for discovery of candidate serum biomarkers and are a spear-point of our biomarker research. The samples are profiled using two complementary platforms for the discovery of diagnostic, predictive and drug response peptide patterns and biomarkers: 1. An automated magnetic bead-based biofluid peptide capture method coupled to high-throughput MALDI-TOF/TOF mass spectrometry and 2. Nano-liquid chromatography on-line coupled to LTQ-FT mass spectrometry for in-depth profiling of sub-fractionated samples. In 2009, with support from the CTMM-DeCoDe project, the mass spectrometry infrastructure will be extended with a dedicated system for candidate-based proteomics. This system will allow for high-throughput validation of candidates from discovery proteomics of genomics.

Additional tasks of the OPL are facilitation and coordination of collaborative proteomics research projects with CCA-V-ICI researchers (and beyond), training and to obtain funding. Guidelines for the sample and project submission procedures can be found on our website (www.oncoproteomics.nl).

OPL activities
The projects of the OPL broadly can be subdivided into 3 categories: 1. collaborative research projects, 2. 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) and 3. service projects (protein identification work on a fee-per-sample basis).

Information exchange

- In weekly meetings (every Friday), proteomics data are discussed with the people working in the lab.
- In bi-monthly lunch meetings (every 2nd and 4th Tuesday, cancer proteomics topics of general interest are discussed in a broader group of CCA-V-ICI researchers.
- All OPL people attend and participate in the weekly Medical Oncology Dept. seminars.

Organisation of the OPL

The OncoProteomics Laboratory is a facilitating center where most projects are shaped in close interaction with the collaborators. Moreover, a substantial part of the activities is core research to develop and implement proteomics methods of general interest for cancer researchers and clinicians.
Head of the unit is a scientist/coordinator (1 fte, Dr. CR Jimenez) who is leading a team of scientists (3 fte, a mass spectrometrist, Dr. S. Piersma; a computer scientist, Dr. T. Pham; and a biochemical technician, Dr. J. Knol) as well as co-workers on temporary projects and PhD students, post-docs and technicians on collaborative projects.
The OPL is housed at the Dept. of Medical Oncology. The head OPL is assisted by a 'Program-Advisory-Committee' with representatives from the major CCA/V-ICI departments (Medical Oncology, Hematology, Pathology, KNO, Molecular, Cellular Biology and Immunology, and Epidemiology and Biostatistics) who meet every 6 months with the head of the OPL to discuss progress, developments and assist in prioritizing projects. Furthermore, the head OPL is assisted by Division V in organizational and financial matters. See figure 1 for an outline of the organizational structure. Currently there has been no substantial secretarial assistance.

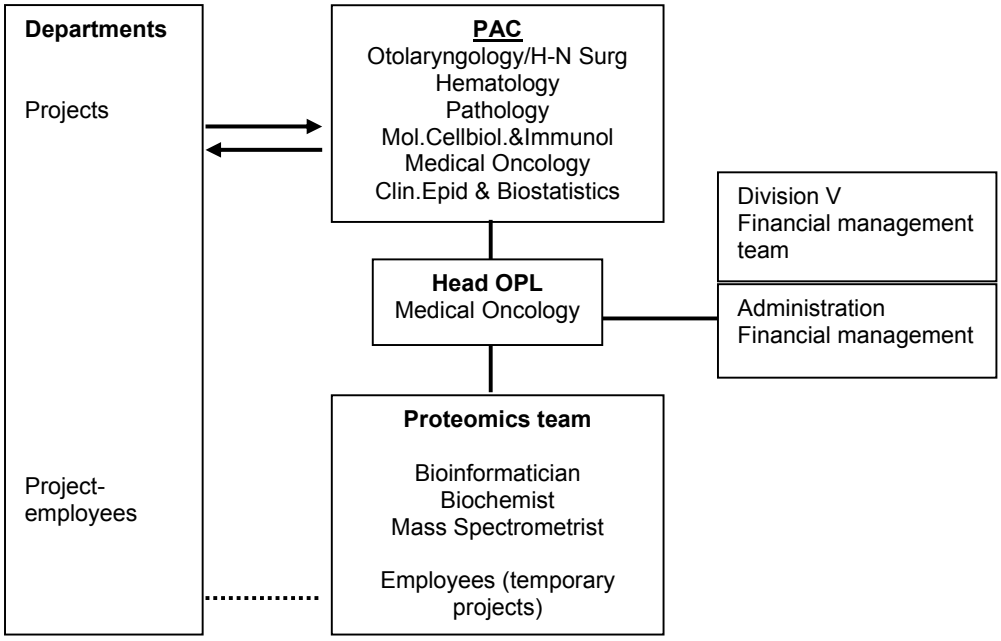


Figure 1. Schematic representation of the organisational structure of the OPL. PAC, Program Advisory Committee.

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 2008-2009 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 expanded in 2008 and luckily is sufficient to accommodate everyone together on the first floor.

PLATFORMS The mass spectrometry lab houses two state-of-the art tandem mass spectrometers: the 4800 MALDI-TOF/TOF (left picture) and the LTQ-FTMS (right picture), a hybrid iontrap-fourrier transform mass spectrometer. A nanoLC system together with a chip-interface is coupled to the LTQ-FTMS. A second micro-LC system is present to fractionate samples prior to nanoLC separation.

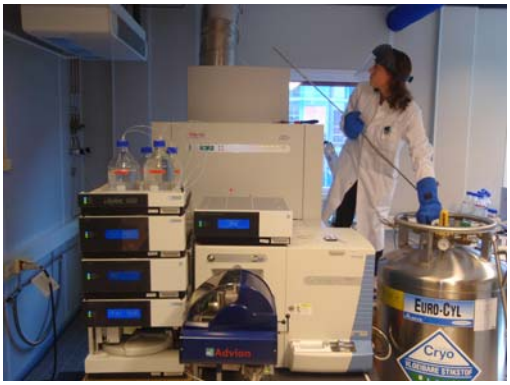


Figure 2. Tandem mass spectrometers at the OPL (CCA 1-47).

Left: 4800 MALDI-TOF/TOF (Applied Biosystems). Jaco Knol is sitting at the computer that controls the instrument.

Right: nanoLC system (Ultimate3000) coupled to a Triversa Nanomate (Advion) and LTQ-FTMS (ThermoElectron). Inge is filling the magnet with liquid nitrgen

After data acquisition and database searching, all data are imported into the software tool 'Scaffold'. Scaffold is a user-friendly web-based tool that aids in confidence assignment to protein identification as well as in data organisation and visualization. All our collaborators can view their data using this tool. Data exports to excel are used to perform further statistical analyses, which is also facilitated by the OPL.

In the past 3 years, the emphasis has been on in-depth proteomics projects, resulting in heavy use of the nanoLC-FTMS workflow (with sometimes a few weeks of waiting-time), while the MALDI-TOF/TOF was not used at its maximum due to low requests for serum peptide profiling projects. To cope with the increasing request in 2009-2010, we are gathering money to purchase a second nano-liquid chromatography system coupled to a MALDI-plate spotter to create a second, independent workflow for in-depth proteomics.

Upgrade of the IT infrastructure in 2009 and desired upgrade in 2010

NanoLC-FTMS/MS experiments create large volume datasets (~ 0.2 Gb/ injection, ~ 2 Gb per biological sample), therefore the data production per day may get as high as 2-4 Gb. In 2009 OPL has bought an additional Mascot licence to switch from Sequest to the Mascot search engine for LC-MS/MS data and purchased three servers :(1) one server for data storage (>1 Tb, RAID 5 storage) (2) a dual quad-core server for protein database searching of MS/MS spectra using Mascot and (3) an optimized server to run data analysis and integration using Scaffold. See figure below.

The three servers are in a local network with the FTMS data acquisition PC and are connected to the VUmc network via a switch. In addition to the servers, multiple external 1Tb hard-discs are used for raw data back-up. The hardware/software upgrade was financed with the money that remained from the CCA start-up grant. We have not set-up a -much needed- database in conhunction with a Laboratory Information Management System (LIMS) yet, funding for this hardware/software solutions still needed.

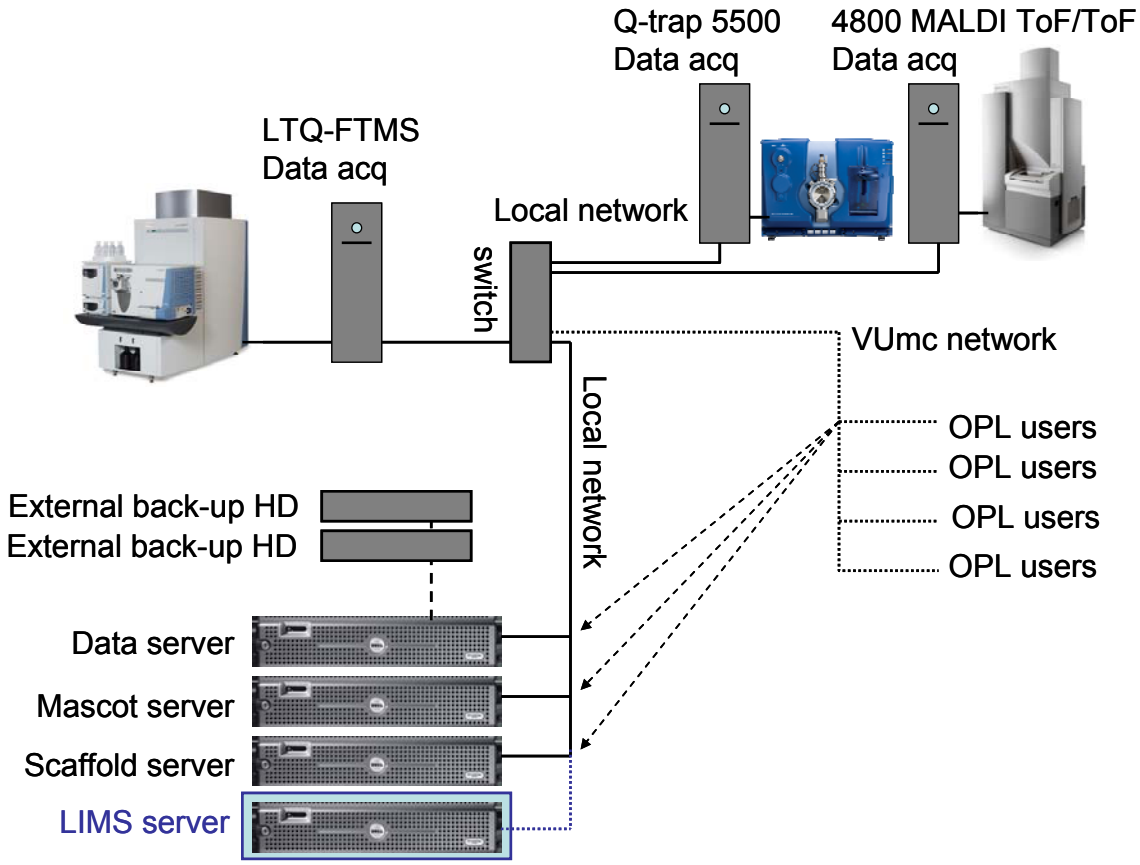


Figure. In grey and black the 2009 OPL-IT situation with in blue the LIMS system that is currently lacking

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

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, detection and (prediction of) drug response (Fig. 3). Candidate-based proteomics is anticipated to speed up the process of candidate validation as up to 200 candidates can be multiplexed in a single analysis. From these targeted analyses validated candidates may be prioritized for development of antibody-based tests. In 2009, the OPL mass spectrometry infrastructure will be complemented with a dedicated mass spectrometry system for candidate-based proteomics.

Cancer-related proteins validated in large cohorts may provide novel drug targets and candidate biomarkers for development into non-invasive (multiplex) antibody-based assays.

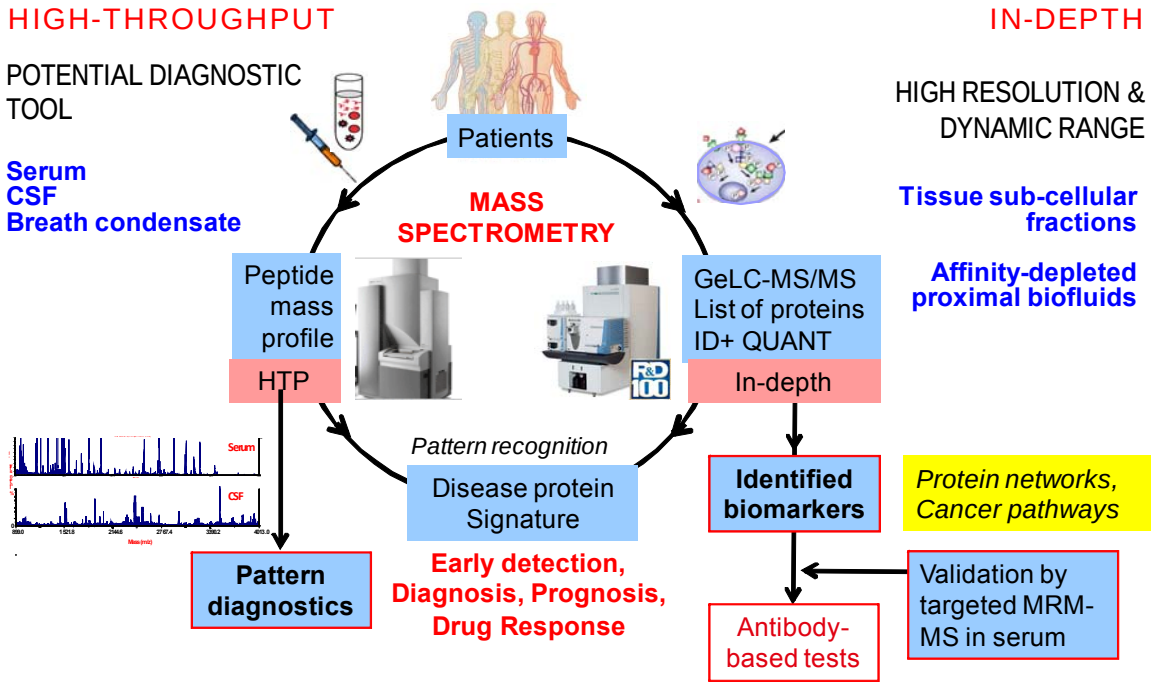


Figure 3. Complementary mass spectrometry-based proteomics approaches for discovery and validation of cancer signatures and biomarkers at the OncoProteomics Laboratory.

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 to amino acid sequence databases
- A wide range of new MS–based analytical platforms has been developed, including hybrid instruments such as the MALDI-TOF/TOF and iontrap-fourrier transform MS.

1. Pattern diagnostics by MALDI-TOF-MS-based high-throughput peptide profiling

BIOFLUIDS. Human body fluids that can be collected non-invasively have been an attractive source for biomarker discovery approaches. Especially, blood samples are easy to obtain, and blood is likely to reflect the physiological and patho-physiological state of the whole human body: blood reaches all organs and tissues in the body that through secretion or shedding enrich the total spectrum of proteins present in the bloodstream.
Indeed, in recent studies we and other have successfully combined serum peptide profiling by mass spectrometry with bioinformatics and established distinctive serum polypeptide mass spectral patterns that correlated with clinically relevant outcomes. Moreover, they provided a direct link between peptide marker profiles of cancer and differential protease activity (Villanueva et al., JCI, 2007, Voortman and Jimenez, 2009), suggesting that the patterns may have clinical utility as surrogate markers for detection and classification of cancer.

Biofluid peptide profiling. We have shown that automated magnetic-particle-assisted peptide capture coupled to MALDI-TOF-MS provides a fast and reproducible profiling platform for measuring peptides in the low molecular mass range of the serum and CSF proteomes (Fig. 4; Jimenez et al., 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 μl is enough). In 2007, we have optimized the method to the analysis of endogenous peptide profiles in cerebrospinal fluid (CSF) and 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 abstract section).

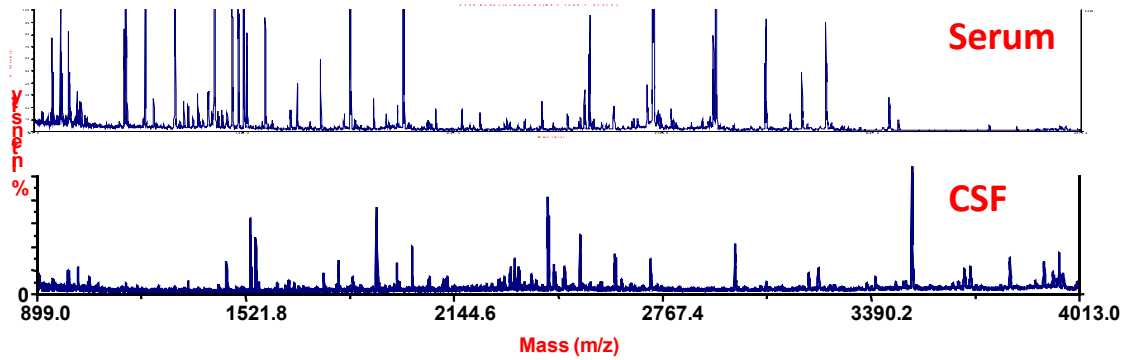


Figure 4. Serum peptide profiling of biofluids using MALDI-TOF mass spectrometry. Biofluid peptides are captured using C18-coated magnetic beads and off-line coupled to peptide profiling by 4800 MALDI-TOF/TOF-MS. The resultant representative mass spectra of serum and CSF peptides are shown. Intra-exp %CV of relative peptide intensities is 5-16% and inter-exp %CV 10-20%.

Prediction of clinical outcome and treatment response monitoring. We applied serum peptide mass profiling to examine the alterations in the serum profiles across sample sets from healthy subjects and cancer patients in a phase I trial in order to assess the feasibility of the current platform in combination with decision algorithms to detect biomarker signatures associated with drug response and clinical outcome (collaboration G. Giaccone, K. Hoekman, MedOnc). This work was selected for an oral presentation at the ASCO2008 meeting in Chicago and was recently published (Voortman et al.,2009). See also abstract section.

Exoprotease activity profiling In the past year, in collaboration with Ing. Dorothe Linders and Dr. Olaf van Tellingen (group of Prof. Hans Bonfrer, NKI), we have started exploring a new avenue for serum peptide pattern diagnostics using stable isotope-labelled peptides spiked into serum followed by analysis of proteolytical breakdown products (see abstract section). The peptides we received from Dr. Josep Villanueva and Prof. Paul Tempst of the Memorial Sloan Kettering Cancer Center in New York. We are currently applying the approach to the analysis of groups of cancer sera with different storage times.

2. Biomarker discovery and cancer pathways by targeted nanoLC-MS/MS-based proteomics using the LTQ-FTMS

For in-depth quantitative analysis of peptides and proteins in patient samples, we focus on sub-proteomes (looking at less to see more). Which sub-proteome depends on the sample type and research question. Moreover, we focus on sub-proteomes that are amendable to analysis by at maximum a two-dimensional separation (usually 1D gel electrophoresis followed by nanoLC) or a single dimension nano-liquid chromatography (LC) separation followed by on-line detection and MS/MS sequencing of the peptides in LTQ-FTMS (Fig.5). Together this will ensure a large dynamic range of detection (~ 10^6) at intermediate throughput (2 hrs- 15 hrs per sample). Because this approach yields identified proteins, the data can be used for pathway analysis and candidate biomarkers can be more easily coupled to antibody-based screening.

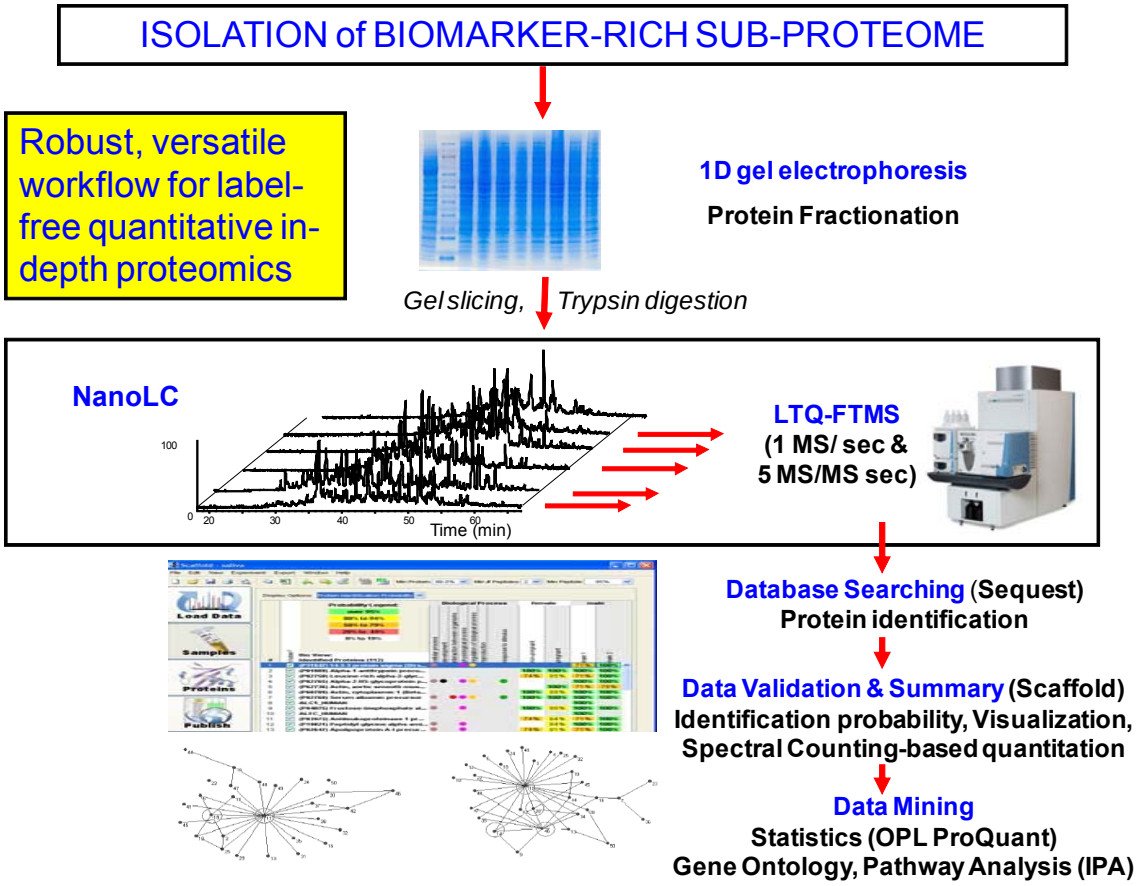


Figure 5. In-depth proteomics workflow at the OPL.

BIOFLUIDS For nanoLC-FTMS profiling of proximal biofluids such as CSF, we apply affinity-based abundant protein depletion to enrich for low abundant target proteins (in CSF, typically brain-derived plasmamembrane and secreted proteins). In 2009 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).
Other biofluid avenues with potential for biomarker discovery that are being explored together with Prof. Henk Verheul are platelets and platelet-derived releasates (Piersma et al., 2009) and plasma microparticles/ exosomes.

TUMOR TISSUE If well-characterized tumor tissue is available for proteomics, we prefer to use tissue as the starting point for discovery. For in-depth analysis of total tissue lysates 1-10 mg is enough. If a large quantity is available (>50-100 mg, along with man-power), fractionation into tumor sub-proteomes may enhance the sensitivity of detection of selected proteins of interest. Typically, proteins isolated from total tissue lysates or subfractions are subjected to 1D gel electrophoresis and nanoLC-FTMS analysis.
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 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 to identify candidate biomarkers that have an increased chance to be detected in serum as well. In hybrid core-collaborative projects, we have explored the above listed subproteomes using colorectal tumor tissue as the model. For this cancer, 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). Cell surface proteomics of colon cancer cell lines (work of PhD student Meike de Wit) is further described in research highlight 1 (pages 16-17).
A less complex experiment is the analysis of total cancer cell and tumor tissue lysates. The sensitivity of those analyses in tumor tissue obtained from mouse models and primary patient material has been much better than anticipated. Therefore, when sample amount is limited or for first explorations, we simply use whole tissues or cells for analysis. One example of a project that employed proteomics of total tissue lysates is the comparative analysis of breast cancer tissue of three different genetic mouse models (collaboration Dr. Jos Jonkers). This work is described in research highlight 2 on pages 18-19.
Colorectal tumor growth is driven by a subset of tumor cells with tumor-initiating capacity, the cancer stem cells. The cancer stem cells are not only responsible for the growth but also for therapy resistance and recurrent tumor growth in patients with metastatic colorectal tumors. We have applied proteomics in two cancer stem cell projects using patient tumor tissue-derived colon cancer spheroids as the model system. The project with Prof. Onno Kranenburg and Benjamin Emmink at the UMCU is described in research highlight 3 (pages 20-21). The project with Dr. Simone Niclou, Prof. Bjerkvig and Uros Raijcevic is described in the abstract section on page 53. In both projects, proteins associated with stemness have been identified.

3. Mining of multi-dimensional proteomics datasets

MASS SPECTROMETRY DATA PREPROCESSING & QUANTITATION
Except for one iTRAQ-based study (Rajcevic et al., 2009), all our quantitation has been performed label-free because of satisfactory results in our pilot studies. We further stream-lined the label-free workflow by testing and implementing the 'whole gel' protocol for parallel sample processing prior to mass spectrometry. Thang Pham optimized and implemented dedicated beta-binomial-based statistics for the analysis of spectral count data (the quantitative measure of protein abundance). Spectral counting turned out to be a robust, reliable approach. In addition, to exploit MS1 data in LC-MS datasets for quantitation, we have implemented MSInspect and PEPPeR, tools that allow for quantitation of peptide ion abundance (Figure 6).
For MALDI-TOF-MS data, MarkerView in conjunction with the OPLAnalyzer tool box, developed by Thang Pham (Pham et al., 2008), has proven to be a reliable data preprocessing and visualization tool.

DOWN-STREAM DATA MINING FOR PATHWAY AND NETWORK ANALYSIS. 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 and pathway analysis to go from large-scale data to new molecular knowledge about cancer pathways. In 2007, Scaffold has been obtained for validation of protein identification data, data organization and visualization. In 2008, for more down-stream data mining, a license for Ingenuity Pathway Analysis was obtained. Other tools include FatiGO, STRING and sequence motif tools like SecretomeP and SignalP.

II. Listing of projects

Below all running projects are summarized in tables. In the appendix you can find abstracts of the majority of the listed projects.

a. OPL core research 2008-2009

A list of specified OPL core projects can be found in Table 1. Core projects are typically initiated by the OPL but may be carried out jointly with collaborators.

Table 1. OPL-core projects		
Name	**Department**	**Project**
Piersma/ Jimenez	OPL	Kinome profiling: bead synthesis and evaluation in cancer cell lines
Jimenez	OPL	Exosome proteomics as new avenue for biofluid biomarker discovery
Jimenez/ Boven/ Jonkers	OPL/Onc/NKI	Proteomics in breast cancer
Jimenez	OPL	CSF proteomics in Mild Cognitive impairment and Alzheimer's Disease
Jimenez/ Meijer	OPL PA	Sub-Nuclear proteomics in CRC adenome-carcinome progression
Jimenez/ Fijneman	OPL/ PA	CRC secretome proteomics for biomarker discovery in genetic mouse and human
Schaaij-Visser/ Berns	OPL/ NKI	Proteomics of genetic mouse models for lung cancer
Jimenez/ Tellingen/ Bonfrer	OPL/ NKI	Serum profiling -activity test
Jimenez/ Peerdeman	OPL/Neurosurgery	Meningeoma proteomics in correlation with growth rate

Yellow: See research highlights below.

b. Projects completed in 2008-2009

Table 2 contains the list of studies that were completed in 2008-2009. All projects resulted in (co-) authorships (see publications) and submitted manuscripts.

Table 2. Collaborative projects completed in 2008 and 2009		
Name	**Department**	**Project**
Jimenez/ Broxterman/Hoekman	OPL/Onc	Proteomics of platelet releasates for early detection of cancer (method optimization)
Jimenez/Piersma/ Fiedler	OPL/ ProQinase	Secretome proteomics as a new avenue for serum-based candidate biomarker discovery
Jimenez/ Piersma	OPL	Methods optimization for proteomics of cancer-cell-conditioned media
Jimenez/ Kruyt	OPL/Onc	Proteomics of caspase-9 interactome
Voortman/ Giaccone	OPL/Onc	Fase 1B studie met Velcade/ Gemcitabine/ Cisplatin
Niclou, Bjerkvig	NorLux Neuro-Oncol.	Membrane proteomics of differentially invasive brain tumor sferoids
Luurtsema	NucMed/PET	Structural analysis of verapimil metabolites
Gast/ Beijnen	apotheek SLZ	Identificatie differentele SELDI signature peptides in sera of breast cancer patients
Engwegen/ Beijnen	apotheek SLZ	Identificatie differentele SELDI signature peptides in sera of colon cancer patients

c. Collaborative projects CCA-V-ICI:

Table 3 summarizes the list of on-going collaborative projects with CCA-V-ICI researchers.

Table 3. collaborative projects CCA/V-ICI		
Name	**Department**	**Project**
Fijneman/ de Wit	PA	Cell surface proteomics for identification of new imaging markers for colon cancer
Fijneman/ Boven	PA/Onc	Secretome proteomics of co-cultered fibroblasts and colon cancer cells vs monocultures
De Winter	KlinGen	Analysis of FANCM prot-prot interactions in the Fanconi anemia pathway
Dorsman	KlinGen	Analysis of PALB2 prot-prot interactions in the Fanconi anemia pathway
Schuurhuis/ Zweegman	Hema	Tumor microenvironment proteomics in AML in relation to apoptosis resistence
Middeldorp/ Pegtel	Pathologie	Exosome proteomics in EBV+ tumor immune escape
Schilte/ Beelen	MCBI	Identification of new biomarkers during peritoneal dialysis
Van Die	MCBI	Mass spectrometry of glycoconjugates
Lind/ Smit	Pulm.	Serum profiling in phase II study of Erlotinib and Sorafenib in Patients with Advanced NSCLC
Posthuma de Boer/ Helder	Orhtopaedic Surgery	cell surface and secretome proteomics in metastatic and non-metastatic osteosarcoma cells
Bitter	Med.Microbio	Mycobacterium mutant screening
Van Dongen	KNO	Determination of number of labels on antibodies

d. Collaborative projects VU/VUmc:
Table 4 summarizes the list of non-cancer collaborative projects with researchers from various departments.

Table 4. collaborative projects VU/VUmc		
Name	**Department**	**Project**
Teunissen	MCBI/ Klin. Chem.	Biomarkers discovery for axonal damage in Multiple Sclerosis by CSF proteomics
Pals	Klin.Gen	Mapping of hydroxyproline residues on collagens isolated from patients
Van der Velden/ Stienen	Med. Physiol	Phosphorylation Site Mapping on Human Cardiac Troponin I and T
Vd Berg/ Van Dijk	Anat.	Proteomics of post-mortem brain in Parkinson's disease
De Vies	PA	Proteomics of post-mortem brain in Alzheimer's disease
Van Elk /Veerhuis	Klin Chem	b-amyloid peptide fingerprinting in CSF of dementia patients by ImmunoMS
VU		
M. Smit/ Slinger	Pharm.Chemie-VU	Phosphoproteomics of viral chemokine signaling networks in oncogenesis & metastasis
De Boer	VU	13-3-3 interactors in cancer cell lines

e. External collaborative projects:
Table 5 shows the list of external collaborative projects.

Table 5. External collaborative projects		
Name	**Department**	**Project**
Emmink/ Kranenburg	UMC	Proteomics of colon cancer stem cells
Jos Jonkers/ Jaspers	NKI	Proteomics of drug treatment in genetic mouse models for breast cancer
Fitzimonos/ Vreugdenhil	LUMC	Proteomics of hippocampus in rats with seizures and learning deficits
Saydam	Harvard	Proteomics of miRNA ko meningioma and arachnoidal cell lines
Raijcevic, Niclou	NorLux Neuro-Oncol.	Differential proteomics of colon cancer tissue and derived spheroids
Brunagel	Univ. Bonn	Identification of nuclear matrix antigens with application in colorectal cancer screening
Albrethsen/ Rosenkrands	Statens Serum Institut	Secretome proteomics of Mycobacterium Tuberculosis H37Rv
Berven	Univ. Bergen -Norway	Quantitative CSF proteomics for biomarker discovery in MS

f. Service projects:
Table 6 lists the service projects in which routine proteomics tasks were performed on a fee-for-sample basis without special input of intellectual property in the project (other than doing the job well). In these types of projects, in publications only acknowledgement for mass spectrometry services is requested.

Table 6. Service projects		
Name	**Department**	**Project**
Ligtenberg/ Bolscher	Med. Microbio	Massabepaling synthetische peptiden and saliva peptides
Westerveen	ID Lelystad	Proteomics of tuberculosis bacteria

g. New projects (other than above) planned for 2009-2010:
Several new exploratory meetings between the head OPL and interested VUmc researchers took place in the past 1, 5years and many collaborators were trained. Table 7 lists all the new projects planned for 2009-2010. A major focus in 2009-2010 will be on miniaturization (analysis of cancer stem cells), kinome profiling and phosphoproteomics for personalized medicine and candidate-based proteomics.

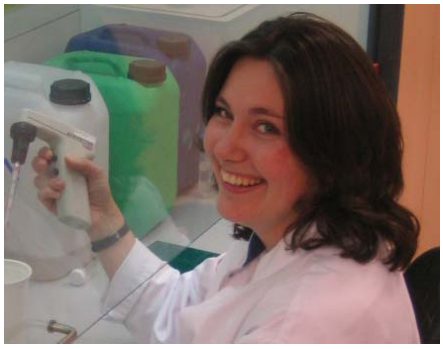
Table 7. Planned for 2009 and 2010		
Name	**Department**	**Project**
Piersma/ Verheul	OPL/Onc	Kinome profiling for patient selection
Jimenez/ Broxterman/ Verheul	OPL/Onc	Platelet proteomics for therapy monitoring in cancer patients
Jimenez/ Verheul	OPL/Onc	Proteomics of serum-derived exosomes isolated from cancer patients
Jimenez/ Fijneman	OPL/ PA	Validation of colon cancer candidates using nanoLC-MRM-MS
Schaaij-Visser/ Snijders	OPL/ PA	Proteomics of (pre)malignant tissues in NSCLC
Hozemans/ De Vies	OPL/ PA	Kinase activity profiling of CSF kinases identified by discovery proteomics
Jimenez/ Heutink	OPL/ Klin.Gen.	CSF proteomics in Parkinson's disease
Diosdado/ Meijer	PA	Proteomics of miRNA ko cell lines
Dijkmans/ Jansen	rheumatology	Serum/synovial fluid profiling in rheuma artritis
Meisch/ Niessen	PA	Proteomics of heart failure
Rodenberg/ De Vries	RIVM	Proteomics of genetic mouse models for breast cancer
Winden/ Van Gils	UMC	Serum profiling in breast cancer
Smit	MCN-VU	Serum profiling in depression

Research highlight I

Cell surface proteomics for the identification of candidate imaging biomarkers

New targets are needed for detecting cancer through molecular imaging and for treating cancer through directed delivery in vivo. Selectively targeting a solid tumour in vivo remains a challenge in molecular cancer medicine. The difficulty lies in the fact that most tissue- and cancer-associated proteins are expressed by cells inside tissue compartments not readily accessible to intravenously injected biological agents such as antibodies. This inaccessibility hinders many site-directed therapies and imaging agents. Proteomics strategies that selectively can enrich for accessible proteins at the cell surface hold great promise for discovery of candidates most relevant to targeting, imaging and treating disease.

The OPL is involved in 2 cell surface proteomics, one carried out by PhD student Meike de Wit (with Dr. Remond Fijneman and Prof. Gerrit Meijer) which is described in this research highlight. The other project focuses on the cell surface proteome of (non-)metastatic osteosarcoma cell lines and is described in the abstract section on page 44.
The research in the highlight described here focuses on colon cancer cell lines. In the context of the CTMM-DeCoDe project we will expand this line of research and include mouse models and human tissue in our discovery efforts.



Meike de Wit is a biologist with a specialization in Oncology. After obtaining her bachelor degree in Biology at the University of Utrecht in 2003 she followed a master course specialized in Oncology at the Faculty of Medicine of the VU University of Amsterdam which was completed in 2006. Currently she is working on her PhD project titled "Molecular imaging of colorectal cancer". This work is carried out at the Tumor Profiling Unit of Prof Gerrit Meijer (Dept. Pathology, VUmc) under the supervision of Dr. Remond Fijneman.
The aim of this project is the identification and validation of biomarkers that can distinguish low-risk colon adenomas from high-risk colon adenomas and CRC and that could be applied for molecular imaging. In this project a combination of state-of-the-art proteomics and genomics techniques is used to identify cell surface proteins that could serve as molecular imaging biomarkers.

Identification of new Biomarkers for Colon Cancer using Genomics and Cell Surface Proteomics

BACKGROUND: Detection of colorectal cancer (CRC) at an early stage of disease is the most realistic approach to reduce the currently high mortality rates. Molecular imaging with biomarkers that discriminate colon adenomas with low-risk of progression from adenomas with high progression risk and CRC could be an effective approach for early detection. Based on microarray expression analysis, we previously have identified genes whose mRNA expression is significantly increased in adenocarcinomas compared to low-risk adenomas. For most of the proteins encoded for by these genes the intra-tumoral (stromal or epithelial compartment) and sub cellular localization is unknown. Among them genes encoding proteins with extracellular domains are the most promising targets for molecular imaging.

AIM: The aim of the present study is to identify plasma membrane proteins with extracellular domains in CRC cell lines that are coded for by genes found to be over-expressed in high-risk adenomas and CRCs compared to low-risk adenomas.

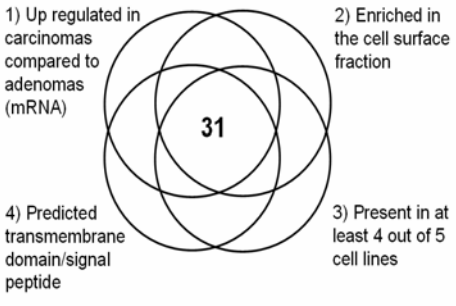


Figure 1

APPROACH: Colon cancer cell lines (COLO 205, HT-29, RKO, Caco-2 and HCT 116) were cultured until 70-80% confluency and incubated with sulfo-NHS-SS-Biotin to biotinylate cell surface proteins, which were then isolated from the whole cell lysate. Both the biotinylated and the non-biotinylated protein samples were fractionated by gradient 1D SDS-PAGE and further processed for in-depth proteomics analysis by liquid-chromatography followed by tandem mass spectrometry (LC-MS/MS). For validation of candidate markers, FACS analysis was done and Immunohistochemistry was performed on formalin fixed paraffin embedded (FFPE) colon carcinomas and adenomas.

RESULTS:
Biotinylated protein fractions were collected for the five cell lines and western blotting detecting E-cadherin confirmed that these were enriched for cell surface proteins. A total of 2987 proteins were identified with LC-MS/MS, of which 684 were identified only in the combined cell surface fractions, 1925 proteins were found in the cell surface fractions as well as in the pools of non-biotinylated proteins and 377 were found only in the non-biotinylated protein pools. A combination of criteria, including presence in at least 4 out of the 5 cell lines, enrichment in the biotinylated protein fractions compared to the non-biotinylated protein fractions and overexpression in CRC lead to the selection of the 31 most promising biomarkers (see figure 1).
From these overlapping proteins a sub selection was made based on information in the current literature and antibody availability for validation by FACS analysis of the cell lines and immunohistochemistry on tissue micro arrays containing adenomas and CRCs. We have been able to show for several candidates that the proteins are expressed on the plasma membrane and/or on the luminal side of the tumors see figure 2.

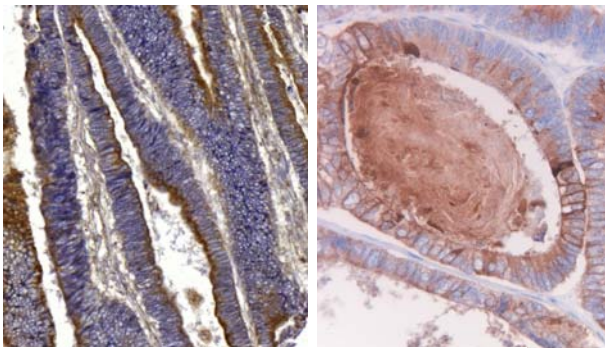


Figure 2

OUTLOOK:
Integrative analysis of mRNA expression data from primary colorectal tumor tissue samples and cell surface proteome data from cell lines successfully identified cell surface proteins as candidate molecular imaging biomarkers for high-risk adenomas and CRCs.

Research highlight II

Comparative Proteomics of Genetic Mouse Models for Human Breast Cancer: Identification of BRCA1-associated Proteins Involved in DNA-repair

Breast cancer is the most common malignancy in women in the western world, constituting more than 25% of cancers in this group. The outcome of breast cancer would be strongly improved if patients could be diagnosed and treated early. This especially hold s for patients with hereditary breast cancer who are hit at a for breast cancer young age.

Our starting point for discovery were three elegant genetic mouse models for breast cancer of which the $Brca1^{-/-};p53^{-/-}$ model exhibits high resemblance at the histological and molecular levels to human BRCA-related breast cancer. The mouse models were developed by Jos Jonkers at the NKI and the work is done in collaboration with him.

AIM: Identification of novel biomarkers for early detection of DNA-repair deficient ($BRCA1^{-/-}$) hereditary breast cancer.

APROACH: Comparative proteomics of 3 genetic mouse models of breast cancer. To this end, total tissue lysates were analyzed from breast tumor tissue of three genetic mouse models: 1.the $Brca1^{-/-};p53^{-/-}$ mouse that develops breast tumors that share histopathological and molecular features with BRCA1-deficient basal-like breast cancers in women, 2. the $ECAD^{-/-};p53^{-/-}$ mouse with metastasizing mammary tumors resembling human invasive lobular cancer and 3. the $p53^{-/-}$ mouse that develops diverse mammary tumors.

Marc Warmoes got both his B.Sc. in Chemistry and his M.Sc. in Biotechnology at Ghent University. After his second M.Sc in Bioinformatics at the University of Leuven he moved to Amsterdam where he started working at Agendia, a company who brought the first FDA approved multivariate microarray breast cancer diagnostic test to the clinic. Currently he works full time at the OncoProteomics Lab as a PhD student under the supervision of Dr. Connie Jimenez and Prof. Epie Boven. His PhD project focuses on the identification of biomarkers of women with BRCA1 hereditary breast cancer. The goal is to identify biomarkers that will allow early detection of breast cancer, crucial in good treatment outcome and survival.

METHODS: Total tissue lysates of the tumors were fractionated using SDS-PAGE followed by tryptic in-gel digestion and peptide analysis by nanoLC-MS/MS and database searching. Normalized spectral counting was used for protein quantification. Statistical testing was applied to discover significantly regulated proteins.

RESULTS: Analysis of technical replicates showed good reproducibility throughout the entire workflow. Comparative analysis of five DNA-repair-deficient ($BRCA1^{-/-};p53^{-/-}$) tumors and five DNA-repair-proficient ($ECAD^{-/-};p53^{-/-}$ or $p53^{-/-}$) tumors identified a total of 3836 proteins of which 804 were significantly regulated between the two groups ($p<0.05$). Unsupervised clustering could already identify two main biological groups, a separation that was further enhanced in the supervised clustering (fig. 1). Among the proteins upregulated in BRCA1 tumors, we identified markers for basal breast cancer, the histopathological type were the bulk of BRCA1 deficient tumors belongs to.

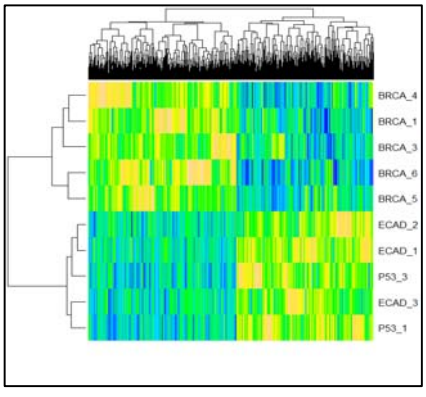


figure : Supervised clustering clearly separated the BRCA1 deficient tumors, from the non-BRCA1 deficient tumors

Why proteomics of a genetic mouse model for candidate biomarker discovery?

- No genetic heterogeneity between samples
- Mouse $BRCA1^{-/-};p53^{-/-}$ breast tumors resemble human tumors both at the molecular and histological level

Functional pathway analysis using the software tool Ingenuity and literature mining, revealed that many of the differential proteins were involved in DNA-repair and several implicated in BRCA1 breast cancer. The majority (> 90%) of differential DNA repair proteins were upregulated in the BRCA1 deficient tumors.
Ingenuity pathay analysis generated “DNA Replication, Recombination and Repair” as the top molecular and cellular function. The protein network in the figure also contains many up-regulated drug targets. Furthermore, many members of back-up DNA repair complexes like Single Strand Break (SSB)-repair and Non-Homolgous End Joining (NHEJ) were up-regulated in BRCA1 breast tumors.

Also the BRCA1 associated stem cell marker ALDH1A was uniquely identified in the BRCA1 deficient tumors which is in line with data published for human BRCA1 breast tumors.
Together, these findings give us confidence in the novel candidates of which 3 are indicated in the DNA repair network.

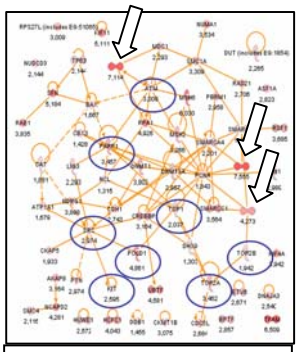


Figure: Protein network associated with ”DNA Replication, Recombination and Repair” (61 proteins), is the number 1 molecular and cellular function associated with proteins up-regulated in BRCA1 breast tumors. Encircled are drug targets. Novel candidates for follow-up are indicated by the arrows

OUTLOOK
We have identified established and promising novel candidate BRCA1 DNA-repair deficiency proteins using differential proteome analysis on genetic mouse models. Combined with in-depth statistics and functional pathway analysis we selected a subset not only showing strong up-regulation in BRCA1 breast tumors but also biological relevance.

These candidates will be further validated in human breast cancer tissues, and followed up in nipple aspirate fluid and serum using targeted mass spectrometry and immuno assays. Finally, DNA repair proteins up-regulated in BRCA1 breast tumors markers may have use for early detection, but may also identify tumors that are sensitive to PARP inhibitors, a family of small molecule drugs recently proven to be highly effective in BRCA hereditary breast cancer.

Acknowledgements: *This research was supported by CenE Van/ Lanschot*

Research highlight III

Proteomics of cancer stem cell-enriched colon cancer spheroids

Colorectal tumors contain a small subpopulation of cells that has 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. However, the relationship between chemoresistance and cancer stemness is poorly understood.

The OncoProteomics Laboratory is involved in 2 cancer stem cell projects: 1 with Benjamin Emmink and Onno Kranenburg of the research group Surgical Oncology of the University Medical Center Utrecht (UMCU) (this research highlight) and 2. With Uros Raijcevic and Simone Niclou of the NorLux Oncology Laboratory in Luxemburg (see abstracts).

To study the relationship between cancer stem cells and chemoresistance the Kranenburg group has established 8 cancer stem cell-enriched tumor cell populations from primary human colon tumors and their metastases. These cell populations are highly clonogenic and as little as 200 cells initiate tumor formation in immune-deficient mice. In addition, they are capable of multi-lineage differentiation which is accompanied by loss of tumorigenic potential.

AIMS
(1) identifying pathways that promote loss of the cancer stem cell phenotype, (2) identifying cancer stem cell mediators of resistance or sensitization to anti-cancer drugs, and (3) identifying novel secreted cancer stem cell markers as potential novel circulating biomarkers.

APPROACH
We used the culture system of cancer stem cells and isogenic differentiated tumor cells to identify differentially expressed and secreted proteins by a comparative proteomics approach which was developed at the OncoProteomics Lab (OPL) of the VUmc. After separating proteins on a 1D gel and performing in-gel digestion, the resulting peptides were analysed using the nanoLC MS/MS. Database searching, validation and mining resulted in data sets which were used to identify factors that were commonly expressed and secreted by cancer stem cells but not by their differentiated progeny.

Benjamin Emmink, MD, completed his medical study at the University of Utrecht in 2008. The proteomic analysis of colon cancer stem cells and their isogenic differentiated cells was performed as part of his final research internship at the OncoProteomics Lab (OPL) of the VUmc in 2008. Currently he is performing his PhD research on 'cancer stem cells in colorectal cancer' at University Medical Center in Utrecht.

Dr Onno Kranenburg obtained is PhD degree at Leiden University in 1995, studying cell cycle changes during oncogenic transformation. He then moved to the Netherlands Cancer Institute for a post-doc on signal transduction by the Ras and Rho small GTPases. In 2000 he moved to the University Medical Center Utrecht where he first investigated the mechanism of action of anti-angiogenic drugs. In 2003 he moved to the Department of Surgery, and became staff member and head of the Lab Surgical Oncology. In 2006 he was appointed Associate Professor. His research interests are focussed on liver metastases of colorectal cancer. In particular, his lab studies the mechanisms that drive tumor recurrence in the liver following chemotherapy and surgery.

RESULTS

The above-mentioned analysis resulted in the identification of 3048 (proteome) and 1536 (secretome) different proteins. After applying the Fisher's exact test, Mantal Haenszel test and fold change cutoffs on the 4 isogenic pairs we identified 105 significantly up-regulated and 92 significantly down-regulated factors in the CSC proteome. Similar lists were generated for the secretome. One of the most significantly up regulated proteins in the proteome was aldehyde dehydrogenase 1 (ALDH1). This was validated by Western blot analysis (Fig 1). ALDH1 was recently identified as a breast and colon cancer stem cell marker (Huang, E. H. et al. Cancer Res 2009), demonstrating the validity of our proteomics approach. We are currently validating novel spheroid-associated proteins.

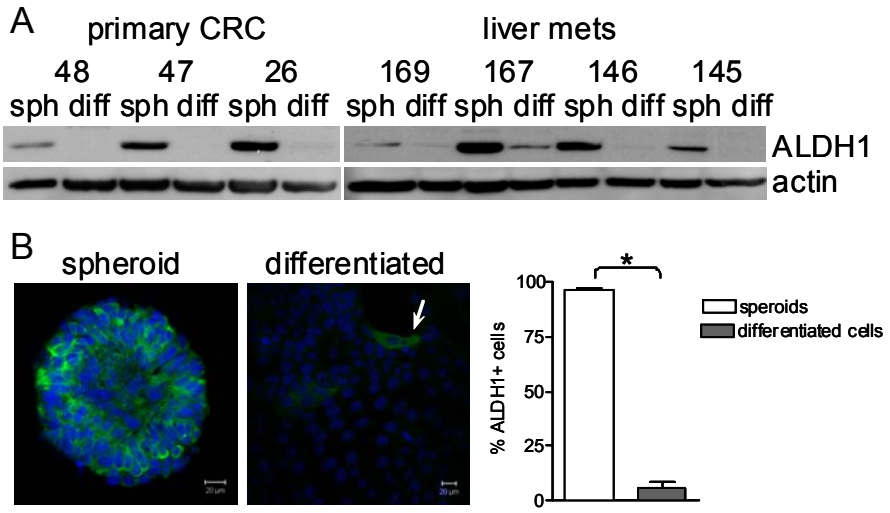


Figure 1. Validation of ALDH1 as cancer stem cell marker

A) Lysates of paired spheroid and differentiated cultures derived from primary colorectal tumors and liver metastases were analyzed for the expression of aldehyde dehydrogenase (ALDH1), a marker for colorectal CSCs, by Western blotting.

B) The percentage of ALDH1-expressing cells in spheroids, and their isogenic differentiated derivatives was determined by immunofluorescence analysis. The left panel shows representative images of L145 cultures. The right panel shows quantification on the basis of 5 random fields of four paired cultures. *denotes statistical significance $p<0.0001$, student's t-test).

OUTLOOK

The four sets of proteins which are significantly up-regulated and down-regulated in the proteome and the secretome of a series of colorectal cancer stem cells provide a solid basis for follow-up research. Based on these lists and on the available literature we are currently studying selected pathways and proteins for their involvement in controlling cancer stem cell identity, in mediating resistance to chemotherapy, and for their potential usefulness as circulating biomarkers.

EDUCATION

In 2008-09 several lectures were given to bachelors, masters and PhD students and 2 practical lab courses 'Protein identification by mass spectrometry and database searching', each of 3 days, were organized to train both internal and external collaborators to prepare their own samples for mass spectrometry. See table. In addition, two masters students performed their internship at the OPL in 2008 and 2009 on the analysis of cancer cell line exsomes and human colon tissue secretomes. Also we hosted 2 HLO students for short-term internships and 4 bachelor students from the Hogeschool Leiden for 4-week and 10-week bioinformatics projects.

Academic teaching

Course	**Class**
Dr. CR. Jimenez, Dr. Fratantoni and Dr. S. Piersma	
Collaborator training- JUNE08	3-day proteomics lab course
Collaborator training- FEB09	3-day proteomics lab course
Dr. CR. Jimenez	
AIO cursus LACDR	Phospho)proteomics for target and pathway discovery
ITCB bachelor course tumor biology:	Clinical proteomics
Bachelors Oncology	Short proteomics lecture and tour of lab
Masters Oncology, Tumor Biology	Mass spectrometry-based proteomics for cancer biomarker discovery
Bachelor course	Proteomics technieken in de immunologie en kanker
Bachelor course, FEW-VU	Introduction to proteomics
Dr. Thang Pham	
Computational Genomics & Proteomics, FEW-VU	Statistical and pathway analysis of proteomics data
Computational Genomics & Proteomics, FEW-VU	Applications of machine learning in proteomics
Bioinformatics and Large Datasets, FEW-VU	Proteomics

SCIENTIFIC OUTPUT 2008-2009

Peer-reviewed Publications 2009

1. Van Dijk FS, Nesbitt IM, Zwikstra EH, Nikkels PGZ, Smit M, Morsman AC, van Roij MH, Elting M, Cobben JM, Verbeke JIML, Piersma, SR., Jimenez CR, Fratantoni S,Wijnaendts LCD 7, Shaw NJ 8, Hogler W 9, McKeown C, Sistermans EA, Dalton A, Meijers-Heijboer EJ, Pals G. PPIB mutations causes lethal/severe osteogenesis imperfecta . Am. J. Clin. Gen. 2009. In press.
2. Marie-Christine W. Gast, Eric J. van Dulken, Thea K.G. van Loenen, Florine Kingma-Vegter, Johan Westerga, Claudie C. Flohil, Jaco C. Knol, Connie R. Jimenez, Carla H. van Gils, Lodewijk F.A. Wessels, Jan H.M. Schellens, and Jos H. Beijnen. Detection of breast cancer by SELDI-TOF MS tissue and serum protein profiling. International Journal of Biological Markers, 2009. In press.
3. Johannes Voortman, Thang V Pham, Jaco C Knol, Giuseppe Giaccone and Connie R Jimenez. Prediction of outcome of non-small cell lung cancer patients treated with chemotherapy and bortezomib by time-course MALDI-TOF-MS serum peptide profiling. Proteome Science. 2009. 2009, 7:34.
4. Abdallah M. Abdallah,1 Theo Verboom,1 Eveline M. Weerdenburg,1 Nicolaas C. Gey van Pittius,2 Phetole W. Mahasha,2 Connie Jiménez,3 Marcela Parra,4 Nathalie Cadieux,4 Michael J. Brennan,4 Ben J. Appelmelk1 and Wilbert Bitter1*. PPE and PE_PGRS proteins of Mycobacterium marinum are transported via the type VII secretion system ESX-5mmi. Mol. Microbiology, 2009, in press.
5. Rajcevic U, Petersen K, Knol JC, Loos M, Bougnaud S, Klychnikov O, Li KW, Pham TV, Wang J, Miletic H, Peng Z, Bjerkvig R, Jimenez CR, Niclou SP. iTRAQ based proteomic profiling reveals increased metabolic activity and cellular crosstalk in angiogenic compared to invasive Glioblastoma phenotype. Mol Cell Proteomics. 2009 Aug 12. [Epub ahead of print]
6. Tefsen B, van Stijn CM, van den Broek M, Kalay H, Knol JC, Jimenez CR, van Die I. Chemoenzymatic synthesis of multivalent neoglycoconjugates carrying the helminth glycan antigen LDNF. Carbohydr Res. 2009 Aug 17;344(12):1501-7. Epub 2009 Jun 6.

7. Jiménez CR. Sorting and zooming: subcellular proteomics is booming! J Proteomics. 2009 Feb 15;72(1):1-3. Editorial.
8. Rajcevic, U., Niclou, S., Jimenez, CR. (2009) Proteomics strategies for target identification and biomarker discovery in cancer. Frontiers in Bioscience 14, 3293-3303. Review
9. Checińska A, Giaccone G, Rodriguez JA, Kruyt FA, Jimenez CR. Comparative proteomics analysis of caspase-9-protein complexes in untreated and cytochrome c/dATP stimulated lysates of NSCLC cells. J Proteomics. 2009 May 2;72(4):575-85. Epub 2008 Dec 7.
10. Sander R. Piersma, Henk J. Broxterman, Muhammed Kapci, Richard R. de Haas, Klaas Hoekman, Henk M.W. Verheul, Connie R. Jiménez (2008) Proteomics of the TRAP-induced platelet releasate. J Proteomics. 2009 Feb 15;72(1):91-109. Epub 2008 Nov 8.
11. Albrethsen, J., Knol, JC, Jimenez, CR., (2008) Unravelling the nuclear matrix proteome. J Proteomics. 2009 Feb 15;72(1):71-81. Epub 2008 Oct 14. Review.
12. Thang V. Pham and Connie R. Jimenez (2008) OplAnalyzer: a Toolbox for MALDI-TOF Mass Spectrometry Data. Int. Conference on Mass-Data Analysis of Images and Signals in Medicine, Biotechnology, Chemistry
13. M. Hamacher, C. Stephan, T. Hardt, M. Eisenacher, A. Henkel, J. Wiltfang, CR. Jimenez, YM Park, K. Marcus and HE. Meyer (2008). Applications in Brain Proteomics: 8th HUPO Brain Proteome Project Workshop, 7 October 2007, Seoul, Korea. Proteomics
14. Li, KW and Jiménez, C.R (2008) Synapse proteomics current status and quantitative applications Expert Rev Proteomics. 2008 Apr;5(2):353-60. Review.
15. Pham, TV., Van der Wiel, M., Jiménez, C.R (2008) Support Vector machine approach to separate control and breast cancer serum spectra. Stat Appl Genet Mol Biol. 2008;7(2):Article11. Epub 2008 Feb 21.
16. Judith Y.M.N. Engwegen, Marieke Alberts, Jaco C. Knol, Connie R. Jimenez, Annekatrien C.T.M. Depla, Henriëtte Tuynman, Henk A. van Heukelem, Pleun Snel, Marianne E. Smits, Annemieke Cats, Jan H.M. Schellens, Jos H. Beijnen (2008) Influence of variations in sample handling on SELDI-TOF MS serum protein profiles for colorectal cancer. . Prot. Clin. Applic. Volume 2, Issue 6, Date: No. 6 June 2008, Pages: 936-945.

Submitted papers:

1. Okay Saydam, Ozlem Senol, Tieneke B.M. Schaaij-Visser, Thang V Pham, Sander R. Piersma, Anat O. Stemmer-Rachamimov, Thomas Würdinger, Saskia M Peerdeman and Connie R. Jimenez. Proteomics protein profiling reveals minichromosome maintenance (MCM) proteins as novel potential tumor markers for meningiomas. J. Prot. Res. 2009. Submitted.
2. Winan J. van Houdt, Benjamin L. Emmink, Robert G. Vries, Frederik J.H. Hoogwater, Connie Jimenez, Hans Clevers, Inne H.M. Borel Rinkes & Onno Kranenburg. Differentiated tumor cells protect colorectal cancer stem cells from irinotecan. Cancer Stem Cell, 2009. Submitted.
3. Sander R. Piersma, Ulrike Fiedler, Simone Span, Andreas Lingnau, Thang V. Pham, Steffen Hoffmann, Michael HG Kubbutat and Connie R. Jiménez. Workflow comparison for in-depth, quantitative secretome proteomics for cancer biomarker discovery: Method evaluation, differential analysis and verification in serum. J. Prot. Res. 2009. Submitted.
4. Silvina A. Fratantoni, Sander R. Piersma, Connie R. Jimenez. 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. 2009 Submitted.
5. Thang V. Pham, Sander R. Piersma, Marc Warmoes, Connie R. Jimenez. On the beta binomial model for analysis of spectral count data in label-free tandem mass spectrometry-based proteomics. Bioinformatics. 2009 Submitted.

Thesis chapters:

1. Marie-Christine W. Gast, Eric J. van Dulken, Thea K.G. van Loenen, Florine Kingma-Vegter, Johan Westerga, Claudie C. Flohil, Jaco C. Knol, Connie R. Jimenez, Carla H. van Gils, Lodewijk F.A. Wessels, Jan H.M. Schellens, and Jos H. Beijnen. Detection of breast cancer by SELDI-TOF MS. PhD Thesis Marie-Christine Gast. 2009.
2. Marie-Christine W. Gast, Marc Zapatka, Harm van Tinteren, Marijke Bontenbal, René Q.G.C.M. van Hoesel, Paul N. Span, Vivianne C.G. Tjan-Heijnen, Jaco C. Knol, Connie R. Jimenez, Jan

H.M. Schellens, and Jos H. Beijnen. Post-operative serum proteomic profiles may predict recurrence free survival in high-risk primary breast cancer. PhD Thesis M.C. Gast. 2009.

Book chapters:

1. S. Fratantoni and CR. Jimenez. Abundant protein depletion sample pretreatment method for in-depth analysis of the cerebrospinal fluid proteome and biomarker discovery. Springer Protocols. Neuroproteomics issue. 2009. Submitted.
2. T. Pham, S. Piersma, M. Warmoes, CR. Jimenez. Statistical analysis of spectral count data in label-free tandem mass spectrometry-based proteomics. Springer Protocols. Neuroproteomics issue. 2009. Submitted.

INDICATORS of ESTEEM

Obtained grants

2009 EU kp7 MEFOPA. Research technician/budget for CSF proteomics in Parkinson's Disease
2008 CTMM-DeCoDe. PhD student for colon cancer proteomics and nanoLC-MS/MS system (QTrap 5500, AB) for targeted analyses
2008 CTMM-AIRFORCE. Post-doc for lung cancer proteomics (3 years)
2008 CCA-V-ICI. Research technician (2 years) for a collaborative project on the AML microenvironment in relation to therapy/apoptosis resistence
2008 Avanti. Post-doc 2 years. Proteomics of lung cancer.

Invited lectures at international conferences (Jimenez)

2008

- 15 feb. 2008, Uppsala, Sweden. Title: 'Mass spectrometry-based sub-cellular proteomics for cancer biomarker discovery'. Symposium Uppsala University and Applied Biosystems
- 7 maart 2008, Amsterdam, ISCO meeting. Title: 'Proteomics targeted to sub-cellular compartments and secretomes for candidate biomarker discovery in colorectal cancer'
- 13 juni 2008, NUBIN symposium, Amsterdam. Title: 'Evaluation of in-depth, multi-dimensional nanoLC- MS/MS-based proteomics for biomarker discovery in CSF'
- 20 aug. 2008, HUPO2008, RAI, Amsterdam. Title: 'Proteomics targeted to subnuclear compartments and secretomes for pathway discovery in colorectal cancer'
- 9 okt. 2008, Dusseldorf, Germany. Title: Evaluation of CSF biomarker discovery workflow by in-depth proteomics. Mid-term EU cNeuPRO consortium meeting

2009

- 17 march, Brussel, EORTC PAM group meeting. Title: 'Proteomics targeted to sub-cellular fractions for biomarker discovery in colorectal cancer'
- 21 april, 2009, Denver, AACR. Title: 'Targeted proteomics of sub-nuclear compartments for discovery of signaling pathways and candidate biomarkers in colorectal cancer'
- 5 may, 2009, Dusseldorf, cNeuPRO general assembly. Title: 'CSF proteomics for candidate biomarker discovery in Mild Cognitive Impairment and Alzheimer's Disease'
- 7 may, 2009, Bonn, Novel tool for cancer risk meeting, German Cancer Aid, Title:'Proteomics targeted to sub-nuclear compartments and secretomes for biomarker discovery in colorectal cancer'
- 9 june 2009. EMBO Cancer Proteomics 2009, Dublin. Title:'Proteomics targeted to sub-cellular fractions for biomarker discovery in colorectal cancer'
- 24 aug. 2009. 9th Mass spectrometry in the health and Life sciences, San Francisco, USA. Title: 'Proteomics targeted to sub-cellular fractions for biomarker discovery in colorectal cancer'
- 29 sept. 2009.33th meeting of the international Society of Oncology and Biomarkers, Amsterdam. Tiltle: 'Proteomics targeted to sub-cellular compartments for candidate biomarker discovery in colorectal cancer'.

Lectures at departments / work group meetings (national and abroad, Jimenez)

- 19 feb,2008. NCI-medical oncology, Bethesda. Title: 'High-throughput and in-depth mass spectrometry-based proteomics for cancer diagnostics & candidate biomarker discovery'
- 25 feb, 2008. Mass Spectrometry Resource, USCF, San Francisco. Title: Cancer biomarker discovery by MS-based proteomics.
- 6 feb. 2009. VUmc-Dept. Nuclear Medicine. Title:'Mass spectrometry-based proteomics for serum pattern diagnostics and (imaging) biomarker discovery'
- 7 april 2009, VUmc-Dept.Pulmonology. Title:'Mass spectrometry-based proteomics for serum pattern diagnostics and (imaging) biomarker discovery'
- 8 mei 2009. Annual VUmc-CRC meeting. Title:'Colon cancer proteomics'
- 16 juni 2009, VUmc-NUBIN group meeting. Title: Identification of novel candidate biomarkers for Alzheimer's Disease by CSF proteomics'

Other academic activities

Participation and function in scientific societies

Function	Organisation
Dr. Connie Jimenez	
Session Chair Cancer Proteomics	International Society of Oncology and Biomarkers ISOBM 2009 meeting
Session Chair Cancer Proteomics	Human Proteome Organisation HUPO2008 meeting
Guest editor special issue 2009	Journal of Proteomics (issue 'Cancer Proteomics')
Guest editor special issue 2008	Journal of Proteomics (issue 'Organelle Proteomics')
Editorial board member	Journal of Proteomics
Coördinator	Netherlands Proteomics Platform
General Council Member	European Proteomics Association (EuPA)
Member	EuPA-HUPO interactions committee
Work Package Leader	EU kp6 STREP cNeuPro
Proteomics coördinator colon team	International Cancer Biomarker Consortium
Member	WP1 CTMM-Airforce consortium
Member	WPB CTMM-DeCoDe consortium
Member	Human Proteome Organisation
Member	American Society for Mass Spectrometry
Member	Dutch Society for Biochemistry and Molecular Biology
Member	Dutch Society for Mass spectrometry
Dr. Sander Piersma	
Member	American Society for Mass Spectrometry
Member	Netherland Proteomics Platform
Member	Dutch Society for Biochemistry and Molecular Biology
Member	Dutch Society for Mass spectrometry
Dr. Thang Pham	
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Member	Netherlands Proteomics Platform

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- Dr. Matthew Fitzgibbon, Fred Hutchinson Cancer Research Center, Seattle, USA
- Dr. L. Pasa-Tolic, Mass Spectrometry Facility, Pacific Northwest National Laboratory, USA
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- Dr. Simone Niclou, NorLux Neuro-Oncology Laboratory, Luxembourg
- Prof. S. Gammeltoft, Department of Clinical Biochemistry, Glostrup Hospital, Denmark
- Prof. dr. AL Burlingame, Mass Spectrometry Resource, University of California San Francisco, USA
- Dr. D. Gillooly (Invitrogen, Finland)
- Dr. M. Hornshaw (London, ThermoElectron)
- Dr. Matthias Glueckmann and Christie Hunter (Applied Biosystems)
- Dr, Gisela Brunagel, Univ. Bonn, Germany

National

- Dr. Monique Slijper, University of Utrecht, Netherlands Proteomics Center
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Local

- VUmc: See collaborator lists
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- Dr. Ka Wan Li, Prof. dr. Guus Smit, Dept. Molecular & Cellular Neurobiology, VU

APPENDIX: PROJECT SUMMARIES

High-Throughput biofluid peptide profiling using MALDI-TOF-MS

Prediction of outcome of non-small cell lung cancer patients treated with chemotherapy and bortezomib by time-course MALDI-TOF-MS serum peptide profiling

Johannes Voortman[1, 2*], Thang V. Pham[1*], Jaco C. Knol[1], Giuseppe Giaccone[1, 2], Connie R. Jimenez[1]
[1]OPL [2]Medical Oncology Branch, National Cancer Institute, NIH, USA

Background Only a minority of patients with advanced non-small cell lung cancer (NSCLC) benefit from chemotherapy. Serum peptide profiling of patients treated with gemcitabine, cisplatin and bortezomib was performed to discover patterns associated with treatment outcome.

Methods Using magnetic bead-assisted serum peptide capture coupled to matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF-MS), serum peptide mass profiles (spectra) of 27 NSCLC patients were obtained (pretreatment, after two cycles, and at end of treatment). Also, spectra were obtained from 13 cancer-free controls. Algorithms were established to classify for tumor response and progression-free survival and to distinguish NSCLC patients from cancer-free controls.

Results A signature of 47 m/z features could discriminate NSCLC patients from cancer-free controls with 98% accuracy at 100% sensitivity and 96% specificity. Comparing pretreatment sera, a 6 peptide ion signature could distinguish with 82% accuracy, sensitivity and specificity patients with a relatively short vs. long progression-free survival. Inclusion of 7 peptide ions showing differential changes in abundance during treatment led to a 13 peptide ion signature with 86% accuracy, 100% sensitivity and 73% specificity. A 5 peptide ion signature could separate patients with a partial response vs. non-responders with 89% accuracy at 100% sensitivity and 83% specificity. Identification of selected peptides by tandem MS showed (cleaved) derivatives of abundant serum proteins.

Conclusions (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 chemotherapy, potentially enabling patient selection.

Reference

Johannes Voortman, Thang V Pham, Jaco C Knol, Giuseppe Giaccone and Connie R Jimenez. Prediction of outcome of non-small cell lung cancer patients treated with chemotherapy and bortezomib by time-course MALDI-TOF-MS serum peptide profiling. Proteome Science. 2009. 2009, 7:34.

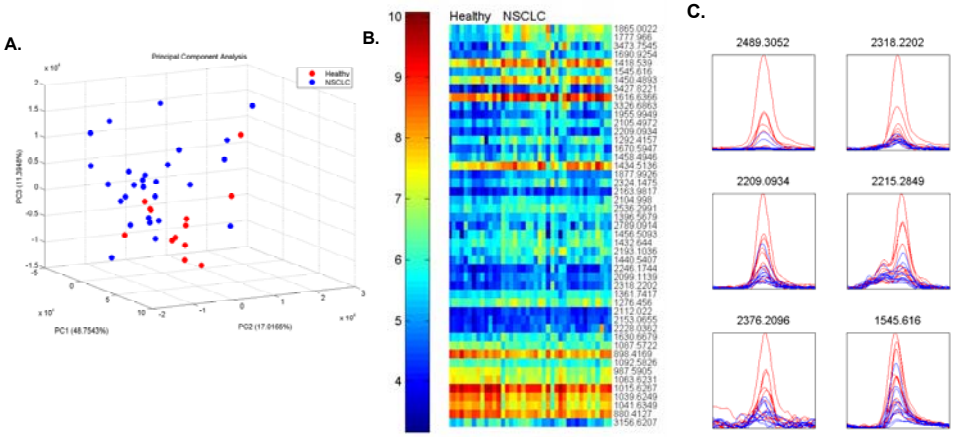

Figure . A, Principle Component Analysis (PCA) healthy vs. NSCLC comparison. B, heatmap of the 47 differential peaks in the healthy versus NSCLC comparison shown in the natural log scale. The peaks are ordered by median fold change between the two groups. C. Spectral overlay of ion intensities of the 6 peptides underlying the progression-free survival (PFS) signature: short PFS (red) and long PFS (blue).

Oral presentation at ASCO2008.

This research was supported by the VUmc Cancer Center Amsterdam

Time-course serum profiling in non-small cell lung cancer patients treated with sorafenib and erlotinib

Joline S.W. Lind [1], Thang V. Pham[2], Jaco C. Knol[2], Anne-Marie C. Dingemans[3], Harry J.M. Groen[4], Pieter E. Postmus[1], Connie R. Jimenez[2], Egbert F. Smit [1]

Departments of Pulmonary Diseases [1]VU University Medical Center Amsterdam, [3]Maastricht University Medical Center and [4]University Medical Center Groningen , [2]OncoProteomics Laboratory, Department of Medical Oncology, VU University Medical Center Amsterdam

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.

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.

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- Jiménez, CR, El Filali, Z, JC. Knol, Li KW, Hoekman, K, FAE Kruyt, Giaccone, G., Smit AB. Automated serum peptide profiling using novel magnetic C18 beads off-line coupled to MALDI-TOF mass spectrometry. (2007) Prot. Clin. Applic. 1(6), 598-604.

Serum Protease Activity Profiling coupled to MALDI-TOF mass spectrometry

Jaco C. Knol[1], Dorothe Linders [2], Olaf Tellingen [2], Hans Bonfrer [2], Connie R. Jimenez[1]

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With the advent of powerful mass spectrometric platforms affording swift, sensitive, and high-throughput analyses - such as present-day MALDI MS - large sets of small clinical blood samples can be mutually compared with multivariate statistics to discern a subset ("signature") of peptides and (small) proteins discriminating, e.g., healthy from diseased subjects. These peptides/proteins show up as m/z peaks in mass spectra ("profiles") after their capture from blood serum with chromatographic beads.
Although this promising technique of "classical" MS-based profiling has spurred a huge interest from the clinical world, it has not yet come to full fruition due to some complicating factors in serum constitution as well as sample collection, handling, and storage.
First, due to the huge dynamic range (~10^{11}) of serum protein concentrations, disease-specific traces are obscured by some 20 high-abundance proteins such as albumin. In fact, it has been found that disease signatures obtained in this way are largely made up of peptides generated by the action of (disease-specific) sets of exoproteases on abundant serum constituents, especially clotting and complement factors. Hence, profiling in this way does not uncover the culprits, i.e. the biomarker proteins themselves, but rather identifies their substrates within the normal serum peptidome/proteome, which can serve as "surrogate biomarkers".
Second, the formation of these surrogate markers is sometimes affected in cancer patients (malignant coagulopathy), and, more importantly, their formation/degradation is highly dependent on clinical sample collection and handling variables (clotting time, number of freeze-thaw cycles) as well as storage temperature and time. This renders a wealth of archived samples useless for profiling, and complicates clinical sampling procedures.

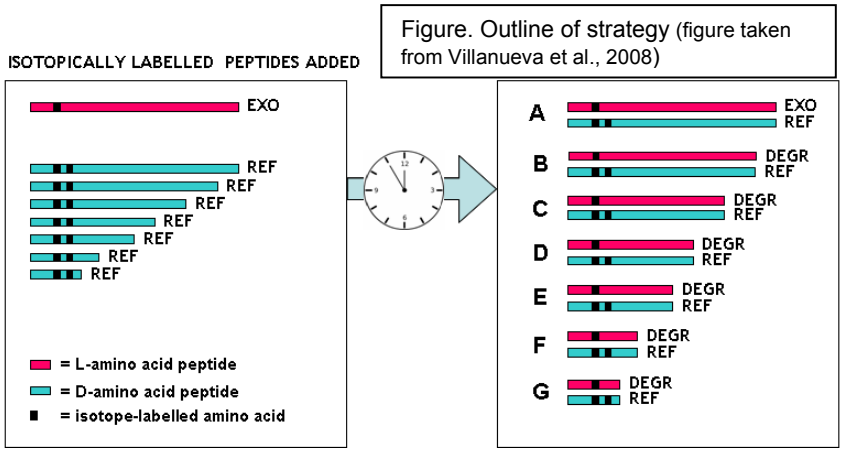
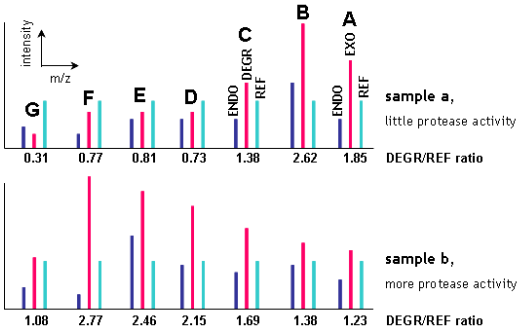


Figure. Outline of strategy (figure taken from Villanueva et al., 2008)

Recently, a novel strategy has been presented (SSEAT, for Sequence-Specific Exopeptidase Activity Test) by Villanueva et al (2008). Here, we do not look at the endogenously formed peptide products ("ENDO"), but rather at the action of the still-active proteases on synthetic peptides added to the serum. An isotopically labelled full-length peptide serves as a degradable protease substrate ("EXO"), and is added together with a set of non-degradable, doubly labelled synthetic peptides representing a ladder of degradation products ("REF"). The latter serve as internal ratioing standards for quantitation of the various EXO degradation products (DEGR) generated by the action of endogenous proteases on the EXO peptide after a specific incubation time (yielding multiple DEGR/REF ratios). The ENDO, DEGR, and REF peptides of the *same* sequence are separated in MS spectra due to the different degrees of isotopic labelling.

The SSEAT approach, spiking labelled substrates for trace amounts of enzymatic activities, may allow profiling in a background of high-abundant proteins, also using archived material which has largely retained its protease activities, irrespective of the way the samples were handled. The assay can be fully controlled and tuned (peptide concentrations, incubation times), although it requires tuning for each new



sample set.
It has been shown by Villanueva et al (2008) that, when using labelled EXO/REF sets for three serum components (C3f, FPA-A, and clusterin), multivariate analysis on all DEGR/REF ratios can generate a significant signature for thyroid cancer with high sensitivity (94%) and specificity (90%).

RESULTS We are in the process of testing these same tools on fresh and archived cancer sera. The first step is to optimize the assay for the particular serum set that will be analyzed. The figure below shows the results for testing the C3f substrate that was spiked and incubated for different time periods in serum prior to analysis by MALDI-TOF-MS. Measurement of peak intensities of substrate, breakdown products and reference peptide allows to create a kinetics profile (see figure).

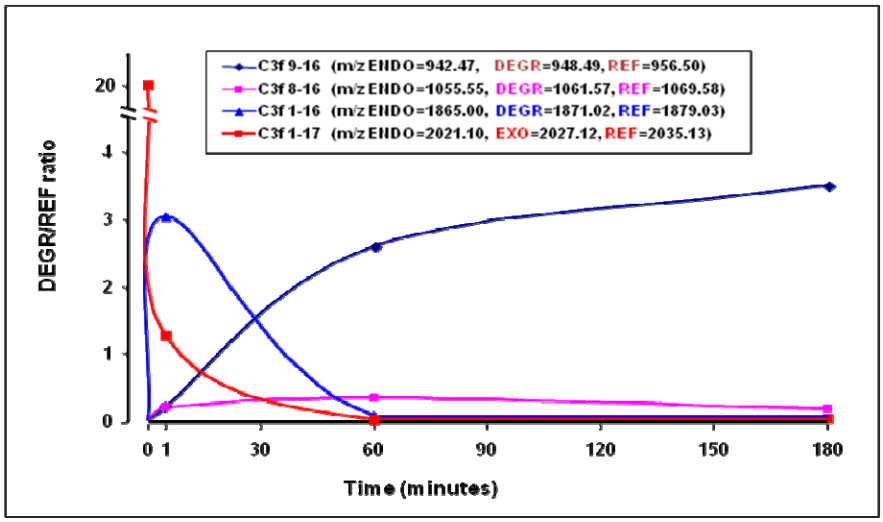


Figure. SSEAT monitoring of time-dependent proteolytic activity on an isotopically labelled EXO peptide added to human serum. The t=0 time points are the input values, the other time points were determined by ratioing the MS intensity of the respective DEGR and REF peptides. In this example, only the added EXO peptide (C3f 1-17, SSKITHRIHWESASLLR), the first degradation product (C3f 1-16), and the two smallest degradation products (C3f 8-16 and C3f 9-16) of the MALDI-detectable degradation ladder were followed. A swift carboxypeptidase-mediated removal of the C-terminal arginine residue of the EXO peptide is followed by slower aminopeptidase-mediated truncation at the other end of the peptide.

CONCLUSIONS The SSEAT assay generates reproducible kinetic profiles.Moreover, the assay can be applied to archived samples with very long storage times. Apparently, the endogenous proteases in serum are very stable.

OUTLOOK We intend to apply the SSEAT assay to our lung cancer serum sample collections which have already yielded signatures in traditional profiling experiments. This may also provide a way of validating the "classically" obtained signature. Furthemore, the SSEAT assays will allow for profiling precious archived materials for which clinical follow-up information is available that are not amendable to ‘classical’ serum profiling due to the long storage times.

Acknowledgments: *We are grateful for the SSEAT peptides received from Dr. Josep Villanueva and Prof. Paul Tempst of the Memorial Sloan Kettering Cancer Center and Josep for the useful discussions .*

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Identification of candidate biomarkers for axonal damage in Multiple Sclerosis by MALDI-TOF-MS-based CSF peptide profiling

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a NUBIN, Dept. of Molecular Cell Biology and Immunology, VUmc, Amsterdam. bOncoProteomics Laboratory, Department of Medical Oncology, c Dept. of Neurology , VU University Medical Center, Amsterdam, The Netherlands.

BACKGROUND: Body fluid biomarkers for clinical subtyping and monitoring and predicting disease progression are of considerable interest in MS. However, only few candidate biomarkers have been identified so far. In view of the heterogeneity of mechanisms in the MS pathology, several biomarkers reflecting several aspects of the pathology should be analysed simultaneously to accurately monitor ongoing pathology. Proteomics tools are the method of choice for the unbiased simultaneous detection of large series of peptides and proteins.

AIM: To identify novel candidate biomarkers discriminating RRMS from SPMS patients using a high-throughput MALDI-TOF/Mass spectrometry method which reproducibly detects native peptides and proteins < 20 kD.

METHODS: Paired CSF and serum of 40 RRMS, 28 SPMS patients, 13 PPMS patients and 12 patients with inflammatory neurological diseases (IND) and 23 patients with non-inflammatory other neurological diseases (OND) were analysed using MALDI-TOF-MS.

RESULTS: Comparative analysis of CSF and serum mass profiles of native small proteins and peptides between the clinical subgroups revealed peptide signatures that discriminated MS patients from the OND control group. In addition, other profiles of native small proteins and peptides in both serum and CSF discriminated RRMS from SPMS. Protein/peptide identification showed that among the candidate markers were fragments of secretogranin III, chromogranin A and proteolytic fragments of complement C3. The peak intensity a serum peptide with m/z value of 872.429 correlate to disease progression measures.

OUTLOOK: Using a high-throughput body fluid profiling by MALDI-TOF Mass spectrometry, promising candidate biomarker peptides were identified that were related to different disease stages of MS. The relation of some small proteins/peptides with different stages of relapse-onset MS patients and their relation with neuronal functioning warrant further studies into the use of these markers in relation to disease progression.

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- [P-WED-069] CSF PROTEOMICS TO IDENTIFY CANDIDATE BIOMARKERS FOR MULTIPLE SCLEROSIS. Authors: Charlotte Teunissen, Marleen Koel-Simmelink, Thang Pham, Laura van der Voort, Connie Jimenez Date: Wednesday, August 20, 2008. Poster Session: Biomarkers III

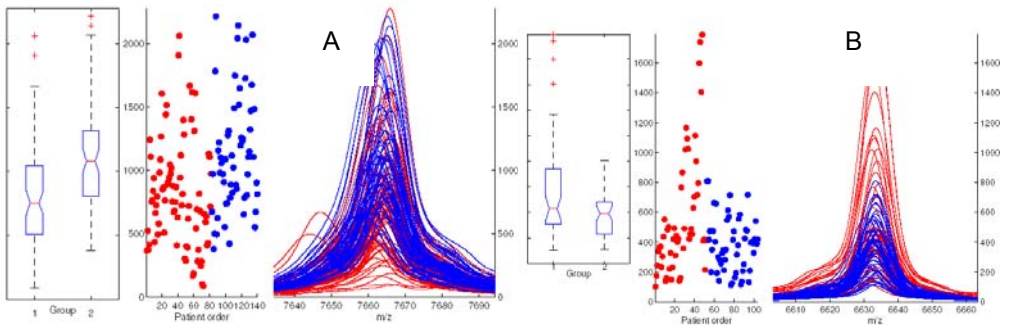

Figure. Examples of discriminating protein mass peaks in CSF (A) and serum (B) of MS patients (red) and neurological controls (blue).

Acknowledgements: Dutch MS Research Foundation, grant number 05-559 and VUmc Cancer Center Amsterdam

APPENDIX: PROJECT SUMMARIES

In-depth proteomics by nanoLC-LTQFTMS

Label-free quantitative secretome proteomics for discovery of serum-based cancer biomarkers

Workflow evaluation and proof-of-concept analysis in model system

Sander Piersma1, Ulrike Fiedler2, Michael Kubblat2 and Connie Jiménez1

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BACKGROUND

The secretome consists of proteins that are secreted, shed from the cell surface and intracellular proteins 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. Insulin and Insulin-like growth factors are well known as regulators of energy metabolism and growth. There is more and more evidence that both molecules play also a key critical role in neoplasias, such as diabetes and obesity but also in cancer. IGF1R expression was detected in human cancers and showed to have transforming activities. IGF1R signaling influences expression and signaling of numerous growth factors like VEGF and EGF associated with tumor growth.

AIMS

- Optimization of the proteomics workflow for secretome analysis, especially comparison of first-dimension separation methods prior to nanoLC-MS/MS in terms of number of identified proteins, reproducibility of identification and reproducibility of label-free protein quantitation.
- Identification of IGF1R-signalling associated proteins in the secretome of mouse embryonic fibroblasts transformed with IGF1R and stimulated by IGF1, and subsequent candidate marker verification in mouse serum.

APPROACH

- Three first-dimension protein/peptide separation strategies were evaluated for secretome nanoLC-MS/MS analysis. 1D Gel electrophoresis, protein hydrophobic interaction chromatography and peptide strong cation exchange chromatography were used to prefractionate H460 non-small cell lung cancer cell secretomes prior to (digestion and) nanoLC-MS/MS on an LTQ-FTMS.
- GeLC MS/MS was used for the differential analysis of the secretome of engineered mouse fibroblasts with controlled expression of IGF1R (Mef/Toff/IGF1R). Label-free quantitation was performed using spectral counting (the #MS/MS spectra per identified protein). Selected candidate IGF1R signalling-related proteins were verified in cell secretomes and in mouse sera by ELISA.

RESULTS

WORKFLOW EVALUATION

For a systematic comparison, the secretome of H460 non-small cell lung cancer cells was fractionated by each workflow in a biological triplicate analysis. 1D gel electrophoresis 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 for proteins detected in 3/3 replicates). Since small proteins are important in signalling (e.g. IL8, CXCL2 and cystatinB), 4-12% gradient gels are preferred over 10% homogeneous gels for optimal capture of proteins across the protein Mw range .
To identify secretome-enriched proteins, the ratio of normalised spectral counts for secretome and the

corresponding cell lysate was determined using the gradient GeLC-MS/MS method. At a cut-off of a secretome/lysate spectral-count ratio > 3, 568 secretome-enriched proteins were obtained (from 1092 secretome-identified proteins and 1736 lysate-identified proteins) that were enriched for GO annotations 'extracellular space' (28%) and 'plasma membrane' (18%), moreover, 62% had a predicted signal peptide for protein secretion. Having established a reproducible and sensitive workflow for quantitative secretome analysis that yields a sub-proteome data set enriched for secreted proteins, we applied the method for discovery of IGF1R signalling markers in a mouse model.

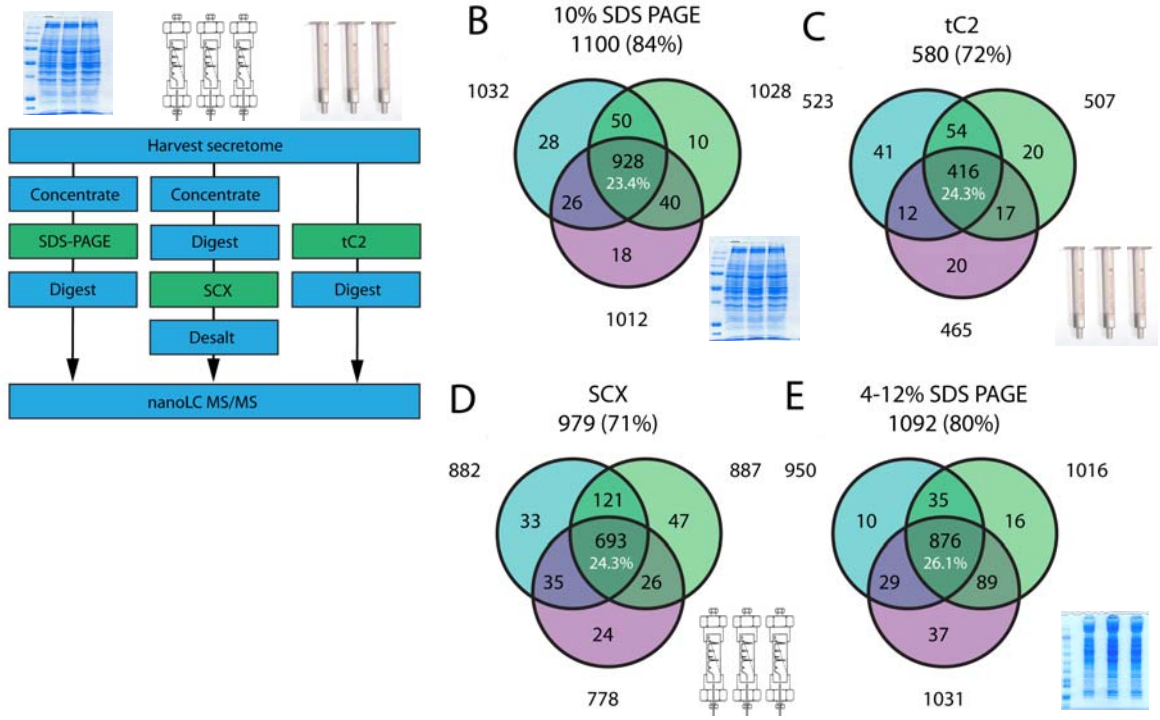

Figure. Evaluation of first-dimension separation strategies for secretome analysis by nanoLC-MS/MS. A. 3 first dimension separation workflows evaluated for secretome analysis. **B, C, D and E**. Venn diagrams of identified proteins in the secretome of H460 lung cancer cells N=3. total # IDs, # IDs/replicate, reproducibility(%) and Avg %CV on spectral counts of IDs in 3/3 replicates (in white).

PROOF-OF-CONCEPT IN MODEL SYSTEM

In order to identify IGF1R signalling-specific biomarkers, we applied a GeLC-MS/MS workflow to secretomes of mouse embryonic fibroblasts (MEFs) with IGF1R protein kinase expression switched on and off using a TET-inducible system. By stable transfection of IGF1R into MEFs, cells become transformed and grow tumors in mice. For differential secretome analyses MEF/Toff/IGF1R cells were grown in the presence and absence of doxycycline to modulate IGF1R expression, and in the presence and absence of IGF1 to stimulate IGF1R signalling. As controls the parental cell line MEF/Toff was grown in the presence or absence of doxycycline and in the presence or absence of IGF1. In total 1435 proteins were identified in the seven different secretome samples (MEF/Toff+Dox, MEF/Toff-Dox, MEF/Toff-Dox+IGF1, MEF/Toff/IGF1R-Dox MEF/Toff/IGF1R+Dox, MEF/Toff/IGF1R- Dox, MEF/Toff/IGF1R+Dox-IGF1, MEF/Toff/IGF1R-Dox+IGF1).

Analysis of MEF/Toff cells expressing IGF1R and stimulated with IGF1 to initiate IGF1R signaling yielded 136 regulated proteins, including 72 up-and 64 down-regulated proteins (p <0.01). Examples of up-regulated, secreted proteins from MEF/Toff/IGF1R cells during IGF1R signaling are laminin B (Fc 4.9 P0.001), VEGF (Fc>100, P0.0045), cathepsin B (Fc 3.6 P0.0012), TGFß (Fc>100 P0.0045), TIMP1 (Fc 15.5 P0.005), TIMP2 (Fc 4.0 P0.004), cathepsin L (Fc 9.2 P0.0028), carboxypeptidase E (Fc 1.9 P0.0067), and osteopontin (Fc>100 P0.0022). VEGF and osteopontin were verified in secretomes by ELISA. The results corroborate the LC-MS/MS data.

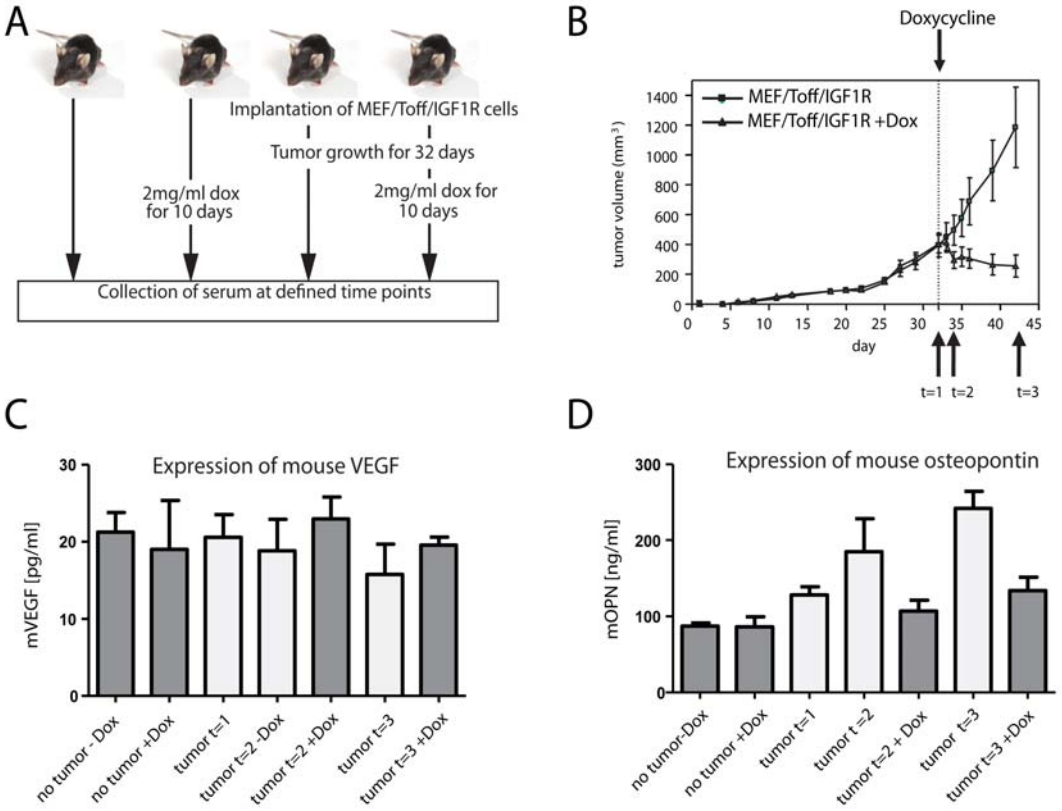

Figure: Quantitative secretome proteomics as a candidate blood-based biomarker discovery strategy in a mouse tumor model.
A MEF/Toff/IGF1R cells were grown in NMRI nu/nu mice. After establishment of tumors half of the tumor bearing mice received doxycycline via the drinking water for 10 days to inhibit IGF1R expression in the tumors. **B** The tumor regressed. 200 µl blood was taken at day 32 (t=1), before addition of doxycycyline to the drinking water, at day 34 (t=2) when tumors went into regression phase and at the end of the experiment when mice were sacrificed (t=3. Additionally, sera of mice without tumor and of mice without tumor but treated with doxycycline for ten days were collected. **C** Serum levels of mouse VEGF and **D** mouse osteopontin were analyzed by ELISA.

Follow-up in serum of mice 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 (in response to doxycyclin administration) and the tumor regressed. VEGF levels in serum were very low, this can most likely be attributed to active sequestration by platelets.

OUTLOOK
Verification of osteopontin in serum in an *in vivo* IGF1R model system illustrates the potential of secretome proteomics in model systems for candidate biomarker discovery. Additionally, it shows that the label-free GeLC-MS/MS strategy yields rich and reproducible data that can be followed-up successfully in a targeted analysis of candidate biomarkers.

REFERENCE
[P MON-158] THE SECRETOME OF H460 NON SMALL CELL LUNG CANCER CELLS: COMPARING WORKFLOWS Authors: Sander Piersma, Simone Span, Connie Jimenez Date: Monday, August 18, 2008, Session: Poster Session: Innovation In Peptide & Protein Seperation Technologies

This work was supported by the EU fp6 Angiotargeting project and has been submitted to J. Prot. Res.

Secretome proteomics of stimulated platelets

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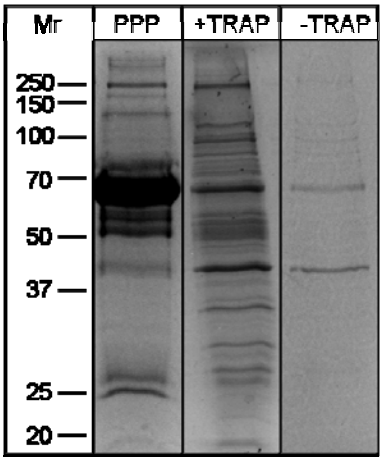
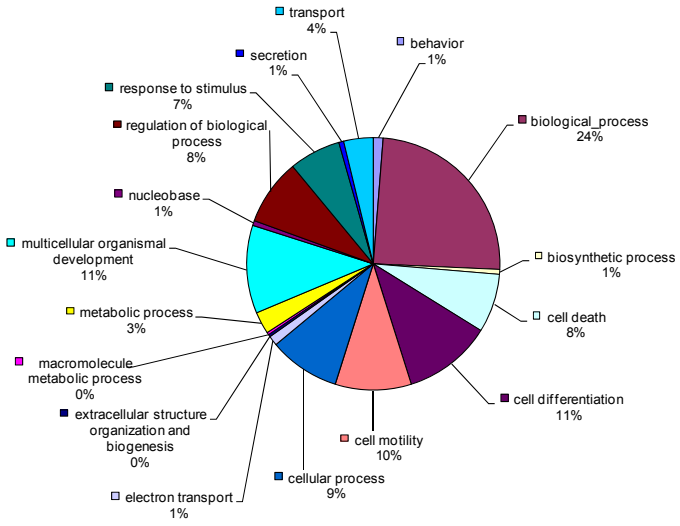
BACKGROUND. Upon stimulation, platelets release the soluble content of their cytoplasmic granules. This sub-proteome is of interest since many of its constituents are associated with coagulation, (tumor) angiogenesis, cell growth and adhesion. Previously, differential –antibody-based- serum analysis has yielded information on the proteins released from platelets upon stimulation. A promising alternative strategy is formed by identifying the proteins released by freshly isolated platelets from blood using proteomics.

APPROACH Here we report on the analysis of the thrombin receptor activating peptide (TRAP)-induced releasate (secretome) from 3 different volunteers using high resolution, high mass accuracy hybrid LTQ-FT mass spectrometry in a GeLC-MS/MS workflow.

RESULTS. We obtained a core platelet secretome of 225 proteins present in the releasate of 3/3 volunteers from a total of 716 identified proteins. This core dataset is characterized by gene ontology mining and signal peptide analysis. Compared with the platelet-secretome published previously, this high-accuracy platelet secretome represents by far the largest and most comprehensive analysis to date.
Our study highlights the much higher sensitivity in detection of platelet-derived proteins when the background of abundant plasma proteins is avoided.

OUTLOOK. This approach offers unique possibilities to analyse the role of platelet-secreted proteins in physiology and in diseases such as atherosclerosis and cancer and may provide candidate biomarkers.





Reference

Sander R. Piersma, Henk J. Broxterman, Muhammed Kapci, Richard R. de Haas, Klaas Hoekman, Henk M.W. Verheul, Connie R. Jiménez (2008) Proteomics of the TRAP-induced platelet releasate. J Proteomics. 2009 Feb 15;72(1):91-109. Epub 2008 Nov 8.

This research was supported by the VUmc Cancer Center Amsterdam

Identification of new serum biomarkers for Colon Cancer by analysis of secreted proteins from CRC cell lines

M. de Wit[1], RJA. Fijneman[1], S. Piersma[3], MP. van Berkel[2], E. Boven[2], GA Meijer[2] and CR. Jimenez[3]

Depts. of [1]Pathology, [2]Medical Oncology, [3]OncoProteomics Laboratory, VU University Medical Center, Amsterdam, the Netherlands.

BACKGROUND: Detection of colorectal cancer (CRC) at an early stage of disease is the most realistic approach to reduce the currently high mortality rates. Molecular imaging with biomarkers that discriminate CRC from healthy colon could be an effective approach for detection. Proteins that are secreted from a tumor could be imaged showing a "hotspot" of protein secreting cells and the surrounding (new) vessels of the tumour. For example, the well known tumor marker CEA is produced by the tumor cells and secreted into the bloodstream. This protein is now used in the clinic as a marker to monitor treatment response and there is a PET tracer developed targeting CEA (IMMUNOMEDICS, arcitumomab) to detect distant metastasis or local recurrence of CRC see figure 1.

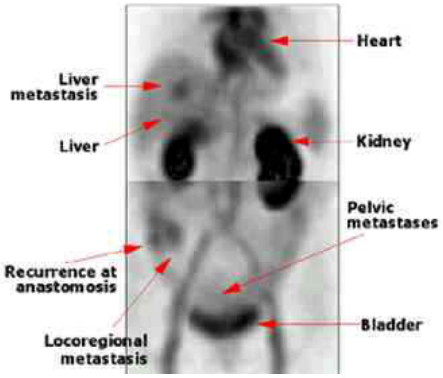


Figure 1

AIM: The aim of this project is to identify biomarkers for CRC. These biomarkers are secreted and can therefore be detected in the tissue surrounding the tumor, the stroma, or even in the bloodstream.

APPROACH: Adherent colon cancer cell lines (HT-29, SW1398, SW480, Caco-2 and HCT116) were cultured until 70-80% confluency. The cells were 24 hours prior to harvesting washed and cultured on serum-free medium. Medium was collected and concentrated by ultrafiltration (10 kDa cut-off). Equal amounts of secreted proteins as well as a pool of cell lysates were fractionated by 1D gradient SDS-PAGE (see figure 2). This gel was then further processed for in-depth proteomics analysis by liquid-chromatography followed by tandem mass spectrometry (nanoLC-MS/MS).

RESULTS: A total of 3020 proteins were identified, of which 565 were identified only in the combined secreted protein fractions, 1933 proteins were found in the secreted protein fractions as well as in the pool of cell lysates and 522 were found only in the cell lysates.
A gene ontology search indicated that the secreted protein fractions were significantly enriched for extracellular space annotation and extracellular region part, whereas the annotations for mitochondrion and organelle part were depleted.

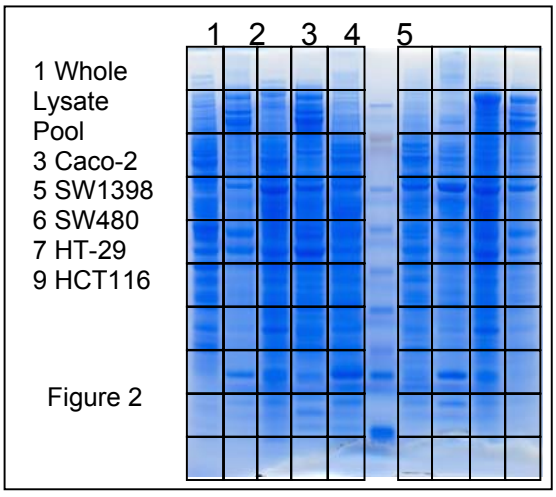


Figure 2

OUTLOOK: Currently we are extending these experiments to tissue secretomes using CRC tissue and corresponding healthy mucosa. A combination of these datasets will lead to the selection of candidate markers of which we would then like to confirm the presence in blood of CRC patients.

This research was supported by the VUmc Cancer Center Amsterdam

Proteome profiling of mouse colon tumor proximal fluids reveals candidate biomarkers for colorectal cancer screening

Remond J.A. Fijneman[1,2], Meike de Wit[1], Maral Pourghiasian[1,2], Sander Piersma[2], Marc Warmoes[2], Pien M. van Diemen[1], Chloe Piso[1], Els C. Robanus-Maandag[3], Ron Smits[4], Riccardo Fodde[4], Victor W.M. van Hinsbergh[2,5], Gerrit A. Meijer[1] and Connie R. Jimenez[2]

1Dept. of Pathology, 2Dept. of Medical Oncology, 5Dept of Physiology, VU University Medical Center, 3Dept. of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands 4 Dept. of Pathology, Josephine Nefkens Institute, Erasmus MC, Rotterdam, the Netherlands

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 novel protein biomarkers that can be used for development of a screening test for early diagnosis of CRC.

Approach: Straightforward biomarker discovery from human blood by proteomics is hampered by sample complexity and heterogeneity. The tumor “secretome” is a rich source of tumor-derived proteins. It comprises proteins that are either secreted, shed by membrane vesicles (exosomes), or externalized due to cell death, and provides a new avenue for discovery of cancer biomarkers. Mouse models for human disease lack genetic heterogeneity and allow immediate analysis of freshly collected tumor- and matched control samples. Therefore, to maximize sample biomarker concentrations and minimize sample heterogeneity, proximal fluids were collected from colon (tumor) tissues obtained from a mouse model for sporadic CRC. Three control and three tumor samples were subjected to in-depth proteome profiling by a GeLC-MS/MS workflow.

Results: A total of 2172 protein IDs were identified from proximal fluids of colon (tumor) tissues. Spectral counting-based quantitative comparison revealed 99 proteins with at least 3-fold higher excretion by tumors (FDR<0.10), many of which were connected to molecular pathways underlying CRC development (Figure 1).
To enrich for biomarker sensitivity and specificity, candidates were restricted to proteins identified in every tumor sample while being undetectable in controls, and acute phase response proteins were excluded. This stringent selection procedure yielded 50 candidate CRC biomarkers. Five of these proteins were previously published as human blood- or stool-CRC-biomarkers, supporting the validity of our approach. For two proteins, S100A9 (Figure 2) and MCM4, differential expression between tumor samples and control tissues was visualized by immunohistochemistry.

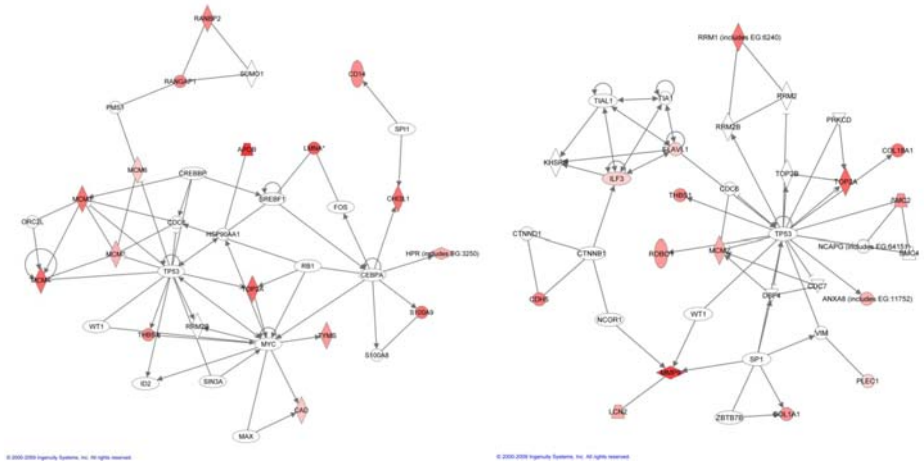


Figure 1: IPA-retrieved cancer-related Top Networks.
Network 1 (left) and Network 2 (right) represent candidate biomarkers (red, intensity dependent on fold-difference compared to control tissue) that were molecularly connected to each other through IPA pathway analysis. Non-identified interconnecting proteins are blanc.

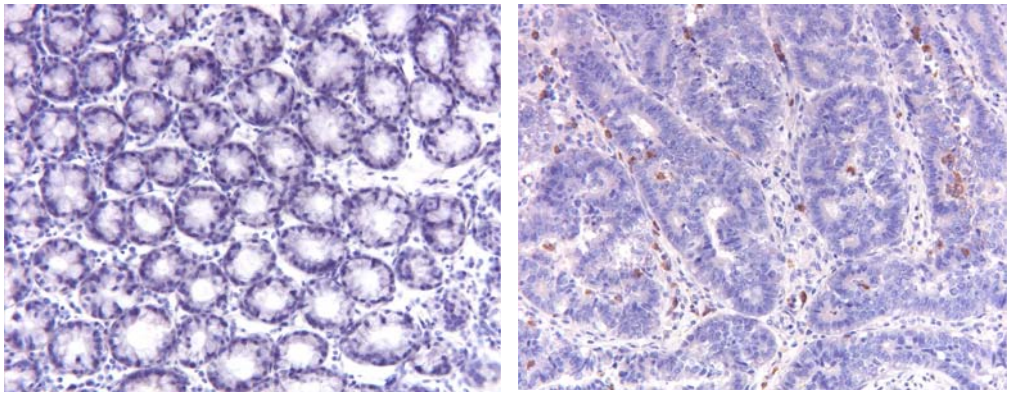


Figure 2: Immunohistochemical staining for S100A9.
Normal colon (left) is negative, while colon tumor (right) shows S100A9 positivity in some cells within tumor stroma and in necrotic debris.

Outlook We conclude that combining mouse models for human disease with in-depth proteomics of proximal fluids is a powerful strategy to discover novel candidate biomarkers for CRC from a limited number of samples. This strategy may be generally applicable to boost biomarker discovery for several types of cancer and other complex diseases.

Reference

[P-MON-046] IDENTIFICATION OF COLORECTAL CANCER BIOMARKERS FROM MOUSE COLON TUMOR SECRETOME Authors: Remond J.A. Fijneman, Meike de Wit, Maral Pourghiasian, Sander Piersma, Pien M. van Diemen, Victor W.H. van Hinsbergh, Ron Smits, Riccardo Fodde, Gerrit A. Meijer, Connie R. Jimenez Date: Monday, August 18, 2008. Session: Poster Session: Biomarkers I

Manuscript to be submitted to JCI

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Proteome profiling of human colon (tumor) tissue proximal fluids reveals candidate biomarkers for colorectal cancer screening

Huub Kant[1], Meike de Wit[2], Sander R. Piersma[1], Thang V. Pham[1], Gerrit A. Meijer[1], Remond J.A. Fijneman[2], Connie R. Jimenez[1]

[1]OncoProteomics Laboratory, Dept. of Medical Oncology, [2]Tumor Profiling unit, Dept. of Pathology, VU University Medical Center, Amsterdam, The Netherlands

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 novel protein biomarkers that can be used for development of a screening test for early diagnosis of CRC.

Approach: The tumor "secretome" is a rich source of tumor-derived proteins. It comprises proteins that are either secreted, shed by membrane vesicles (exosomes), or externalized due to cell death, and provides a new avenue for discovery of cancer biomarkers. Fresh human normal colon, colon adenoma and carcinoma tissue samples were obtained directly following surgery. Upon a brief washing procedure, tissues were incubated in PBS at 37°C for one hour to collect proximal fluids containing the tumor secretome. Proximal fluids of normal and tumor tissues from 4 patients were subjected to in-depth proteome profiling by a GeLC-MS/MS workflow.

Results: A total of 2898 proteins were identified from proximal fluids of normal colon, colon adenoma and carcinoma tissues. A total of 399 proteins were consistently more secreted by tumor samples than by normal colon control samples. Of these, 166 proteins were previously identified in the secretome of multiple CRC cell lines, confirming that many of these proteins are derived from neoplastic cells.

Outlook We conclude that proximal fluid protein profiling of human colon tumor tissues and patient-matched normal colon controls is a powerful strategy to discover novel candidate biomarkers for CRC. A selection of candidate biomarkers will be used for validation studies, using large series of blood and stool samples from CRC patients and control subjects.

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

Proteomics of exosomes secreted by cancer cell lines

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1OncoProteomics Laboratory, Dept. Medical Oncology, 2Dept. Pathology, VU University Medical Center, Amsterdam, The Netherlands

BACKGROUND Exosomes are 10-120 nm microvesicles originating from the plasma membrane of cancer cells, as well as a variety of normal cells. After the fusion of multivesicular bodies with the cell membrane, exosomes are released into the blood and into the lumen of luminal organs. Exosomes contain membrane, cytoplasmic and nuclear proteins and are detectable in all body fluids. In most cases exosomes carry tumor-specific antigens.

AIM The aim of our project is biomarker discovery in human colorectal cancer, using tumor-derived exosomes as platform.

APPROACH Our approach is a high-throughput mass spectrometric-based qualitative and quantitative comparison analysis of the protein profiles of exosomes released by several human colorectal cancer model cell line, as well as a number of other human model cancer cell lines and human primary and benign cells. These include human colorectal cancer cell lines HT29, HCT116, SW480, SW1398 and CaCo2, human lung cancer H460, human breast cancer MCF-7 and human prostate cancer cell lines PC3 and LNCaP. The primary human cells are endothelial cells and keratinocytes. We will also include a benign human intestinal epithelial cell line (HIEC) in our experiment.

METHODS As the first step we established a reproducible exosome isolation procedure that guaranties high purity of our exosome samples. To this end we use differential centrifugation including two ultracentrifugation steps at 100.000 x g force. For the validation of purity of our exosomes, besides searching for specific exosome markers in our dataset, we also use electron microscopy and western blot (see the slide).
We compare the exosome protein profile of each cell type with the protein profile of the cell lysate and secreted soluble proteins from the same cell type, as well as with the exosomes from other cell types. Our criteria for the recognition of a protein are: detection of 2 peptides with ≥ 95% probability per protein and overall protein probability of ≥ 99%.

RESULTS At the moment we are still in the measurement and preliminary analysis phase. Up until now we have been able to detect an average of 1300-1400 exosome proteins per cell type. This number is 2-4 times larger than the most of published data by other groups.

RESULTS: The preliminary results are promising and show:
1- Our exosome isolation procedure is reproducible and guaranties high purity of samples.
2- The number of detected exosome proteins by us is very large.
3- Exosomes could be considered as a relevant new platform for biomarker discovery in colorectal cancer because:

- Universally accepted tumor-specific antigens are present in exosomes.
- Proteins belonging to well-established oncogenic pathways are highly present in tumor-derived exosomes.
- Exosome isolation procedures are highly reproducible.

OUTLOOK In the future, we intend to establish a panel of colorectal cancer-specific proteins present in exosomes. For the validation of these potential biomarkers in vivo we intend to use antibodies against the candidate biomarkers in different body fluids and faeces.

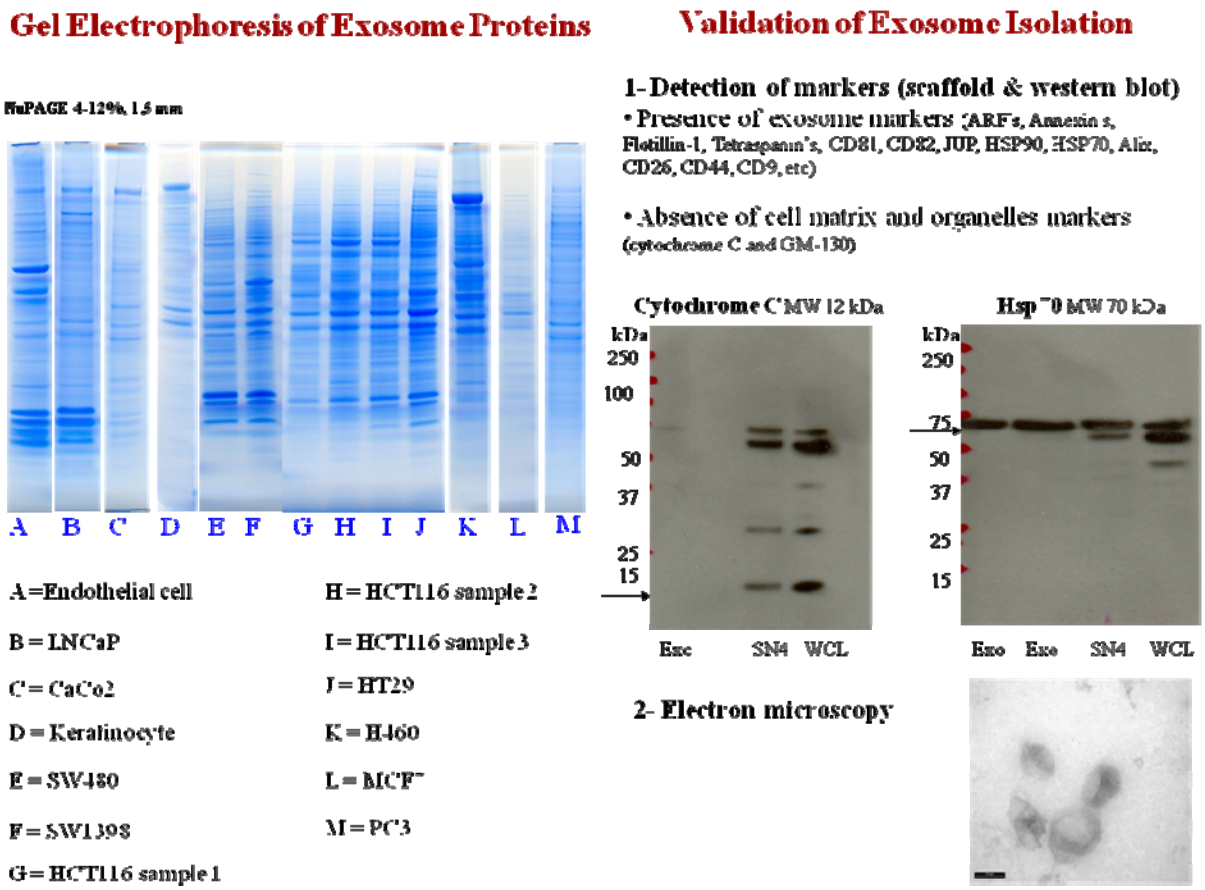


Figure. LEFT: Fractionation of exosome proteins isolated from a panel of cancer and primary cells. Overlapping but different protein band patterns can be seen in each lane.
RIGHT: Validation of the exosomal preparation by western blotting for known markers and contaminant proteins and by electron microscopy. The blot clearly show in the exosome lanes absence of contaminants and presence of known markers, thereby validating the preparation.

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

Subnuclear proteomics of colorectal cancer tissue

Jakob Albrethsen[1], Jaco C Knol[1], Sander Piersma[1], Meike de Wit[2], Sandra Mongera[2], Beatriz Carvalho[2], Gerrit A Meijer[2], and Connie R Jimenez[1]

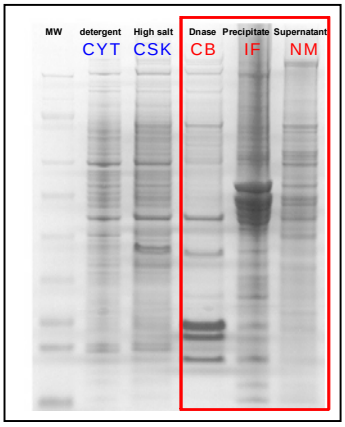
[1]OncoProteomics Laboratory, VUmc-Cancer Center Amsterdam, Dept. Medical Oncology, [2]Dept. Pathology, VU University Medical Center, Amsterdam, The Netherlands. www.oncoproteomics.nl

Background: Abnormalities in nuclear shape and chromosome structure are key features of cancer cells. Protein determinants of nuclear sub fractions may reflect aberrant chromosome function and chromatin organization and in addition yield cancer biomarkers. Here we evaluate a subnuclear fractionation protocol coupled to in-depth proteomics by GeLC-MS/MS for profiling of protein constituents in sub-nuclear domains in colorectal carcinoma tissue samples.

Aims: 1. Establish workflow for subnuclear fractionation in colorectal cancer tissue that yields 3 subnuclear fractions: chromatin-binding (CB), intermediate filament (IF) and nuclear matrix (NM). 2. Assess reproducibility of the isolation procedure. 2. Identification of subnuclear compartment enriched proteins, associated functions and cancer-associated proteins.

Methods: Subnuclear fractionation was employed using an established biochemical protocol for differential extraction of the CB, IF and NM fractions. The proteins were subjected to a label-free proteomics discovery workflow consisting of GeLC-MS/MS using the LTQ-FTMS and database searching using SEQUEST. Scaffold was used to organize the gel-band data and to validate peptide and protein identifications. Protein identifications with a probability of >99% with 2 peptides or more were retained and the total number of MS/MS spectra detected for each protein (spectral counts) was used for relative quantitation for enrichment and differential analysis.

Results: In three pooled sub-nuclear fractions from fresh-frozen CRC tumor tissue, we identified a total of 2,020 non-redundant proteins. Gene ontology mining using differentially enriched proteins revealed specific enriched functions in the 3 fractions. For example, the CB fraction was significantly and uniquely enriched for proteins associated with “Chromatin assembly and disassembly”, the IF fraction was uniquely enriched for proteins involved in “RNA export from nucleus,”, and the NM fraction was uniquely enriched for proteins implicated in “protein-DNA complex assembly”. Importantly, especially the CB and NM fractions contained many proteins previously implicated in oncogenesis. Visualization of the cancer-associated proteins in interaction networks revealed grouped in opposing ends of the network, suggesting that cancer-related nuclear proteins are extracted together with their in vivo interaction partners. Finally, in a triplicate analysis of a single NM fraction, 888 of 1,047 proteins (85%) were reproducibly identified at 28% reproducibility of peptide counts, indicating reasonable reproducibility.



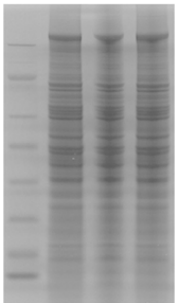


Figure
Reproducible protein band pattern in 3 independent NMP isolations

High (85%) overlap of identified proteins

Conclusions
Sub-nuclear proteomics of tumor tissue is a promising avenue for exploring colorectal oncogenesis and yielded differential enrichment of important cancer-related proteins especially in the chromatin-binding and nuclear matrix fractions.

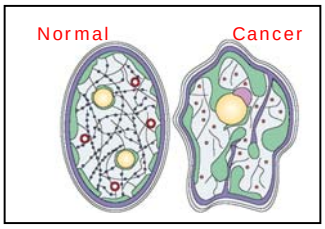
Outlook
Subnuclear proteomics is currently extended to the differential analysis of adenoma to carcinoma progression and comparison of chromosomal instable and microsatellite instable CRC.

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

Sub-nuclear proteomics in colorectal adenoma to carcinoma progression identifies novel candidate biomarkers

Jakob Albrethsen1, Jaco C Knol1, Sander Piersma1, Meike de Wit2, Sandra Mongera2, Beatriz Carvalho2, Gerrit A Meijer2, and Connie R Jimenez1

1OncoProteomics Laboratory, VUmc-Cancer Center Amsterdam, Dept. Medical Oncology, 2Dept. Pathology, VU University Medical Center, Amsterdam, The Netherlands. www.oncoproteomics.nl



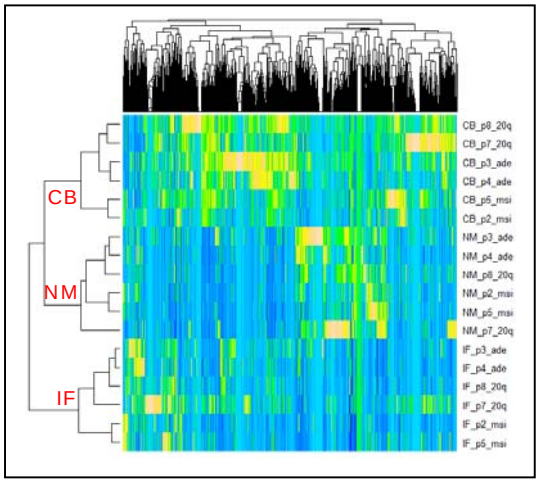
Background: Abnormalities in nuclear shape and chromosome structure are key features of cancer cells. Protein determinants of nuclear sub fractions may reflect aberrant chromosome function and chromatin organization and in addition yield cancer biomarkers. Here, we apply subnuclear proteomics to the analysis of protein constituents in sub-nuclear domains in colorectal adenoma and carcinoma tissue samples.

Aim: Identification of chromatin-binding, intermediate filament and nuclear matrix proteins associated with adenoma to carcinoma progression in chromosomal instable colorectal cancer

Approach: Subnuclear proteomics of well-defined colorectal adenoma and carcinoma tissues (genomic instable 20q+ and microsatellite instable)

Results: Proteomics of colorectal adenoma and the 2 carcinoma types (N=3 per group) yielded a total dataset of 2059 proteins with 1153 proteins in the NM, 1351 in the IF and 1856 in the CB fractions. Beta binomial statistics was applied to identify regulated proteins in adenomas versus both types of carcinomas and versus the CIN and MIN cancers separately.Sofar we focused on the CB and NM proteins. 129 NM and 338 CB proteins are significantly regulated in the 2 group comparison adenoma-carcinoma and 169 NM and 360 CB proteins are regulated in the adenoma-CIN comparison. Many known (colorectal) cancer-associated proteins and drug targets are among the upregulated proteins. The number 1 top networks associated with differential CB proteins is 'Cancer, DNA Replication, Recombination, and Repair, Molecular Transport'. The number 1 top network associated with differential NM proteins is 'Molecular transport, RNA trafficking, cancer'.

Conclusions
Sub-nuclear proteomics of colorectal adenoma and carcinoma tissues has yielded hundreds of proteins associated with genomic instable and microsatellite instable CRC, including known cancer-related proteins as well as novel ones.



Outlook
We are currently integrating different datasets to select candidates for follow-up. A selection of novel proteins up-regulated in chromosomal instable CRC will be validated on in tissue microarrays and followed up in stool and serum using targeted mass spectrometry and antibody-based detection methods. When validated these proteins may find use in CRC screening or patient stratification for therapy.

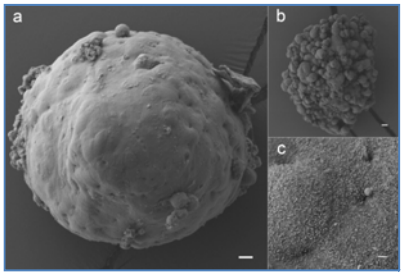
This research is supported by the VUmc-Cancer Center Amsterdam.

Enrichment of tumorigenic stem-like cells in biopsy spheroids from colorectal cancer

Uros Rajcevic1, Sander Piersma2, Sébastien Bougnaud1, Thang V. Pham2, Eirik Sundlisaeter3, PerØyvind Enger3, Rolf Bjerkvig1,3, Simone P. Niclou1 and Connie R. Jimenez2

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

Background. In some tumors, including colorectal cancer (CRC), only a small population of cells has tumor initiating potential. Identification and characterization of colorectal tumorigenic cells may provide novel diagnostic biomarkers and targets for therapy.



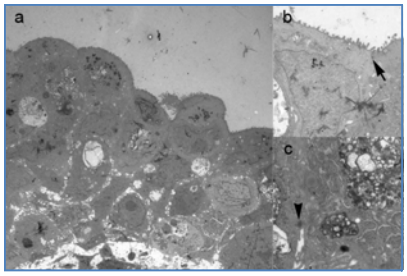


Figure. Scanning electron microscopy (left) and transmission electron microscopy (right) images

Approach. We have developed a culture model that enriches for stem cell-like cells from primary tumor biopsies. Here, we apply this three-dimensional spheroid culture model to colorectal cancer tissue and use it in a large scale proteomics study.

Objective. To identify novel markers of colorectal tumorigenic cells.

Methods. Tumor spheroids were prepared from tissue samples from patients with colorectal carcinoma and were compared to corresponding biopsies in a 'geLC-MS' (gel-liquid chromatography-mass spectrometry) experiment using nano-LC LTQ-FT system. Expression of proteins was assessed by spectral counting and the proteins of interest were validated using immunohistochemistry.

Results. Using a 'geLC'-MS approach, 1366 proteins were identified (at least 2 peptides sequenced, C.I. ≥ 99%) across the samples of the three patients. With the spectral counting quantification of the relative abundance in protein expression 342 proteins were found regulated in 3 patients (± 1.5 fold, $p<0.05$). 169 were upregulated, and 173 were downregulated in the spheroids compared to the corresponding tumor tissue. The list of non-redundant proteins was searched for gene ontology (GO) annotation and KEGG pathway functional annotation of the regulated proteins using FatiGO and IPA. Top significant biological processes terms included: Cell adhesion, Cell Communications, Response to stress. Top significant molecular function terms included: Catalytic activity, Protein binding and Oxidoreductase activity, while top significant cell component terms included: Organelle, Non-membrane-bound organelle, Receptor complex. Expression of several proteins, including those from the Wnt signalling pathways (Rac1), were validated by immunohistochemistry on colorectal tumor tissue and corresponding spheroids.

Conclusion. Enrichment of proteins from non-canonical/planar cell polarity (PCP) 'wnt signaling pathway', as well as proteins from 'colorectal cancer pathway' in the cells forming colon tumor spheroids, compared to the colon cancer tissue, is consistent with an enrichment of colorectal cancer stem cells (Co-CSC) in the spheroids. This points out potential new marker candidates for Co-CSC involved in the deregulation of cancer stem cell signaling that are not a part of the CD marker system.

This work has been accepted for an oral presentation at the HUPO2009 meeting in Toronto.

***Acknowledgments**: This research was supported by the VUmc-Cancer Center Amsterdam*

Comparative analysis of cell surface proteins proteins in closely related metastatic and non-metastatic osteosarcoma cells

Jantine Posthuma de Boer[1], Marco Helder[1], prof B.J. van Royen[1], Connie Jimenez[2]

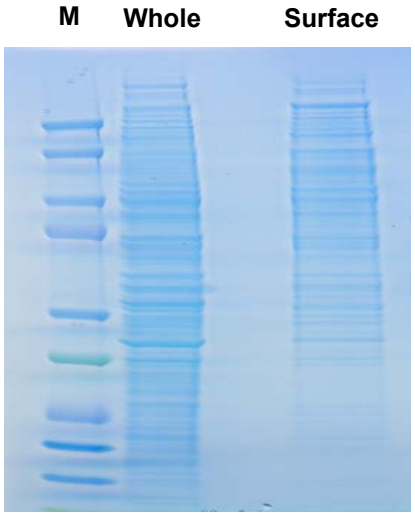
1. Dept. of Orhtopaedic Surgery, VU University Medical Centre, Amsterdam
2. Oncoproteomics Laboratory, VU University Medical Centre, Amsterdam

Background Osteosarcoma is the most common primary bone tumor in children and adolescents. The majority arise in the metaphysis of long bone, mostly in the distal femur and proximal tibia. It has a high tendency to systemic spread, with 80% of all metastases arising in the lungs. 30% of all patients have metastatic disease at time of prognosis. The overall 5y-survival rate is 65%. In patients with spread disease there is a reduced survival rate of only 20-30%. Enhanced treatment of osteosarcoma lung metastases is a valuable step for patients with osteosarcoma.

Aim The aim of this study is to identify proteins that are involved in the process of metastasis, both in the establishment and survival in distant tissue. These molecules could serve as therapeutic target in the treatment of osteosarcoma lung metastases. They could also be of prognostic value.

Approach We cultured human osteosarcoma cells without (SaOS-2) and with high metastatic potential (SaOS-LM7). The cultured cells are pre-treated with Sulfo-NHS-SS-Biotin prior to lysis. Biotinylated (surface) proteins are isolated from whole cell lysates, followed by elution from the biotin. 1D-gel-electrophoresis is used to fractionate the surface protein samples. In-gel-digestion follows and further work-up for Mass Spectrometry.
We verify the biological behaviour of the cells using a nude-mouse model in which human osteosarcoma lung metastases are induced upon tail vein injection of the metastatic cells.
After data analysis and hit-picking, verification of proteins of interest in primary osteosarcoma and metastasis patient samples will be done by immunhistochemistry.

Results A test capture in a non-metastasising osteosarcoma cell line showed enrichment of surface protein in the biotinylated fraction. Among these were well known surface molecules such as integrins, growth factor receptors, MMP's and the CAR receptor. These proteins are known to be expressed on the cell surface of osteosarcoma cells.



Outlook
Our pilot analysis indicated that we successfully captured cell surface proteins from osteosarcoma cell.

We are currently expanding the cells to sufficient amounts for a differential cell surface proteome analysis of metastatic and non-metstatic cells and we will use cells from the same batch in the mouse model.

Protein Composition of EBV-infected B cell secreted Exosomes

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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 antigen presenting B cells have a distinct protein composition comprised of approximately 100 proteins. Most notably, 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 and others have shown that Epstein Barr Virus (EBV)-infected transformed B cells and tumor cells secrete exosomes that suppress T cell function. EBV positive tumors secrete exosomes carrying the virally encoded protein Latent-Membrane Protein 1 (LMP1) and the immunomodulatory protein Galectin 9. Under experimental conditions purified LMP1 inhibits T cell function and thus LMP1 positive exosomes may contribute to immune-escape of EBV positive tumors.

AIM: Define protein composition of immunomodulatory exosomes secreted by EBV-infected B cells

Methods: Using ultra-centrifugation purified fractions of B cell secreted exosomes are analyzed by LC-LTQ-FT Mass Spectometry (MS). Purified viral proteins and exosomes lysates were subjected to a tryptic digest in a single SDS-Page band and analyzed by LC-LTQ-FT MS. Peptides were queried against an extended database containing EBV protein sequences for presence of viral proteins.

Results: We have performed LC-LTQ-FT MS with exosome fractions derived from EBV infected B cells. We detected approximately 350 exosomal proteins including MHC class I and II molecules and the exosomal marker proteins HSP90, HSP70, CD81, CD82, CD9, flotillin-1 and alix. Negative exosomal marker Cytochrome C was not detected. Presence of Hsp70, HLA-DR and Alix and the absence of Cytochrome C were confirmed by western blotting.
We found a near analogous protein profile as published previously using a MALDI-TOF mass spectrometer. Several exosomal proteins such as the tetraspanin CD63 and viral LMP1 and LMP2 were clearly detected on western blot in our exosome preps, but were not yet identified by MS analysis. One reason may be the complexity of the exosome proteome and the fact that in this analysis all exosomal proteins were analysed in one nanoLC-MS/MS run. With more fractionation ~3x more proteins are identified in exosomes (Jimenez and Lavei, unpublished data)..
Furthermore,investigations on predicted trypsin cleavage maps and MS analysis of concentrated EBV proteins have shown that LMP1 and LMP2 are not identifiable with MS when trypsin digestion is used, in contrast to other EBV proteins such as EBNA1 and BARF1. Both LMP1 and LMP2 contain only a limited amount of trypsinization sites, reducing the amount of identifiable peptides for these proteins. For example, LMP1 theoretically contains only three peptide sequences that may be identified by MS after trypsin digestion, considering the optimal length of 10-25 amino acids for MS identifiable peptides. In this case, digestion with alternative enzymes, such as chemotrypsin, may provide a solution.

Conclusions: Our pilot experiments indicate that our exosomal purification method as well as the MS analysis is accurate and reproducible, even when a single SDS-Page band approach is used.

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

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BACKGROUND AND AIM

Apoptosis resistance of leukemic cells at diagnosis has been found to predict outcome in Acute Myeloid Leukemia (AML). In order to overcome apoptosis resistance, responsible mechanisms have to be determined. Proteins released from leukemic cells as aberrant secretion and soluble factors produced by bone marrow stromal cells are known to induce apoptosis resistance. Therefore, we want to apply methods for sensitive and quantitative identification of proteins secreted by leukemic cells and by bone marrow stromal cells that are associated with apoptosis-resistance of AML. Functional studies will provide insight in how these factors lead to apoptosis resistance.
Proteomic profiling of the microenvironment will hopefully provide us with new predictive factors in AML, thereby improving therapeutic decision making. Moreover, proteomics may facilitate the development of novel therapeutic agents by unveiling new therapeutic targets.

APPROACH

Part I: Proteomic analysis of the secretome of leukemic cells in order to identify proteins associated with apoptosis-resistance of leukemic cells
Comparative protein profiling, using GeLC-MS/MS, of the secretome (cancer cell conditioned medium) of apoptosis-resistant and apoptosis-sensitve primary leukemic cells will be performed in order to identify protein profiles associated with apoptosis resistance. Differentially expressed proteins identified in these experiments will provide the basis for *functional* studies in which the responsible molecular pathways in microenvironment-mediated apoptosis resistance will be unravelled.

Part II: Identification of apoptosis-resistance related proteins in bone marrow plasma of AML patients
The identified apoptosis resistance-associated proteins will be searched for in bone marrow plasma of AML patients. It will be determined whether the presence of these proteins is indeed associated with an apoptosis resistant profile of the leukemic cells at diagnosis. Since in a minimal residual disease situation apoptosis-resistant leukemic cell profiles are known to reverse to normal, it will be investigated whether a similar reverse in protein-profile occurs.

PRELIMINARY 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. Forty proteins were upregulated at least 2-fold in apoptosis sensitive blasts and 208 in apoptosis resistant blast ($p<0,05$).

CONCLUSIONS AND OUTLOOK

These preliminary results show that secretome analysis by GeLC-MS/MS is a powerfull approach for reproducible mining of proteins in the bone marrow microenvironment relevant for apoptosis resistance. We are currently performing a literature search to identify which of these 248 proteins might be associated with apoptosis resistance or apoptosis sensitivity. Subsequently, the effect on apoptosis will be validated in functional experiments.

Acknowledgements *This research is supported by CCA-V-ICI.*

Exploring differences in short-term response to cisplatin in sensitive and non-sensitive mouse mammary tumors

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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. Possibly, the expression of genes that are relevant for therapy outcome is very low and difficult to detect. In fact, it may be easier to find a predictive profile shortly after the first treatment, when response-related genes are induced. We tested this approach using genetically engineered mouse models for breast cancer. Spontaneously developing *Brca1*$^{-/-}$*;p53*$^{-/-}$ mammary tumors of our mouse model for hereditary breast cancer (*K14cre;Brca1*$^{flox/flox}$*;p53*$^{flox/flox}$) are very sensitive to the maximum tolerable dose (MTD) 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*$^{-/-}$*;p53*$^{-/-}$ mammary tumors of our mouse model for lobular breast cancer (*WAPcre;Ecad*$^{flox/flox}$*;p53*$^{flox/flox}$) do not shrink, but rather show a short growth delay or stable disease before becoming cisplatin resistant.

Aim
The underlying cause of drug sensitivity is obvious in the case of *Brca1* loss, since BRCA1 function is essential for homology-directed DNA repair. Here we made an attempt to identify distinctive biomarkers indicating (DNA damage) responses to cisplatin treatment in sensitive *Brca1*$^{-/-}$*;p53*$^{-/-}$ tumors versus resistant *Ecad*$^{-/-}$*;p53*$^{-/-}$ tumors 24h after treatment.

Approach
For this purpose, samples of *Brca1*$^{-/-}$*;p53*$^{-/-}$ and *Ecad*$^{-/-}$*;p53*$^{-/-}$ mammary tumors before and 24h after cisplatin treatment have been snap frozen (see figure). We ran total tissue lysates of treated and their untreated control tumors on a SDS-PAGE gel and cut whole lanes into ten pieces for in-gel digestion of the proteins, using trypsin. Extracted peptides were identified with nanoLC-MS/MS.

Results and Outlook
With a total sample size of 12 tumors (three per group) 3486 proteins were detected with high confidence (minimum 2 peptides per protein with 95% CI). The average per sample of the total number of spectral counts for the 3486 proteins was 27018. The coefficient of variation among the triplos per group was 0.29. The highest variation between the triplos was among the low abundant proteins (2-10 peptides per protein).
In both mouse models we observed an increase in expression of DNA damage-related proteins after treatment with cisplatin, such as topoisomerase 2a and PARP1. As differential markers, we found four proteins that were significantly upregulated after treatment in the *Ecad*$^{-/-}$*;p53*$^{-/-}$ model, but significantly downregulated in the *Brca1*$^{-/-}$*;p53*$^{-/-}$ model, for example fatty acid synthase. However, we picked up only very few cell cycle-related proteins, of which many were significantly up- or downregulated in the gene expression analysis. Most likely, the protein levels of these were too low to detect in the total tissue lysate. Another possibility is that the 24 hour time point is too early to measure changes for certain proteins. We are continuing the parallel analysis of gene expression and proteomics data and will verify the results in a larger tumor set by qPCR and Western blotting.

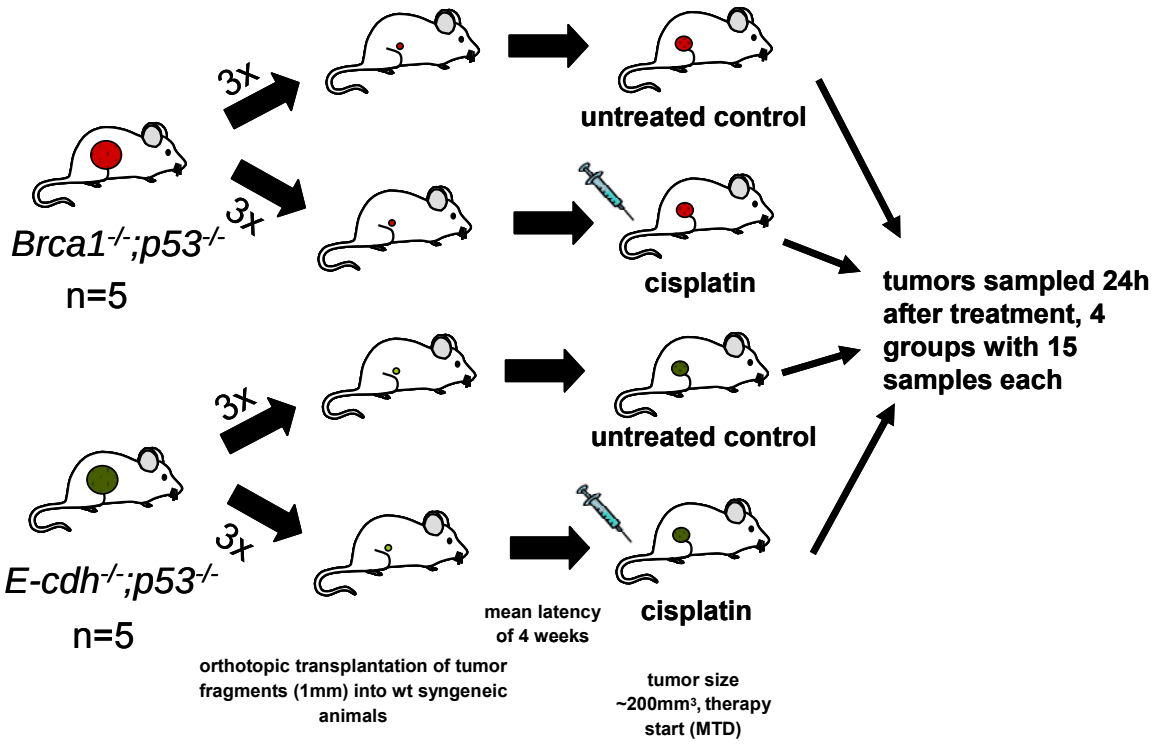

Figure: study outline

Proteomics reveals minichromosome maintenance proteins as novel tumor markers for meningiomas

Okay Saydam1, Ozlem Senol1, Tieneke Schaaij-Visser2, Sander R. Piersma2, Thang V. Pham2, Saskia Peerdeman3 and Connie Jimenez2

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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 identity 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 ninetytwo 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.

This research was supported by the VUmc Cancer Center Amsterdam

J. Proteome Res., 2009, in press.

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.

Objective 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. The figure below illustrates the network of proteins involved in cell-to-cell signaling and cellular morphology as constructed by Ingenuity, containing potential biomarker candidates.

Conclusion: 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.

This research was supported by the VUmc Cancer Center Amsterdam

Abstract accepted for an oral at the 6th International Congress on Meningiomas and Cerebral Venous System, Boston MA, USA, 2008

Molecular Variations in Tumor-Host Interactions between Invasive and Angiogenic Phenotype in High Grade Gliomas

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Background. In solid tumor growth, a crucial turning point is the transition from the avascular to the vascular phase, leading to a more aggressive tumor growth. Anti-angiogenesis therapy is a powerful approach aimed at blocking/reducing tumor growth. The identification of novel angiogenesis-specific proteins is crucial for the development of new anti-angiogenic therapies, and such proteins are potential new biomarkers in cancer.

Approach. A xenograft animal model of human glioblastoma multiforme (GBM) has been developed in our lab. The model includes 4 generations of rats with the GBM tumor phenotype varying from very invasive, non-angiogenic in the first to less invasive, fully angiogenic in the last generation rat. These 4 phenotypes are compared at various levels to study the background of tumor development, invasion and angiogenesis.

Objective. To explain the molecular background of the phenotypic change – the angiogenic switch – at the protein level and to identify potential biomarker and target candidates

Methods. We applied quantitative proteomics based on iTRAQ 2D-LC MALDI TOFTOF and bioinformatics on a membrane enriched fractions of the tumors.

Results. In four generations of tumors, we identified 1038 non-redundant, host or tumor specific proteins (C.I. ≥ 95%). Species specific separation of the proteins allowed tumor-host interaction studies at the proteome level and may reveal novel biomarkers involved in the angiogenic switch.
Bioinformatic analyses over four tumor phenotypes revealed distinct groups of proteins with specific expression profiles that may be involved in the angiogenic switch and tumor angiogenesis.
The expression of particular proteins representing these profiles was validated by non-proteomic methods (e.g. GBM tissue arrays) as well as by functional assays using a novel GFP-expressing NOD/Scid mouse model, recently developed in our laboratory, which will allow us to confirm the tumor/host interactions involved in the angiogenic switch.

Conclusion. It appears from these profiles that many of the regulated proteins are involved in alternative tumor specific metabolic pathways for energy production.

This research has been accepted for publication by the journal Molecular and Cellular Proteomics
This research was supported by the EU Angiotargeting project

Lung cancer biomarker discovery in conditional NSCLC and SCLC mouse models.

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PROJECT SETTING: 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 the most common type of cancer worldwide and in The Netherlands and still has a very poor overall prognosis. Patient care could be greatly improved if high quality biomarkers were available for early diagnosis, staging, personalized treatment and therapy response prediction.

AIM: With this study we aim to discover protein biomarkers for lung cancer by using a combination of conditional mouse models for small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), cell lines, patient tissues and proteomics technology.

APPROACH:
The main source of material for this study will be two different mouse models. The Cre-*loxP* mediated somatic recombination system will be used to sporadically activate or arrest expression of specific genes, which results in a tumorigenesis that mimicks sporadic lung cancer in humans. In mice carrying a conditional K-Ras activating gene combined with a p53 inactivating gene, injection with adeno-Cre virus will induce histopathologically human-like NSCLC. Mice with conditional p53 and RB inactivating genes will develop SCLC that resembles human SCLC. After development of tumors, the tumors will be harvested and tumor-secreted proteins will be collected (tumor secretome).
Further, a series of human and mouse lung cancer cell lines will be cultured to obtain protein extracts. Also tissues of different stages in lung carcinogenesis will be homogenized for protein extraction.
The proteomes of mouse tumor tissue with corresponding secretomes, mouse and human tumor cell lines and human tumor tissues will be quantitatively compared by geLC-MS/MS combined with spectral counting. The beta-binomial test will be employed to find significantly, differentially expressed proteins and the Ingenuity Pathway Analysis (IPA) software will be used to select gene ontologies, networks of proteins and cellular pathways that are overrepresented among the differential proteins. Protein biomarker candidates will be validated in mouse and human tissue and plasma samples by antibody-based, protein-specific detection methods.

OUTLOOK: We expect to find a large number of potential biomarkers for different types and stages of lung cancer. By designing appropriate validation experiments with large series of mouse and human samples, we will identify those that are specific and can be used for clinical applications.

Kinome proteomics for insight into cancer signaling pathways, identification of drug targets and patient stratification

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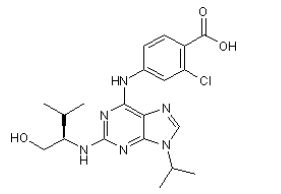
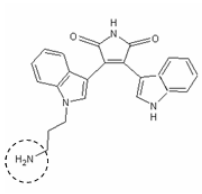
BACKGROUND The human kinome represents a family of 518 protein kinases. Protein kinases, enzymes that catalyze protein phosphorylation, are key regulators of signal transduction and are often implicated in the development of cancer. Moreover, many small-molecule inhibitors have been developed against 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.

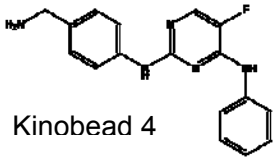
AIM Development of specific protein kinase capturing methods, for enrichment of kinases from complex protein mixtures, prior to identification with mass spectrometry.



Purvalanol B

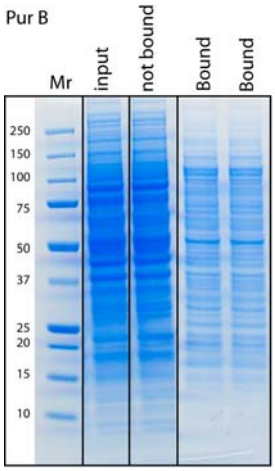


Bisindolylmaleimide



Kinobead 4

Kinobead 8



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 open a detection window for comprehensive profiling and analysis of protein kinases by LC-MS/MS. Using these tools, cell lines can be interrogated using kinobeads and 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 help targeted therapeutic intervention.

This research was supported by the VUmc Cancer Center Amsterdam

Support Vector Machine Approach to Separate Control and Breast Cancer Serum Samples

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This project explores two analyzes of a differential MALDI-TOF mass spectrometry dataset. Both analyzes use the support vector machine as a tool to build a prediction model. The first analysis which is our contribution to the competition uses the given spectra data without further processing. In the second analysis, we employed an additional preprocessing step consisting of peak detection, peak alignment and feature selection based on statistical tests. The experimental results suggest that the preprocessing step with feature selection improves prediction accuracy.

Reference

Pham, TV., Van der Wiel, M., Jiménez, C.R (2008) Support Vector machine approach to separate control and breast cancer serum spectra. Statistical Applications in Genetics and Molecular Biology. (in press).

This research was supported by VUmc Cancer Center Amsterdam

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

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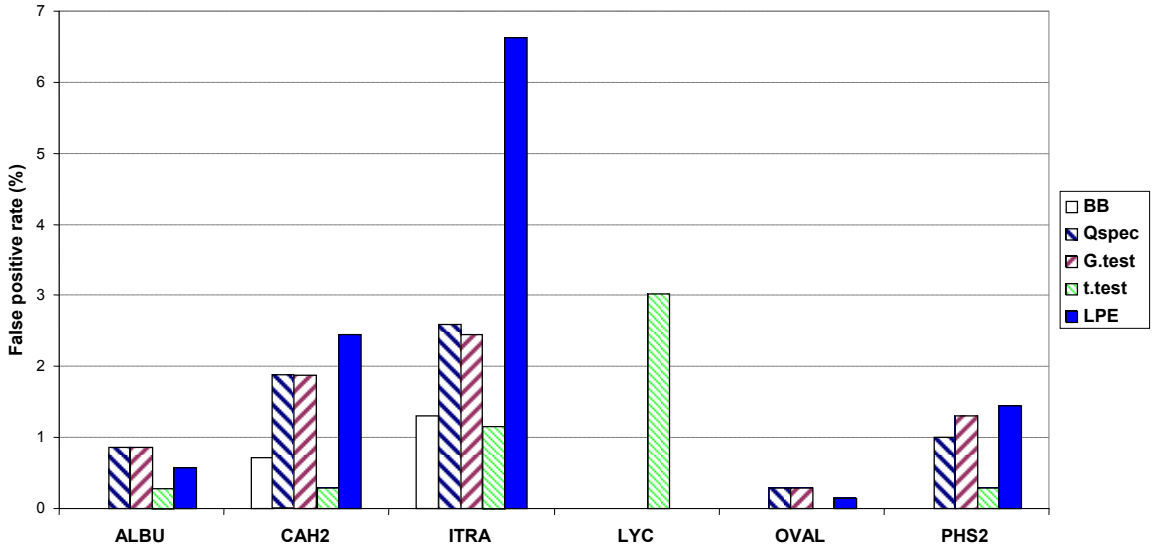
BACKGROUND Spectral count data generated from label-free tandem mass spectrometry-based proteomic experiments can be used to quantify protein's abundances. Comparing spectral count data from different sample groups such as control and disease is an essential step in statistical analysis for the determination 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 We are consolidating the software for the beta-binomial test so that other researchers can easily use the tool for comparative analysis of spectral count data.



Proteomics reveals signaling networks associated with kainic acid-induced changes in the mouse hippocampus.

Fitzsimons CP[1], Fratantoni SA[3], Hubens CJY[1, 2, 4], Piersma S[3], Peifei G[2], Voskuyl R[2, 4], Jimenez CR[3] and Vreugdenhil E[1].

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Background
In human mesial temporal lobe epilepsy (mTLE), seizures typically affect the hippocampus. The kainic acid (KA)-induced model of mTLE in rodents provides a tool to characterize molecular and functional alterations that take place during epileptogenesis in the hippocampus and may mimic the human epileptic condition.

Aim
To determine KA-induced changes in protein expression in the hippocampus.

Approach
Proteomics techniques based on quantitative Nano-LC tandem Mass Spectrometry of hippocampus samples were employed. Mice were subjected to a seizure-inducing dose of KA and 3 days after injection were sacrificed, the hippocampus was dissected, lysed, applied on gel and processed for proteomics analyses.

Results
Out of 2327 proteins confidently identified by our proteomics analysis of tissue lysates, spectral counting-based quantification combined with statistics identified 113 proteins differentially regulated between saline- and KA-treated animal groups (4.86 %). From these, 55 were up- and 58 down-regulated. Within the up-regulated proteins, 43 were de novo expressed and within down-regulated proteins we found 45 proteins whose expression was below detection levels after KA-treatment. We used the Ingenuity Pathway Analysis (IPA) tools to associate KA-induced proteins with biological functions. We identified neurological disease and cellular assembly, growth and proliferation as the most significantly altered biological functions. IPA further showed that the differentially expressed proteins belong to 4 canonical networks associated with NFkB signaling, 1 canonical network associated with P53 signaling and 1 with Fos signaling.

Outlook
Our results suggest a pivotal role for chronic alteration in transcription factor expression in the KA-induced model of mTLE. In addition, neuroplasticity-related proteins were found differentially expressed in KA-treated animals. Our results will contribute to the identification of biomarkers for the development of new anti-epileptic therapies targeting epileptogenesis.

Proteomic analysis to identify differently expressed proteins in the locus coeruleus of Parkinson's disease patients compared to controls

Karin D. van Dijk[1,2], Silvina Fratantoni[3], Thang V. Pham[3], Henk J. Groenewegen[1], Henk W. Berendse[2], Connie R. Jimenez[3], Wilma D.J. van de Berg[1]

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BACKGROUND
Parkinson's disease (PD), a neurodegenerative disorder affecting more than 4 million people worldwide, is pathologically hallmarked by the presence of Lewy bodies and Lewy neurites and the loss of catecholaminergic neurons in the substantia nigra and locus coeruleus. The Lewy body pathology comprises a large variety of aggregated proteins, including alpha-synuclein and ubiquitin, and is thought to play a key role in the pathogenesis of PD. Clinically, diagnosing PD can be challenging, especially in its early stages in which it can be mimicked by essential tremor and atypical parkinsonian syndromes. Identification of proteins related to PD pathology may give more insight into its pathogenesis, and moreover could contribute to the development of biomarkers for PD enabling a more accurate PD diagnosis.

AIM
The aim of the present study is (1) to gain more insight into PD pathogenesis and (2) to contribute to the future development of CSF biomarkers.

APPROACH
Post-mortem material of 6 PD patients and 6 age and gender-matched controls was selected from the collection of the Netherlands Brain Bank. The locus coeruleus was excised, sections for later validation were collected and the locus coeruleus was homogenized in sample buffer. Proteins were fractionated by 1D gel electrophoresis followed by slicing of the whole lane, in-gel tryptic digestion and nanoliquid chromatography (LC) coupled to hybrid iontrap-fourrier transform tandem mass spectrometry (nanoLC-FTMS).

RESULTS
Statistical analysis of the proteomic data is underway. The database 'MASCOTT' and the tool 'Scaffold' will be used for peptide and protein identifications. Proteins will be quantified by the number of spectral counts and will further be classified upon known functions, pathways, motifs and relation with PD pathology using databases and data mining software.

OUTLOOK
Differently expressed proteins will be validated in tissue using antibodies and their relation with PD pathogenesis will be evaluated. In addition, we will examine which of these differently expressed proteins were already demonstrated to be detectable in CSF. Some of these proteins may serve as CSF biomarkers and need further CSF validation.

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

Silvina Fratantoni, Sander Piersma and Connie Jimenez

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Background. Protein profiling of the proximal fluid CSF for biomarker discovery in dementias provides an attractive alternative to serum proteomics because of the proximity to the diseased organ/ tissue. For the comprehensive, global analysis of CSF peptides and proteins, multi-dimensional separation strategies at the protein or peptide level are required to overcome the large dynamic range of expression levels and to simplify the (tryptic) peptide sample introduced into the mass spectrometer. Since 80 % of the protein mass in CSF is serum-derived, removal of major serum proteins is necessary for discovery of low abundant brain-derived biomarkers. In this study, we compared the performance of two multi-affinity depletion methods coupled to coupled to 1D gel separation and nano-liquid chromatography-tandem mass spectrometry (GeLC-MS/MS).

Goals. 1. To create a discovery workflow with acceptable throughput (~1-2 samples/day) that can routinely and reproducibly measure 500-1000 CSF proteins in small volumes of CSF (<1 ml). 2. To evaluate the performance of two multi-affinity depletion methods in spin filter format: MARS Human 14 and Seppro-IgY Supermix coupled to GeLC-MS/MS analysis.

Approach. Here we evaluate two affinity-depletion methods coupled to 1D gel separation and nanoLC-MS/MS (GeLC-MS/MS) for quantitative CSF peptide and protein analysis in terms of the number of proteins identified (depth of analysis) and reproducibility of analysis.

Methods. From one pooled CSF sample (protein concentration 400 µg/ml), six 500 µl aliquots were prepared and applied directly to the two different depletion spin filters (Seppro-IgY Supermix from Genway and Agilent Human 14). Depleted CSF was concentrated further using Amicon filters prior to loading the whole depleted CSF fraction on a 1D gel. The bottom part of the workflow figure displays the coomassie-stained gel band patterns for the 6 depleted fractions as well as for the bound fraction that was only subjected to brief electrophoresis. Subsequently, whole lanes were cut in 10-equal bands and each band is in-gel digested with trypsin. Extracted tryptic peptides were identified by reversed phase nano-liquid chromatography on-line coupled to MS/MS using a LTQ-FTMS platform.

Results. The whole CSF dataset contained 852 proteins that were identified at high confidence (minimum 2 peptides per protein at >95% CI and >99% CI at the protein level). Analysis of the bound CSF fraction revealed that many more proteins are removed than just the 14 abundant target proteins. In total, 56 CSF proteins were detected in the MARS-bound fraction and 63 proteins in the IgY-bound fraction.
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).

Conclusions.
In summary, we have shown that spin filter-based affinity depletion of CSF allows for reproducible and sensitive analysis of the CSF proteome and that the MARS spin filter has a better performance than the IgY-based filter.

Outlook
MARS-based affinity depletion coupled to GeLC-MS/MS analysis provides a promising workflow to biomarker discovery in neurodegenerate and neoplastic disease.

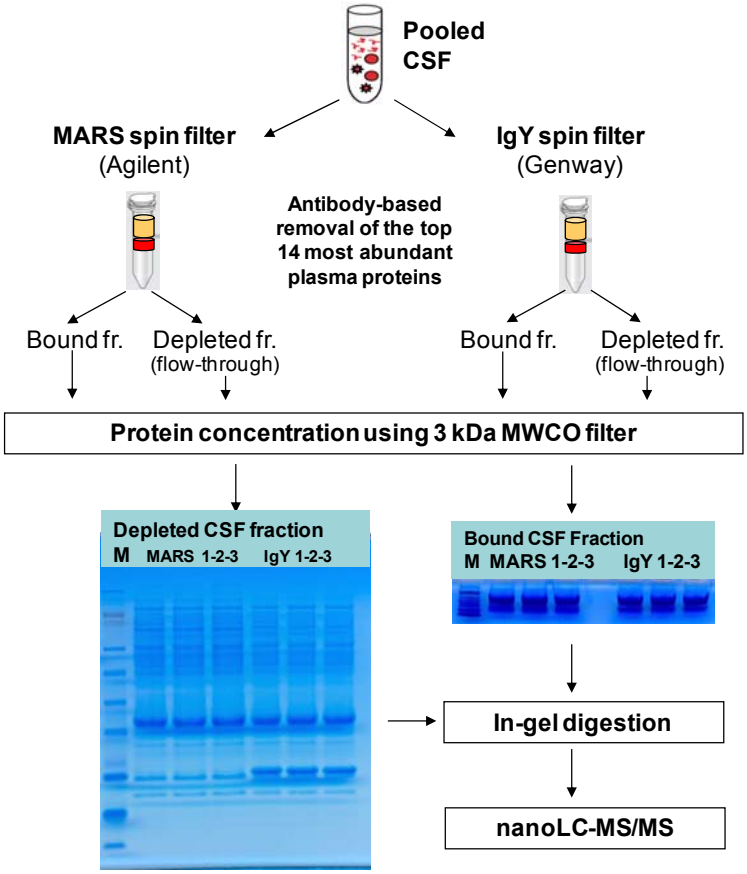

Figure. Workflow for CSF proteomics

Acknowledgements:
This research was supported by the European FP6 project: cNeuPRO. Ing. E. van Elk and Prof. Blankenstein are acknowledged for providing us with CSF and Dr. Thang V. Pham for assistance with statistics.

This work has been submitted for publication in the journal 'Proteomics Clinical Applications'

Novel candidate CSF biomarkers for high risk mild cognitive impairment and Alzheimer's Disease

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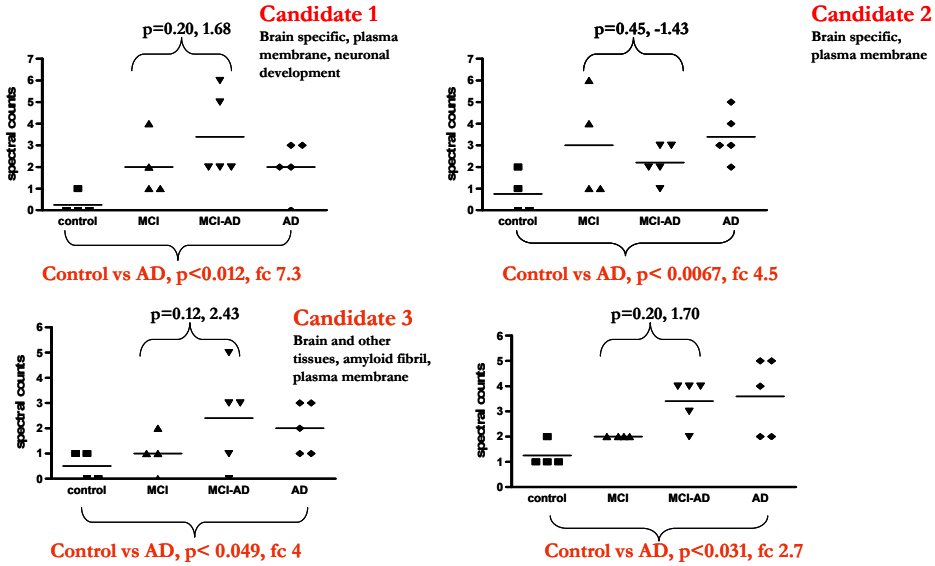
BACKGROUND Protein profiling of the proximal fluid CSF for biomarker discovery in dementias provides an attractive alternative to serum proteomics because of the proximity to the diseased organ/ tissue.

AIM Our primary goal is to find biomarker candidates in CSF of patients with mild cognitive impairment that can predict if they will develop Alzheimer disease.

APPROACH To get as many proteins as possible from a small volume of CSF, we remove the most abundant proteins from the CSF with an affinity column that binds the 14 most abundant proteins. We applied this approach to 20 CSF patient samples, from the VUmc-NUBIN bank. The CSF samples belonged to 4 clinical groups contained 5 AD patients, 5 MCI, 5 MCI-AD and 5 Controls. The fraction of proteins that was not bound to the column was concentrated and applied on gel. The gel bands were trypsin digested and applied in a nanoLC-MS/MS.

RESULTS In total, 1079 proteins were identified with high confidence. Using the beta binomial statistical test, 20 differential proteins were identified that exhibited elevated levels in 1 or more clinical group. Example profiles are shown for 4 selected promising candidates. These candidates are especially interesting for their expression profiles in the 4 clinical groups and because of their association with brain tissue and neurological functions.



OUTLOOK Candidate CSF biomarkers will be followed up in a larger cohort using ELISA. These candidates may ultimately be included in panel for early detection and may aid in AD risk prediction in subjects with MCI.

Acknowledgements: This research was supported by the European FP6 project: cNeuPRO

Mutations in PPIB decrease 3-hydroxylation of Collagen Iα1 and result in severe Osteogenesis Imperfecta

Fleur van Dijk[1], Sander Piersma[2], Silvina Fratantoni[2], Connie Jiménez[2], Gerard Pals[1]

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BACKGROUND Deficiency of cartilage associated protein (CRTAP) or prolyl 3-hydroxylase 1 (P3H1) has been reported in autosomal recessive lethal/severe Osteogenesis Imperfecta (OI). CRTAP, P3H1 and Cyclophylin B(CyPB) form an intracellular collagen-modifying complex that 3-hydroxylates proline specifically at position 986 (P986) in the alpha1 chains of collagen type I. This prolyl- 3-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.

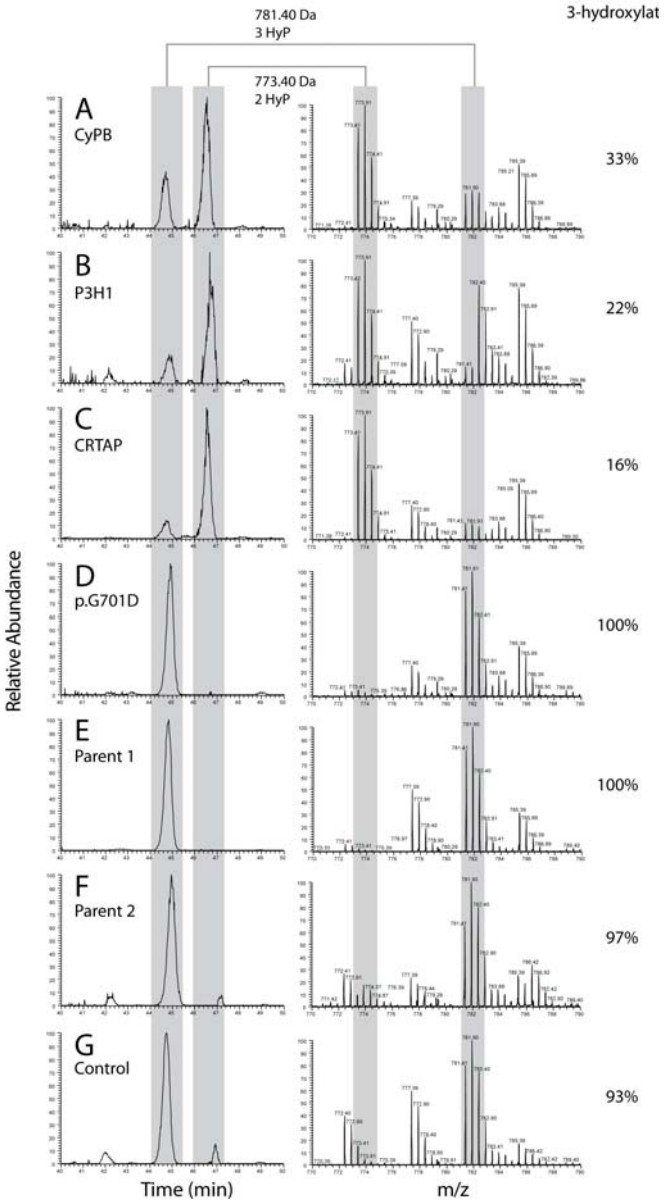
AIM Mass spectrometric quantification of 3-hydroxylation in the P986-containing tryptic peptide of collagen Iα1 in healthy controls and patients with mutations in CRTAP, P3H1 and CyPB.

APPROACH Patient and control Fibroblast culture lysates were preincubated with pepsin/HAc for 24 h. at 4˚C. After concentration proteins were separated by 5% PA-Urea-SDS gel. Collagen Iα1 bands were excised from the urea SDS PAGE gel and were in-gel digested. Peptides were separated by nanoLC (5 µm Reprosil C18) and detected on-line by MS(/MS). Extracted ion chromatograms and mass spectra were generated for the tryptic peptide DGLNGL**P**GPIG**PP**GPR modified with 2 or 3 hydroxyl groups (in **bold** prolines that can be hydroxylated, <u>underlined</u> proline-986, the target for 3-hydroxylation).

RESULTS Collagen Iα1 peptides that show normal P986 3-hydroxylation have an m/z ratio of 781.4 whereas peptides lacking 3-hydroxylation have an m/z ratio of 773.4 (see figure). Controls (D-F) show normal 3-hydroxylation (>90%). Patients with homozygous CyPB mutations (A) shows decreased 3-hydroxylation (33%). Patients with homozygous P3H1 or CRTAP mutations (B and C), however, show even more decreased 3-hydroxylation levels (22% and 16%, respectively).

OUTLOOK LC-MS/MS was used for detection of aberrant collagen Iα1 3-hydroxylation in patients with mutations in the collagen 1α1 3-hydroxylation complex consisting of CyPB, P3H1 and CRTAP.

A manuscript describing this work has been accepted for publication in the American Journal of Human Genetics.



Fishing for new Fanconi genes by proteomics of protein complexes

Anneke Haitjema[1], Davy Rockx[1], Sander Piersma[2], Connie R. Jimenez[2], Josephine Dorsman[1]

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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.

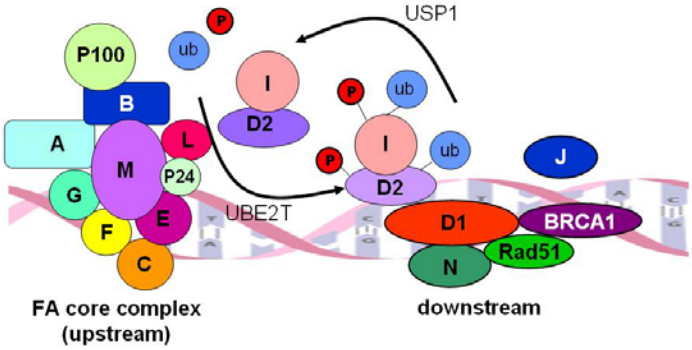

Figure 1. Current model of the FA/BRCA pathway, showing the 13 known FA genes and p24, p100, and BRCA1. The latter 3 proteins formally do not qualify as genuine FA genes, since so far no FA patients have been identified with mutations in these genes. Proteins p24 and p100 are essential components of the core complex, while BRCA1 binds to BRIP1/FANCJ 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 FANCJ (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.

Analysis of 14-3-3 protein complexes in OVCAR3 cells

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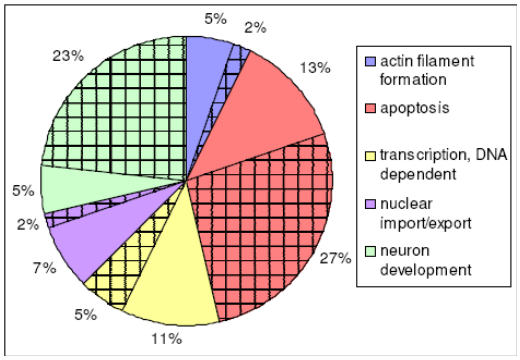
BACKGROUND. In recent years, the 14-3-3 protein family has emerged as a key regulator of a wide range of cellular processes in human cells. Seven mammalian 14-3-3 genes have been identified (β, η, ε, γ, σ, θ and ζ) and their solved crystal structure shows a double-barreled clamp consisting of two 14-3-3 monomers. Each monomer contains a negatively charged groove which can interact with a phosphorylated serine or threonine residue in an α-helix of the target protein. From analysis of plant genomes it is known that about 25% of all genes contain a 14-3-3 interaction motif and up to now more than 200 mammalian targets are known.

AIM One of our goals is to get a better insight into the functions of the 14-3-3 proteins in cancer cells through analysis of the 14-3-3 interactome in the human ovarian tumor cell line OVCAR3.

METHOD To this end, we made a whole cell lysate from OVCAR3 cells with an octyl-glucoside containing buffer. Recombinant GST-14-3-3 proteins of all human isoforms were added and performed affinity chromatography with the GST-tag. Proteins bound in the 14-3-3 groove were specifically eluted with the R18 peptide, eluted proteins were separated on an SDS-PAGE gel and analyzed by LC-MS-MS analysis.

RESULTS We identified more than 300 (co-)target proteins using 14-3-3 affinity chromatography in combination with nanoLC- tandem mass spectrometry, revealing an unprecedented central position for 14-3-3 proteins in cell biology. Intriguingly, further analysis revealed that identified targets have opposing biological functions (See figure). E.g. one group of targets is classified as pro-apoptotic and another group as anti-apoptotic. It is likely, although not required, that certain specificity signals in the 14-3-3 isoforms and/or target proteins are associated with certain functions or cellular localization. In order the understand 14-3-3 ubiquitous functional role, elucidation of their specificities will be essential.



Janus-faced character of 14-3-3s. Identified (co)-targets fall in opposing classes. Clear color: actin growth, pro-apoptotic, stimulation of transcription, nuclear import, neuron development.
Hatched: opposing functions.
100% = 56 proteins.

OUTLOOK. These data demonstrate the great complexity of the global 14-3-3 interactome. As a next step we would like to refine the analysis and determine the isoform specificity of the identified targets by performing affinity chromatography with all GST14-3-3 isoforms separately. Besides this, we would like to get more insight in the cellular localization of the interactions and see if treatment, with the cytokine interferon-α for instance, changes the 14-3-3 interactome.

This research was supported by the VUmc Cancer Center Amsterdam

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

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Background
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 [Maussang et al. Cancer Res, 2009] and to promote tumor growth in vivo [Maussang et al., PNAS, 103(35):13068-73, 2006].

Aim
In order to elucidate the oncogenic signalling pathways activated by the HCMV-encoded chemokine receptor US28, we aim to identify novel signalling partners of US28.

Approach
Immunoprecipitation of the HA-tagged US28 in mammalian cells transfected with US28 is used to isolate US28-complexes that can then be further analyzed by 1D separation on a SDS/PAGE gel. Subsequently, in-gel digestion and LC-MS/MS is used to identify the individual proteins.

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

Outlook
The amount of co-precipitating proteins underlines the necessity of co-precipitating US28 complexes from different cellular compartments (eg. the membrane). Currently, we are exploring a possible way of accomplishing membrane isolation by biotinylating membrane proteins with sulfo-NHS-SS-biotin which can then be purified using streptavidin, thus yielding a membrane specific fraction of US28-complexes.

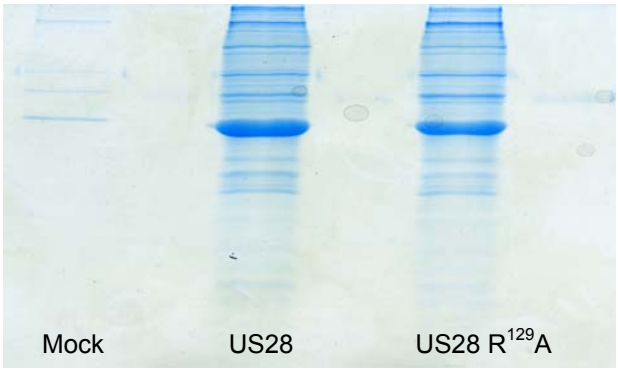


Figure. Typical result showing the different immunoprecipitates obtained from mammalian cells.

Proteomics on the mycobacterial cell envelope

Edith N.G. Houben[1], Aniek van der Woude[1], Maria L. Daleke[1], Roy Ummels[1], Theo Verboom[1] Sander Piersma[2], Connie R. Jimenez[2] and Wilbert Bitter[1]

[1]Dept. of Medical Microbiology & Infection Control, VU University Medical Center
[2]OncoProteomics Laboratory, Dept. Medical Oncology, VUmc

BACKGROUND The bacterium *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is the second most deadly human pathogen, causing ~1.7 million deaths per year. The success of this important pathogen depends on the secretion of virulence factors that manipulate and damage the host immune system. To be effective, these virulence factors need to cross the mycobacterial cell envelope that contains besides an inner membrane a unique outer lipid bilayer, called the mycomembrane. To be able to transport proteins across this highly impermeable mycomembrane, mycobacteria use a specialized secretion system, the type VII secretion system, of which pathogenic mycobacteria have five: ESX-1 to ESX-5. On the outside of the mycomembrane an additional layer is present, known as the capsule that is mainly composed of specific glycans. However, also proteins are present in this outermost layer, of which some are likely to be transported via type VII secretion systems. Mycomembrane and capsular proteins are because of their cell surface localization potential virulence factors and therefore attractive targets for drug and vaccine development.

AIMS In this collaboration we have set out three projects with the aim to identify capsular and mycomembrane proteins, and to investigate which of the capsular proteins dependent on type VII secretion systems (more specifically ESX-1 and ESX-5) for their localization. In addition, we want to get a better understanding of the functioning of type VII secretion by determining the composition of the membrane embedded ESX-5 secretion apparatus.

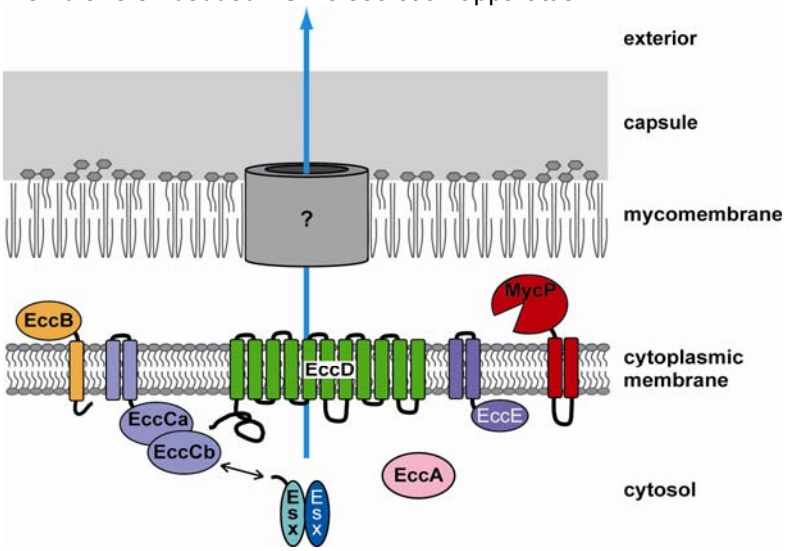


Figure 1. Schematic representation of the mycobacterial cell envelope and the predicted subcellular localization and membrane topology of the known proteins that form a type VII secretion system.

APPROACH Subcellular fractionation, differential solubilisation and pull-down techniques were developed to specifically isolate the proteins of interest, e.g. capsular proteins, mycomembrane proteins and ESX-5 secretion components. In turn, the complete set of isolated proteins were identified by SDS-PAGE gel, coomassie staining and liquid chromatography mass spectrometry (LC-MS).

RESULTS So far, we have identified the major capsular proteins of various mycobacterial species. For the fish-pathogen *M. marinum*, many of these proteins are so called ESX-1 associated proteins, because of the location of their corresponding genes in the same genomic region as genes encoding for the ESX-1

secretion system. We were able to show that these proteins are exported via the ESX-1 secretion system by isolation and identification of the capsular proteins of an ESX-1 secretion mutant. In addition, we have identified a preliminary set of mycomembrane proteins and we are currently verifying their mycomembrane localization by cell surface labeling with biotine and purification using streptavidin beads.
For the ESX-5 secretion system we have been able to determine the secretome. This analysis has shown that a large number of different PE and PPE proteins are secreted via ESX-5. Additional research has shown that ESX-5 effector proteins are involved in modulation of the immune response.

OUTLOOK Identifcation of capsular and mycomembrane proteins will contribute to finding novel targets for drug and vaccine development. In addition, by understanding the transport of capsular protein drugs can be developed to block their surface localisation.

PUBLICATION

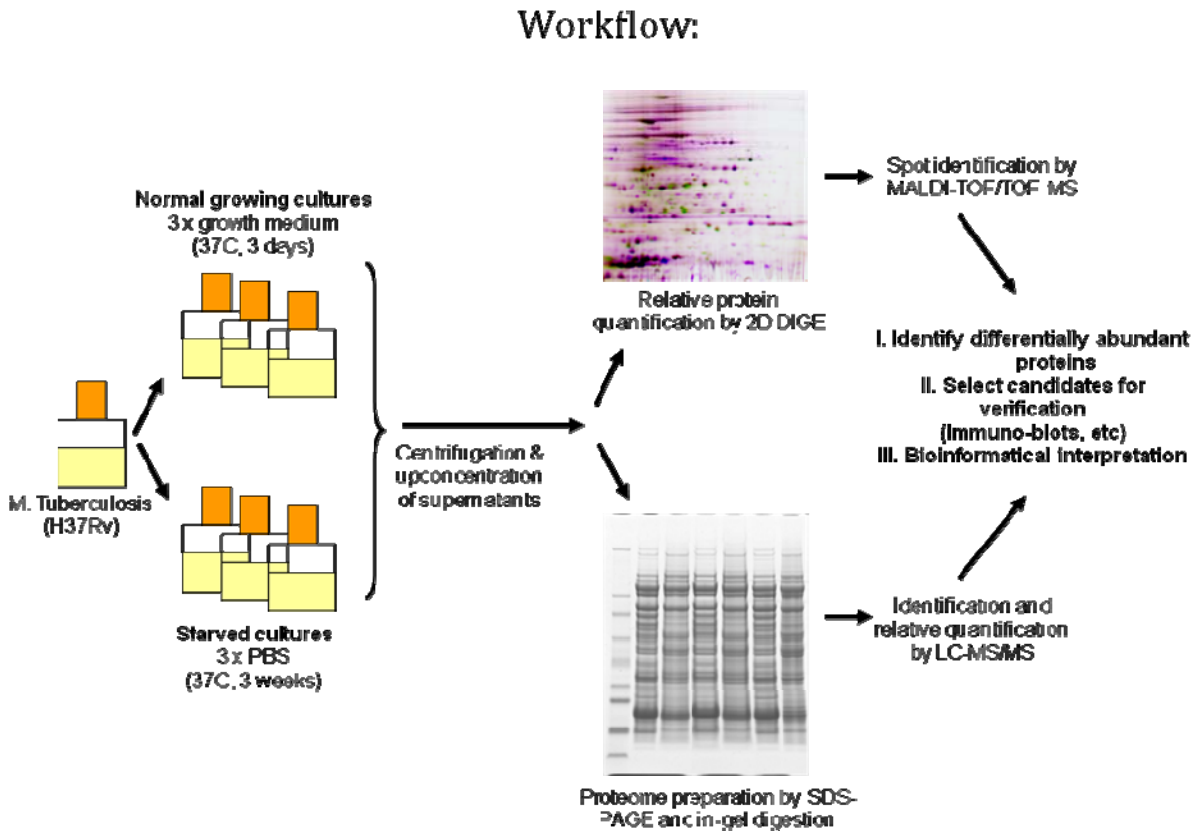
Abdallah AM, Verboom T, Weerdenburg EM, Gey van Pittius NC, Mahasha PW, Jiménez C, Parra M, Cadieux N, Brennan MJ, Appelmelk BJ, Bitter W. (2009) PPE and PE_PGRS proteins of Mycobacterium marinum are transported via the type VII secretion system ESX-5. Mol Microbiol. 2009 Aug;73(3):329-40.

Complementary analysis of proteins released from *Mycobacterium Tuberculosis* H37Rv cultures during starvation by LC-MS/MS and 2D DIGE

Jakob Albrethsen, Sander R. Piersma, Thang V. Pham, Connie R. Jimenez, Ida Rosenkrands

Serum Statens Institute, Copenhagen, Denmark, OncoProteomics Laboratory, Amsterdam, The Netherlands

According to the World Health Organization (WHO) two billion people are currently infected with Tuberculosis (TB) resulting in three million TB-associated deaths annually. It is believed that the success of the *Mycobacterium Tuberculosis* bacteria partly depends on the expression of proteins that can modulate the host-response.
Here, we use label-free LC-MS/MS and 2D DIGE to investigate the culture filtrate proteome of *M. Tuberculosis* H37Rv bacteria during normal log-phase growth and under starved conditions. Previous studies of *M. Tuberculosis* cultures have demonstrated that depletion of nutrients (and/or oxygen) leads to a non-proliferating and drug resistant state that can persist for extended periods of time and this condition is believed to mimic the *in vivo* drug-resistant state of *M. Tuberculosis* bacteria during latent TB. As such, proteins released from *M. Tuberculosis* cultures during starvation may play important roles in establishing latent TB inside the host and, in addition, may constitute a promising source of candidate diagnostic proteins for TB.



Chemoenzymatic synthesis of multivalent neoglycoconjugates carrying the helminth glycan antigen LDNF

Tefsen B, van Stijn CMW, van den Broek, M, Kalay H, Knol JC, Jimenez CR, van Die I

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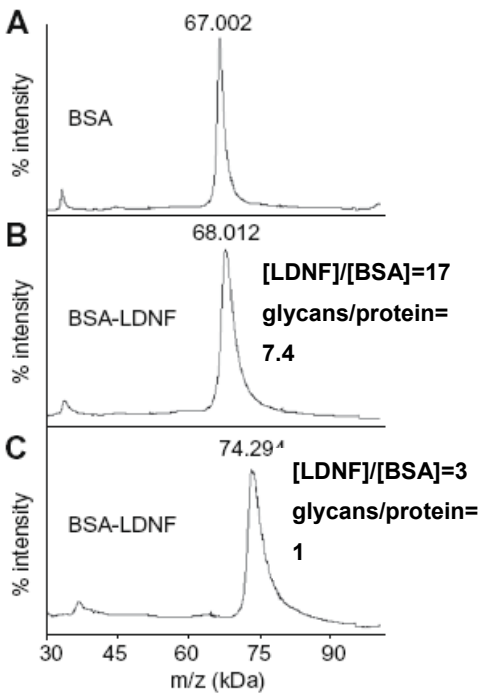
MALDI-TOF MS is a versatile tool to swiftly determine peptide/protein mass shifts, e.g. in order to assess the success and extent of *in vitro* glycoconjugate coupling. This is illustrated by the present study. Several parasitic helminthes, such as the human parasite *Schistosoma mansoni*, express glycoconjugates that contain terminal GalNAc□1-4(Fuc□1-3)GlcNAc□-R (LDNF) moieties. These LDNF glycans are dominant antigens of the parasite and are recognized by human dendritic cells via the C-type lectin DC-SIGN. To be able to study the interactions of LDNF antigens with the host immune system, an easy chemoenzymatic method was developed to synthesize multivalent neoglycoconjugates (ref. 1).
In this procedure, LDNF derivatized with a fluorescent coupling moiety (2,6-diaminopyridine, DAP) was synthesized in three steps from N,N'-diacetylchitobiose (using recombinant glycosyltransferases), and coupled to BSA carrier proteins through diethyl squarate (reacting with amine groups). In order to quickly assess the degree of coupling (and adjust conditions accordingly), mass shifts were determined using linear MALDI-TOF MS (Fig. 1A-C).



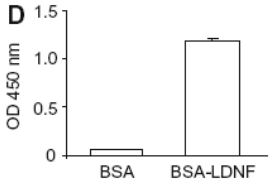


Figure 1. Analysis of BSA–LDNF with MALDI-TOF MS and ELISA. (A) Untreated BSA and (B, C) BSA–LDNF conjugates produced with different molar ratios of sugar (LDNF) to carrier (BSA) were analyzed by MALDI-TOF MS to assess mass shifts and deduce average numbers of glycans per carrier protein molecule. (D) Intact LDNF epitopes were detected on BSA–LDNF conjugates with ~ 1 glycan/protein using an anti-LDNF antibody ELISA. Untreated BSA was used as a negative control.

In figure 1, either a 3-fold or a 17-fold molar excess of LDNF to BSA was used in the coupling reaction. MALDI-TOF MS revealed average peak shifts of ~ 1 kDa and ~ 7.3 kDa, respectively, implicating an average of around 1 LNDF (3-fold excess) or 7.4 LDNF (17-fold excess) moieties per BSA molecule. To investigate whether the LDNF moieties were intact, the binding of an anti-LDNF antibody to BSA–LDNF conjugates was evaluated by ELISA. The antibody bound to both BSA–LDNF preparations (Fig. 1D shows binding to BSA with ~ 1 LNDF moiety/molecule), but not to untreated BSA, confirming the structural integrity of the glycan.

OUTLOOK The availability of neoglycoconjugates with different numbers of glycans per carrier allows the evaluation of glycan density and of the importance of the carrier molecule for biological activity of the conjugates.

Reference: Tefsen B, van Stijn CMW, van den Broek, M, Kalay H, Knol JC, Jimenez CR, van Die I (2009) Chemoenzymatic synthesis of multivalent neoglycoconjugates carrying the helminth glycan antigen LDNF.

Molecular Physiology of Myocardial Troponin I Variants

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Institute for Cardiovascular Research, OncoProteomics Laboratory, VU University Medical Center

BACKGROUND
Heart failure is a leading cause of mortality and morbidity and its prevalence is increasing. Heart failure syndromes include systolic (pump) failure, but diastolic heart failure is said to contribute substantially and therapies for diastolic dysfunction are lacking. Although in both adults and children symptomatic heart failure may present acutely at end stage, more often heart failure in mature and elderly adults results from a long-term process in which systemic hypertension, ischemic disease, genetic and metabolic factors contribute to the complex syndrome. Medical therapy has advanced substantially, *however*, when heart failure is end stage, therapeutic options are limited to transplantation, temporizing devices and potentially novel or experimental therapies such as cellular replacement. In heart failure both calcium dynamics and the response of the myofilaments to calcium are altered. These alterations impair systolic and diastolic function and are potentially reversible.
Our group has a long-term interest in post-translational changes in the myofilament proteins which precede irreversible end stage changes, and which contribute to systolic and importantly to diastolic abnormalities in cardiac disease. We have focused on understanding specific post-translational changes in the myofilament protein troponin I (cTnI), and how they influence function. This study focuses on hypotheses which address the effects of post-translational (phosphorylated) variants of cTnI and their role in heart function. The work does not only utilize murine models, but also more directly relates to human disease by quantitatively examining heart failure associated phosphorylation alterations and linking these directly to function in human cardiomyocytes. The central hypotheses that drive the long-term goals of the studies are that post-translational phosphorylations of cTnI alter cardiac function, and contribute to the pathophysiology of heart failure, and that therapeutic strategies can be designed to target and compensate for these defects.

APPROACH
We will determine the degree and impact of altered site-specific phosphorylation of cTnI in human heart failure by:
A. Use of quantitative phospho-proteomic approaches to determine the pattern and relative abundance of site specific phosphorylation in human cardiac tissue from failing and control donor hearts and,
B. In order to determine the pathologic role of the changes, in vitro replacement of recombinant cTnI, which mimics or corrects the altered phosphorylation will be undertaken in normal and diseased human cardiomyocytes. This approach may directly suggest therapeutic myofilament replacement strategies.

RESULTS
Previous studies have shown increased activity of protein kinase C alpha (PKCα) in human heart failure (PKCα). Force measurements in single human cardiomyocytes revealed that PKCα reduces calcium sensitivity of myofilaments, which may cause reduced systolic performance of the failing human heart (Bassic Research in Cardiology 2009, in press: Figure). Pilot experiments using proteomic analysis revealed that PKCα phosphorylates sites in human cTnI and troponin T (cTnT), which have not been described before in rodent studies.

OUTLOOK
Future studies will be performed to determine if the phosphorylation sites in cTnI and cTnT specific for human troponin underlie the functional effects induced by PKCα (see also Approach B).

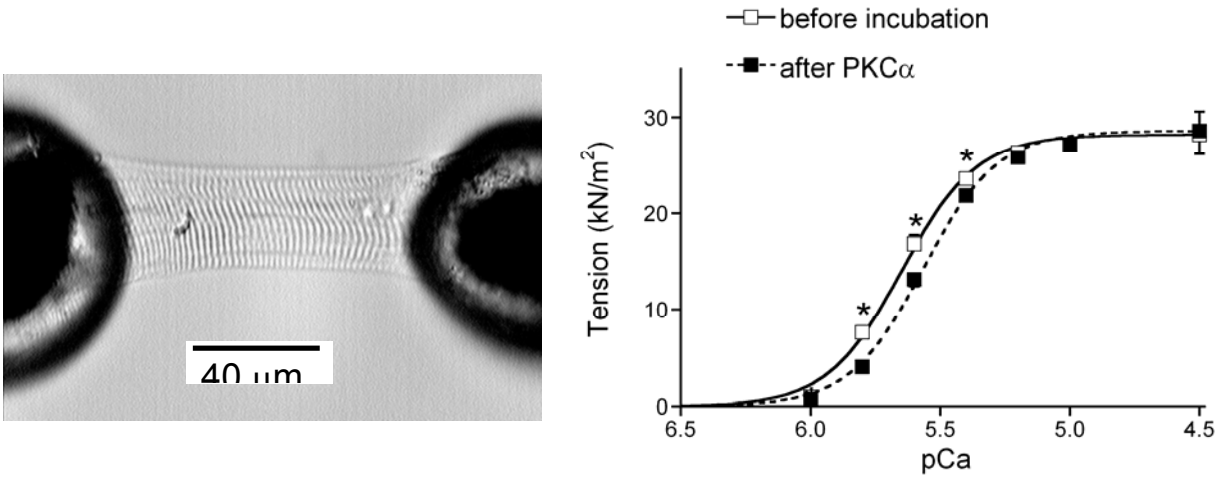

Figure legend: Force (tension in kN/m^2) was measured before and after incubation with PKCα in single human cardiomyocytes isolated from explanted failing hearts. Calcium sensitivity was significantly decreased by PKCα, illustrated by the rightard shift of the force-calcium (pCa) relationship.

Identification of Two Novel Protein kinase Cα Phosphorylation Sites on Human Cardiac Troponin I and T

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Background Protein kinase C α (PKCα) is up-regulated in end-stage heart failure. Rodent studies indicated that PKCα impairs myofilament function by phosphorylation of cardiac troponin I (cTnI) and troponin T (cTnT). However, direct evidence for a detrimental role of PKCα on myofilament function in human heart failure is lacking. Our previous study showed a decrease in Ca^{2+}-sensitivity by PKCα treatment of cardiomyocytes via phosphorylation of cTn and cMyBP-C.

Aim This study aimed to determine the specific sites phosphorylated by PKCα on cTnI and cTnT that could account for the decrease in Ca^{2+}-sensitivity.

Results Western blot analysis showed that PKCα is more active but less expressed in failing compared to donor hearts. A phospho-specific antibody against the conventional PKCα site Thr-143 on cTnI showed no endogenous phosphorylation both in donor and failing tissue. PKCα treatment of donor and failing tissue was, however, able to phosphorylate Thr-143. LC-MS/MS analysis of purified human recombinant cTn complex incubated with PKCα revealed two novel phosphorylation sites, Ser-198 located on cTnI and Ser-189 on cTnT. Both sites are located in conserved regions on cTnI and cTnT.

Conclusion PKCα may lower Ca^{2+}-sensitivity by phosphorylation of Thr-143 and Ser-198 on cTnI and Ser-189 on cTnT.

Outlook Further analysis using cTn exchange experiments with site specific mimicking of phosphorylation will be used to determine the functional consequences of PKCα phosphorylation of these sites.