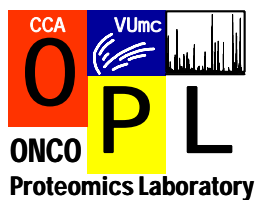
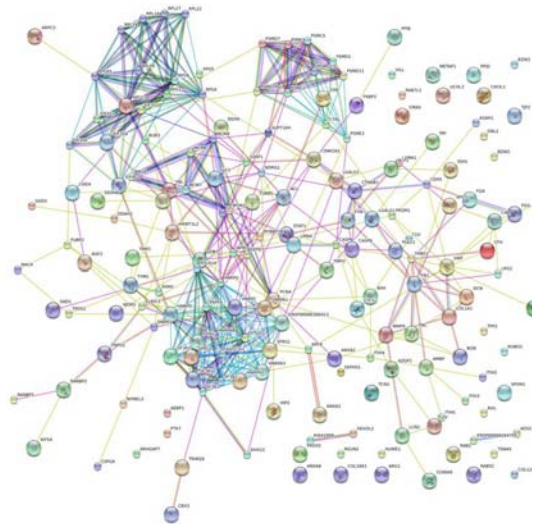


OncoProteomics Laboratory

Annual Report

2007



Annual Report 2007

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Foreword

It is my pleasure to present to you the second annual report of the OncoProteomics Laboratory that was established in April 2006 with support from the VUmc-Cancer Center Amsterdam. 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.



Only 6 months after start-up, the year 2007 was a productive year for the OncoProteomics Laboratory. I am happy to see that we already moved from studies for proteomics method exploration and validation to some successful cancer applications, both in model systems and human material:

- Several papers on high-throughput (HTP) body fluid peptide profiling and mass spectrometry data analysis were published as well as two invited reviews on proteomics technology for biomarker discovery.
- Model studies on subcellular proteomics of colorectal cancer tissue have been initiated in collaboration with the Tumor Profiling Unit (head Prof. Gerrit Meijer, Dept. of Pathology), and have lead to the identification of the first biomarker candidates (patent filed). The results of the sub-nuclear tumor tissue proteomics and tumor secretome projects have yielded two manuscripts in final stages of preparation. Moreover, these results are the basis of new research grant proposals.
- With the funding of 2 grants in 2007, one by the Womens Club of the Hague (research nurse) and one by CenE/Van Lanschot (PhD student), a breast cancer proteomics project has been initiated in Nov. 2007 with the start of the first PhD student of the OPL, Marc Warmoes.

In 2007, several young (foreign) scientists and students joined the OPL for short research projects:

- In the context of the EU Angiotargeting project, Uros Rajicevic (post-doc) worked at the OPL for 6 months on membrane proteomics in glioblastoma
- Anne Kjaergaard was a visiting PhD student from the group of Ole Jensen (Odense, Denmark) that did some training with us in tissue proteomics
- Jakob Albrethsen (post-doc) came to work in the OPL with Danish funding for half a year.
- Maral Pourghasian, a masters oncology student, did her internship on biomarker discovery in colon tumor tissue secretomes in a genetic mouse model
- Muhammed Kapci, a biotechnology master, explored proteomics of platelet secretomes as a new avenue for biomarker discovery

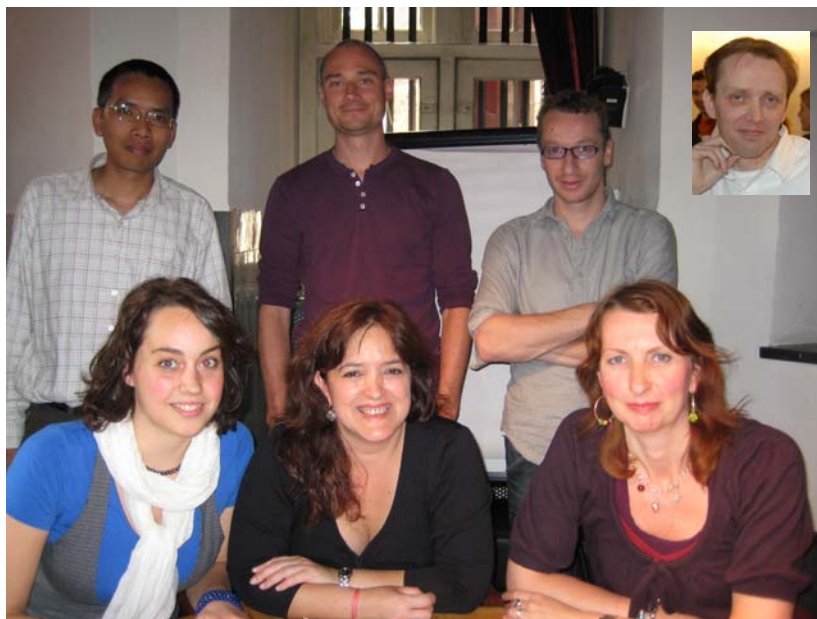
All these short projects form the basis of manuscripts (to be submitted in 2008) that will be important as example studies for other OPL collaborators.

In April 2007, we had our first annual retreat and it was re-encouraging to learn about the grown confidence about the workflows established in 2007 and the high motivation of the OPL team members. In Oct. 2007, the OPL website went live at www.oncoproteomics.nl, thanks to the fantastic job of Drs. Marc Warmoes. This website provides a comprehensive overview of activities, sample/project submission guidelines, current team members, collaborations, publications and protocols.

Furthermore, the OPL was part of two large collaborative grants (submitted Oct 2007), DeCoDe (DeCrease Colorectal cancer Death) and AIRFORCE (lung cancer), that will be funded by the Center for Translational Molecular Medicine (CTMM). The CTMM projects will ensure man-power for state-of-the art proteomics projects as well as provide funding 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.

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

Dr. Connie Jimenez,
Head of the OncoProteomics Laboratory



Group photo OncoProteomics Laboratory. From left to right, top: Thang V. Pham, Sander Piersma, Marc Warmoes; bottom: Inge de Reus, Silvina Fratantoni, Connie R. Jimenez. Insert: Jacob C. Knol.

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. Jacob C. Knol (OPL core: MALDI-TOF/TOF)

Dr. Marleen Koel-Simmelink (end dec 2007)

Dr. Silvina Fratantoni (start jan 2008)

Post-doctoral fellows:

Dr. Jakob Albrethsen (april-oct.2007)

Dr. Uros Rajicevic (visiting jan-june 2007)

PhD students:

Marc Warmoes (start nov 2007)

Anne Kjaergaard (visiting sept-dec 2007)

Internship students

Maral Pourghiasian (feb-juli 2007)

Muhammed Kapci (march-aug 2007)

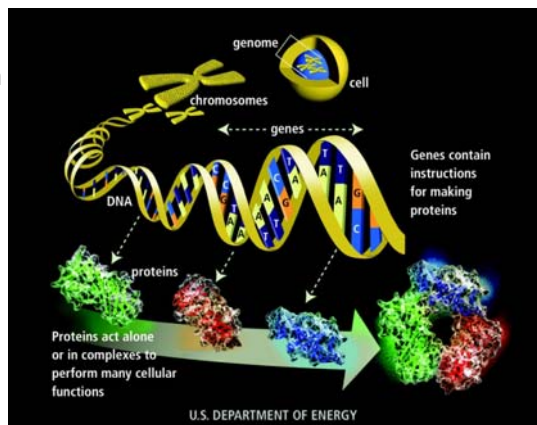
Inge de Reus (feb-aug 2008)

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, a major focus is on developing and implementing robust strategies for biomarker discovery in tumor tissue, in biofluids such as blood-serum/plasma that can be collected non-invasively, as well as in proximal fluids such as cerebrospinal fluid and nipple aspirate fluid. 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.

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 lunch meetings (every Friday), proteomics data are discussed with the people working in the lab.
- In monthly lunch meetings (every 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 2 months with the head of the OPL to discuss progress, developments and assist in prioritizing projects. Furthermore, the head OPL is assisted by Cluster I in organizational and financial matters. See figure 1 for an outline of the organizational structure. Currently there has been no substantial secretarial assistance.

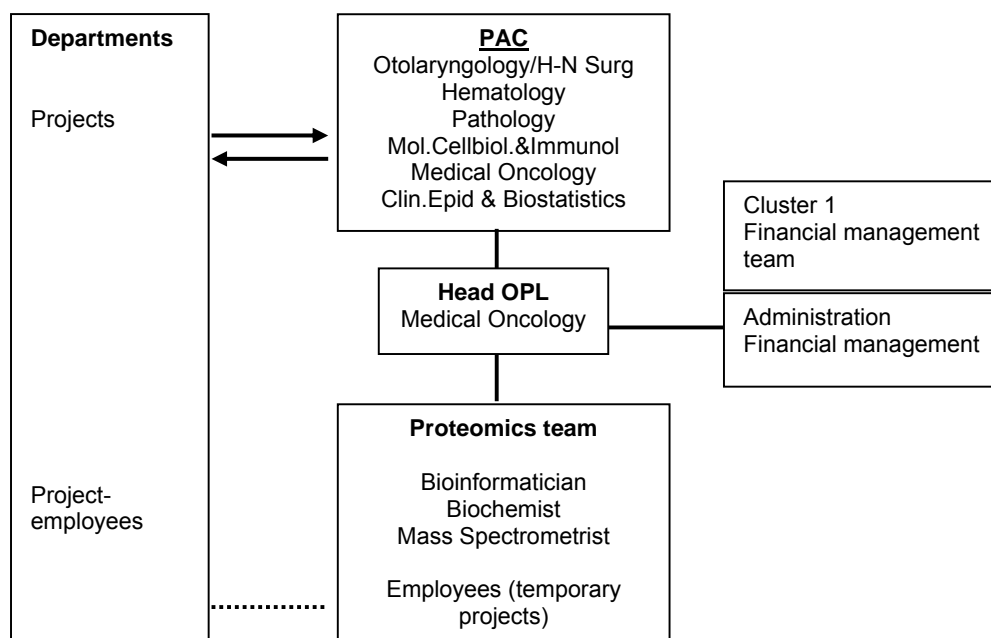


Figure 1. Schematic representation of the organisational structure of the OPL. PAC, Program Advisory Committee.

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 2007 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 2007 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-fourier 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.



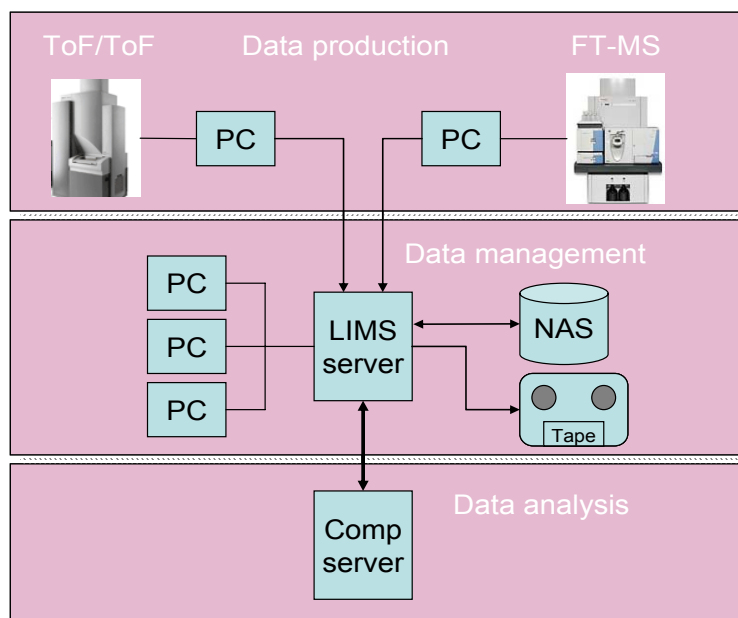
Figure 2. Tandem mass spectrometers at the OPL (CCA 1-47).

Left: 4800 MALDI-TOF/TOF (Applied Biosystems). Right: nanoLC system coupled to a Triversa Nanomate (Advion) and LTQ-FTMS (ThermoElectron).

In 2007, the software tool 'Scaffold' was purchased to aid in confidence assignment to protein identification as well as in data organisation and visualization.

In 2007, 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 2008, 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 2008



NanoLC-FTMS/MS experiments create large volume datasets (~ 0.5-1 Gb/ sample), therefore the data production per day may get as high as 10 Gb. In a typical profiling experiment we may need to compare series of up to 100 LC-FTMS runs, which calls for separate LIMS and data analysis servers. A computation cluster is preferred over a single server. For data storage NAS local storage is more economical and we will opt for a solution of tape storage for finished projects and long-term storage. With the money that remains from the CCA start-up grant, we may cover the hardware expenses but not a Laboratory Information Management System (LIMS). The latter we hope to acquire with the ICT budget in the CTMM DeCoDe grant.

Research strategy and projects

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. In recent years, proteomics has raised the hope of identifying novel biomarkers for cancer diagnosis, detection and (prediction of) drug response. This hope is based on the ability of tandem mass spectrometry, to identify thousands of proteins in complex biological samples such as biofluids, cells and tissues.

A major focus of the OPL is on targeted MS-based proteomics for biomarker discovery in tumor tissue and biofluids. The samples are profiled using two complementary platforms: 1. An automated magnetic bead-based peptide capture method coupled to high-throughput MALDI-TOF-MS and 2. Peptide separation by nano-liquid chromatography (LC) coupled to FTMS for in-depth profiling of fractionated samples (Fig 3). Cancer-related proteins may provide novel drug targets and candidate biomarkers for development into non-invasive (multiplex) antibody-based assays.

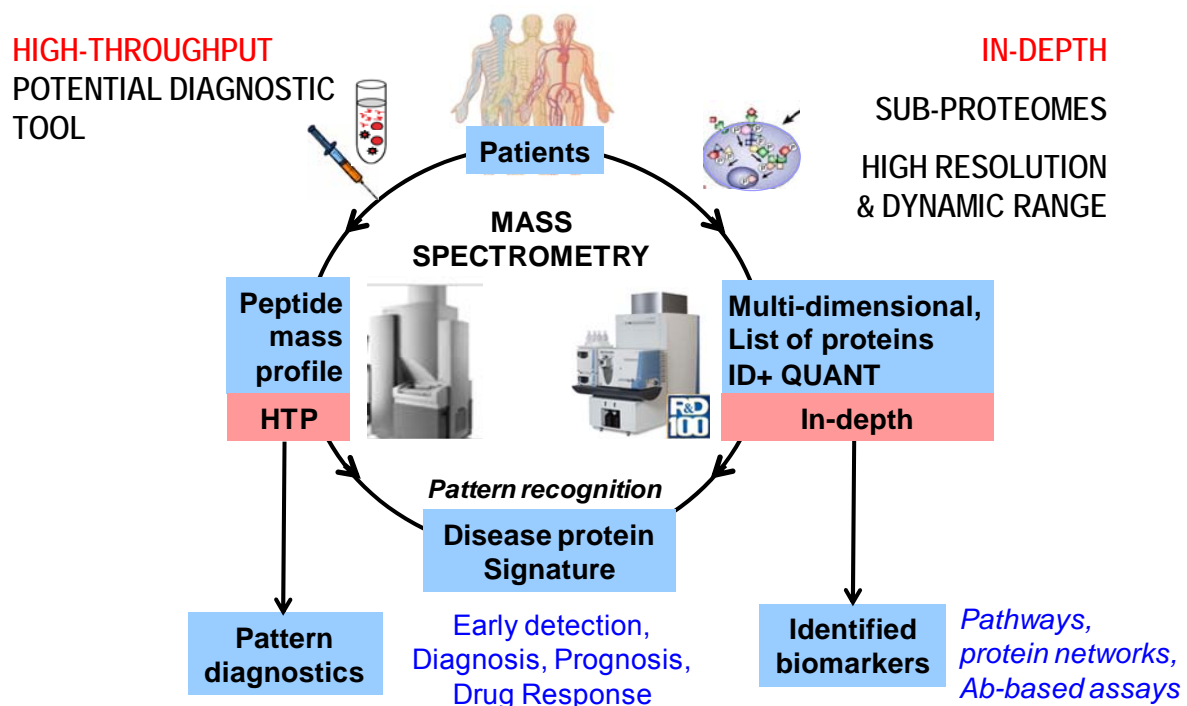


Figure 3. Complementary mass spectrometry-based proteomics approaches for discovery of cancer signatures and biomarkers at the OPL in the VUmc-Cancer Center Amsterdam.

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 (i.e., 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, i.e., 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-fourier 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, 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 et al., in prep), suggesting that the patterns may have clinical utility as surrogate markers for detection and classification of cancer.

Method development. 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 μ l is enough). In 2007, we have optimized the method to the analysis of endogenous peptide profiles in cerebrospinal fluid (CSF) and currently apply CSF peptide profiling to clinical subtype analysis in multiple sclerosis (collaboration Dr. Charlotte Teunissen and Dr. Marleen Koel-Simmelink).

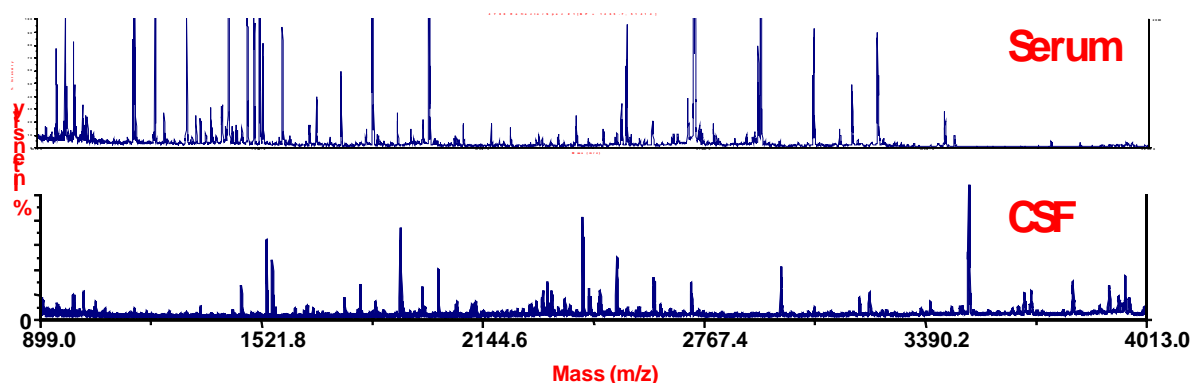


Figure 4. Serum peptide profiling of biofluids using MALDI-TOF mass spectrometry. Biofluid peptides are captured using C18-coated magnetic beads and off-line coupled to peptide profiling by 4800 MALDI-TOF/TOF-MS. The resultant representative mass spectra of serum and CSF peptides are shown. Intra-exp %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; Voortman et al., submitted). This work has been selected for an oral presentation at the ASCO2008 meeting in Chicago. For more information see research highlight I on page 16-17.

Quality control screening tool. MALDI-TOF-MS-based profiling provides a rapid screening tool to drive decisions on sample selection prior to more in-depth analysis by (2D)LC-FTMS. Peptides indicative of serum and CSF sample quality have been identified (Jimenez et al., 2007b). Therefore, MALDI-TOF-MS may be used as an upfront quality control step. Multivariate analysis of the data sets would help reveal potential outliers as a result of either sample handling or intrinsic patient variability. This would aid in the selection of a smaller sample subset for in-depth comparative analysis using lower throughput LC-MS based strategies.

2. Biomarker discovery and cancer mechanisms by targeted LC-FTMS-based proteomics.

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 amenable 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 ($\sim 10^6$) 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.

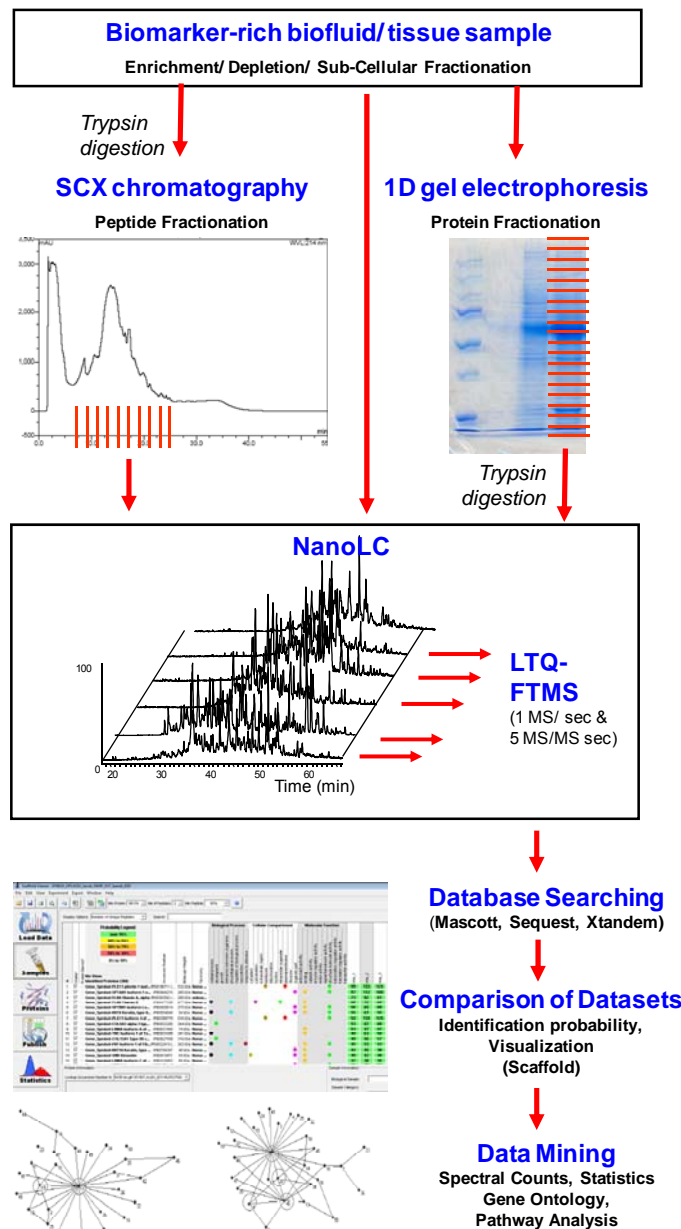


Figure 5. In-depth proteomics workflows at the OPL.

BIOFLUIDS For nanoLC-FTMS profiling of biofluids such as plasma and CSF, a first focus has been on native peptides and protein <30 kD instead of tryptic peptides, because the low molecular weight proteome of biofluids have great diagnostic potential. Moreover, this selection also increases the sensitivity of the analysis as very high abundant proteins such as albumin and IgG are removed. We are currently optimizing existing methods to reproducibly isolate the low molecular weight proteome of CSF while minimizing protein losses (collaboration Frode Berven and EU cNeuPRO project). Moreover, biofluids depleted of abundant proteins using multi-affinity depletion columns create a subproteome enriched for more low abundant proteins. In 2008 this avenue is being employed for mining the CSF proteome (EU cNeuPRO project).

Other sub-proteomes of interest in biofluids are the glycoproteome ('sialome'), platelet-derived releasates and microparticles. The feasibility of proteomics of urine-derived microparticles has been explored in a small-scale study in healthy individuals. Proteomics of fresh platelet-derived TRAP-induced secretomes has been explored in a pilot study performed by a master student, M. Kapci. This analysis yielded 716 proteins of which 225 proteins present in the releasate of 3/3 volunteers, constituting the 'core platelet secretome' (Piersma et al., manuscript in prep; for summary of project see appendix, page 35).

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). Subnuclear proteomics of human CRC and secretome proteomics of mouse CRC are further described in research highlights 2 and 3, respectively (pages 18-24).

3. Mining of multi-dimensional proteomics datasets

MASS SPECTROMETRY DATA PREPROCESSING & QUANTITATION In 2007 most quantitation was done label-free because this approach represents a promising (and economical) avenue for quantitation at high sensitivity.

For MALDI-TOF-MS data, MarkerView has proven to be a reliable data preprocessing tool prior to pattern analysis. In our in-depth LC-MS/MS workflow, we are currently using peptide counts per protein and spectral counts (the total number of times an ion has been selected for MS/MS) as the quantitative measure (Figure 6). This turned out to be a robust, reliable approach though possibly not the most sensitive approach as it relies on MS/MS data.

To exploit MS1 data in LC-MS datasets for quantitation, we are developing robust data analysis methods for label-free quantitation of peptide ion abundance. To this end, we have implemented the open-source tools MzMiner, MSInspect and PEPPer as well as home-made algorithms. See appendix for summaries of these projects that were carried out as part of core research and in collaboration with FEW-VU.

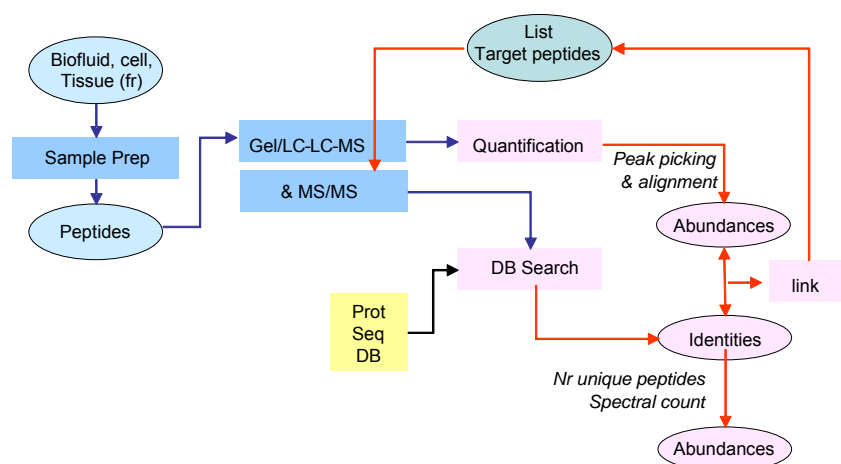


Figure 6. Quantitative LC-MS-based proteomics strategies. In 'shot-gun sequencing-type' LC-MS/MS experiments, peptides are randomly selected for MS/MS. Protein identification is obtained by automated database searching of the acquired MS/MS data. In label-free MS/MS-based quantification, the number of times that a peptide is selected for MS/MS (spectral count) or the number of unique peptides detected per protein give a relative measure of protein abundance. The software tool Scaffold (ProteomeSoftware) facilitates this type of analysis. In addition, the ion intensities in MS1 may be used to quantify the peptides. Peptides with altered expression levels linked to a particular condition, are subjected to data-dependent MS/MS sequencing in a second targeted experiment using an inclusion list resulting from the primary comparative analysis. Our LTQ-FTMS allows for a label-free combination strategy that employs both HTP LTQ MS/MS data with accurate FT MS1-pattern data that were produced in parallel. The freely available PEPPer pipeline that we implemented in 2007 facilitates the automated analysis this type of hybrid identity-pattern approach.

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 evaluating 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 mechanisms. 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 will be obtained.

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 2007

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
Jimenez/ Broxterman/Hoekman	OPL/ Onc	Proteomics of platelet releasates for early detection of cancer (method optimization)
Jimenez/ Piersma	OPL	Secretome proteomics: method evaluation / optimization
Jimenez	OPL	Sub-Nuclear proteomics of human tumor tissue: method evaluation / optimization
Jimenez/ Fijneman	OPL/ Onc	Secretome proteomics for biomarker discovery in genetic mouse model
Jimenez/ Giaccone	OPL/ Onc	Clinical outcome prediction and treatment response serum peptide profiling
Jimenez	OPL	Plasma membrane proteomics of human tumor tissue: method evaluation / optimization
Jimenez	OPL	Clinical Neuroproteomics of Neurodegenerative Diseases, cNEUPRO (EU kp6 STREP)
Jimenez/ Peerdeman	OPL/ Neurosurgery	Meningeoma: comparison of WHO stages and within WHOI

Yellow: See research highlights below.

b. Projects completed in 2007

Table 2 contains the list of studies that were completed in 2007. The projects with Hoekman and Teunissen resulted in two first authorships and with Engwegen and Marchiori in several co-authorships (see publications). The project with Voortman and Giaccone is currently a chapter in a dissertation and will be submitted for publication in 2008.

Table 2. Collaborative projects completed in 2007

Name	Department	Project
Engwegen	apothek SLZ	Identificatie differentele peptides SELDI-TOF-MS
Jimenez/ Hoekman	OPL/ Onc	Method development HTP serum peptide profiling
Jimenez/ Teunissen	OPL/ MCBi	Method optimization HTP CSF peptide profiling and CSF pre-analytics
Jimenez/ Marchiori	OPL/ FEW-VU	Comparison and implementation of LC-MS data analysis tools and algorithms

c. Collaborative projects CCA-V-ICI:

Table 3 summarizes the list of on-going collaborative projects with CCA-V-ICI researchers that have been initiated in 2007.

Table 3. Collaborative projects CCA/V-ICI

Name	Department	Project
Meijer/ Fijneman	PA	Cell surface proteomics for imaging markers for colon cancer.
Lankelma	Onc	Quantitative urinary peptide measurements by fluorogenic labeling
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	Bone marrow and secretome proteomics in AML
Middeldorp, Pechtold	PA	Exosome proteomics in EBV+ tumor immune escape
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 ICEN (cell biology), FALW-VU (molecular neurobiology and plant physiology) and FEW-VU (pharmaceutical chemistry).

Table 4. Collaborative projects VU/Vumc

Name	Department	Project
Teunissen/ Jimenez	MCBI/ OPL	Biomarkers discovery for axonal damage in MS by CSF proteomics
Smit	Pharm.Chemie-VU	Phosphoproteomics of viral chemokine signaling networks in oncogenesis & metastasis
De Boer	VU	13-3-3 interactors & interaction with new anti-cancer agent from plant fungus

e. External collaborative projects:

Table 5 shows the list of external collaborative projects.

Table 5. External collaborative projects

Name	Department	Project
Jimenez/ Niclou/ Bjerkvig	NorLux Neuro-Oncol.	Membrane proteomics of differentially invasive brain tumor spheroids (EU fp6)
Jimenez/ Berven	OPL/ Bergen Norway	Profiling of the of the low molecular weight proteome of serum (CSF)
Jensen, Callesen	Univ.South. Denmark	Serum profiling of breast cancer patients
Kubbutat/ Fiedler	Proqinase, Germany	Secretome analysis for identification of IGFR1-related biomarkers (EU fp6)
Westerveen	ID Lelystad	Shot-gun sequencing by nanoLC-FTMS of mycobacterium tuberculosis

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	Med. Microbio	Massabepaling synthetische peptiden
Bolscher	Med. Microbio	Identification/characterisation HPLC-purified saliva peptides
Luurtsema	NucMed/PET	Structural analysis of verapamil metabolites
Extern		
Van Diepen	Univ. London	Identification of PTEN interactors by tandem MS
Westerveen	ID Lelystad	Protein identification from 2D gel spots
Mohammadi	UvA	Protein identification from 1D gel bands
Kikkert	Sanguin	Protein identification from 1D gel bands

g. New projects (other than above) planned for 2008:

Several new exploratory meetings between the head OPL and interested VUmc researchers took place in 2007. Table 7 lists all the new projects planned for 2007. Some of them are pilot projects where the goal is to obtain data that can be the basis for a grant proposal. A major focus in 2007 will be on miniaturization (analysis of cancer stem cells), kinome profiling and phosphoproteomics for personalized medicine.

Table 7. New projects planned for 2008		
Name	Department	Project
Jimenez	OPL	Exosome proteomics as new avenue for biofluid biomarker discovery
Jimenez	OPL	Serum profiling using the protease activity test -implementation
Jimenez/ Boven	OPL/ Onc	Proteomics for biomarker discovery in (genetic) breast cancer
Jimenez/ Piersma/ Verheul	OPL/ Onc	Kinome profiling for patient selection
Jimenez/ Meijer	OPL/ PA	Sub-Nuclear proteomics in colorectal adenoma to carcinoma progression
Jimenez/ Meijer	OPL/ PA	Candidate-based proteomics by nanoLC-MRM-MS in colorectal cancer (CTMM)
Diosdado/ Meijer	PA	Proteomics of miRNA ko cell lines
Dijkmans/ Jansen	Rheuma	Serum/synovial fluid profiling in rheuma arthritis
Schuurhuis/Ossenkoppele	Hema	Cancer stem cell proteomics in AML
Meisch/ Niessen	PA	Proteomics in cardiac disease
Veerhuis/ Blankenstein	Klin Chem	b-amyloid peptide fingerprinting in CSF of dementia patients by ImmunoMS
Piersma/ Li	OPL/ MCN-VU	Comparison of label-free quantitation methods
Van den Berg, Berendse	NeuroAnat/ PA	CSF biomarkers for Parkinsons' Disease
Extern		
Jimenez/ Giaccone	OPL/ NCI	Serum profiling -activity test for clinical outcome prediction and treatment response in NSCL
Jimenez/ Bonfrer/ Van Tellingen	OPL/ NKI	Implementation of serum profiling -activity test and testing of archived samples
Jimenez/ Berns	OPL/NKI	Proteomics of genetic mouse model for lung cancer (CTMM)
Jimenez/ Jonkers	OPL/ NKI	Treatment response biomarkers discovery in genetic mouse model for breast cancer
Saydam	Harvard	Proteomics of miRNA ko cell lines
Smit	MCN-VU	Serum profiling in depression

Research highlight I

Serum Peptide Classifier for Predicting Treatment Outcome in Lung Cancer Patients

The ability to predict treatment response and clinical outcomes is one of the two "holy grails" of cancer biomarker development, the other being early detection or risk stratification by population screening. Only a minority of patients with advanced non-small cell lung cancer (NSCLC) benefit from chemotherapy. Currently, no biomarkers exist that are useful to select patients more likely to benefit from chemotherapy. Therefore a strongly predictive blood-based test would be a very attractive and patient-friendly method for selection of patients who are most likely to benefit from chemotherapy.

In this study, we applied our newly developed serum peptide mass profiling method for the discovery of serum peptide patterns associated with treatment outcome in patients treated with gemcitabine, cisplatin and bortezomib. During the phase IB clinical trial, Dr. Jens Voortman, with utmost care, collected all the serum samples according to our standardized protocol, to avoid bias from difference in sample handling. Dr. Jaco Knol of the OPL processed all sera with the aid of a liquid handling system, the KingFisher96, for automated magnetic particle-assisted peptide capture from 96 sera in parallel and he performed MALDI-TOF mass spectrometry. Dr. Thang Pham of the OPL used machine learning techniques for the discovery of a combination of peptides, a so-called signature, that classifies patients according to their progression-free survival after chemotherapy. This algorithm was based on 6 peptide ions in the peptide mass spectra of pretreatment sera of patients and was able to distinguish with 82% accuracy, sensitivity and specificity patients with a relatively short vs. long progression-free survival. In addition signatures were identified that separate patients with a partial response vs. non-responders with 89% accuracy at 100% sensitivity and 83% specificity and that could discriminate NSCLC patients from cancer-free controls with 98% accuracy at 100% sensitivity and 96% specificity. Using tandem mass spectrometry, some of the identities of the signature peaks used for the algorithm were elucidated. These signature peptides represented differential cleavage products of abundant blood proteins, underscoring the hypothesis that cancer(-outcome)-specific proteases are important for generating the signature peptides.

OUTLOOK The results of this exploratory pilot study are very encouraging for the further development of serum-based biomarker signatures for prediction of treatment outcome in NSCLC patients. Our study was selected for oral presentation at the 2008 American Society of Clinical Oncology (ASCO) annual meeting (for abstract see text box).



Johannes (Jens) Voortman, MD, PhD, studied medicine and medical biology at the University of Amsterdam. As a MD-clinical research trainee, he completed his PhD at the VU University in 2008. In 2006 he started his residency in internal medicine at the VU University Medical Center, currently interrupted for a post-doc at the National Cancer Institute in Bethesda, USA.
Email: voortmanj@mail.nih.gov



Dr. Jaco Knol joined the OPL as a research technician in April 2006. He studied Biochemistry at the Vrije Universiteit (VU) in Amsterdam, did the military replacement service at the Netherlands Cancer Institute, obtained his PhD in the Dept. of Biochemistry at the VU, and was a post-doc in the Dept. of Biochemistry of the University of Groningen. His research interests are molecular cell biology and mass spectrometry-based proteomics.
Email: i.knol@vumc.nl



Dr. Thang V. Pham received his B.Sc. degree in computer science from the RMIT University, Australia in 1998 and Ph.D. degree from the University of Amsterdam, the Netherlands in 2005. He was a postdoctoral fellow at the Intelligent Systems Lab Amsterdam until he joined the OPL as a research associate in October 2006. He is currently working on pattern recognition techniques for cancer biomarker discovery.
Email: t.pham@vumc.nl

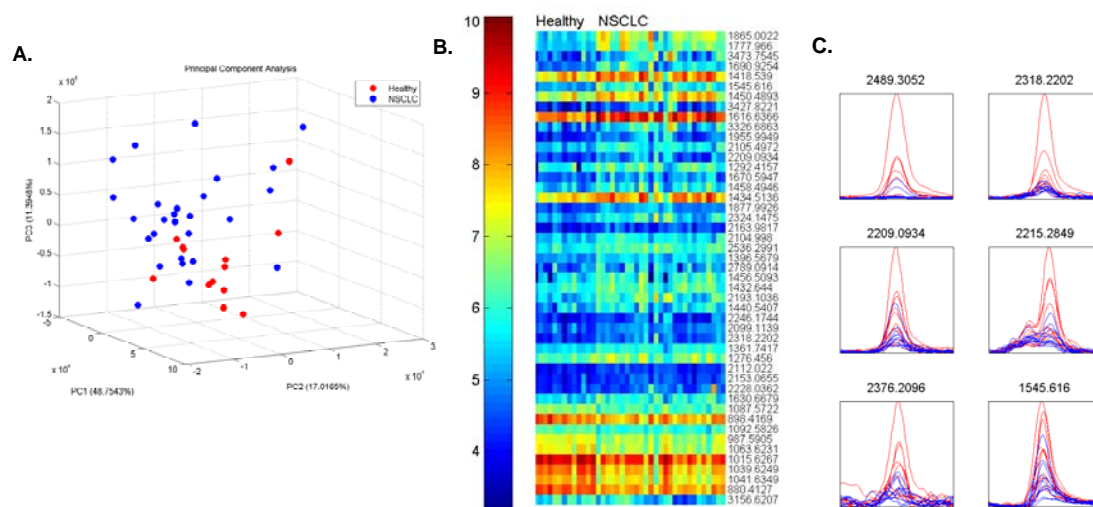


Figure 7. A, Principle Component Analysis (PCA) healthy vs. NSCLC comparison. B, heatmap of the 47 differential peaks in the healthy versus NSCLC comparison shown in the natural log scale. The peaks are ordered by median fold change between the two groups. C. Spectral overlay of ion intensities of the 6 peptides underlying the progression-free survival (PFS) signature: short PFS (red) and long PFS (blue).

In future studies, we plan to validate the signature using independent serum samples and explore serum samples that are currently being collected in clinical trials with new targeted agents (collaboration Prof. Egbert Smit). Moreover, in collaboration with Dr. Josep Villanueva and Prof. Paul Tempst from the Memorial Sloan Kettering Cancer Center in New York, we will explore the use of the newly emerging MALDI-TOF-MS-based 'protease activity test' for prediction of treatment outcome in the serum samples from this study.

Abstract:

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. Knol¹, Giuseppe Giaccone^{1, 2}, Connie R. Jimenez¹
¹OPL ²Medical 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.

Accepted for oral presentation at ASCO2008. This research was supported by the VUmc Cancer Center Amsterdam

Research highlight II

Secretome proteomics for candidate biomarker discovery

Proteins secreted from cancer cells and tumor tissue may provide a new avenue for discovery of blood-based biomarkers of the disease (reviewed by Jimenez et al, Biomarkers in Medicine, 2007). The secretome includes proteins that are either secreted, shed by membrane vesicles (exosomes), or externalized due to cell death. This new source is increasingly being interrogated by the clinical proteomics community, as is apparent from the rapid rise in publications in the past 3 years. This discovery strategy may provide an alternative to serum proteomics that is challenging because of the complexity and large dynamic range of protein expression levels, hampering the detection of low abundant, tissue-specific proteins. Importantly, several secretome studies published to date have validated selected secretome candidates in patient material using antibody-based assays in serum or tissue microarrays, underscoring the potential of this discovery approach.

In 2007 the OPL set out to evaluate this promising biomarker discovery strategy in two studies, one using secretomes derived from cancer cell lines to determine the most optimal proteomics method and the other one analyzing tumor tissue secretomes for candidate biomarker discovery for early detection (in collaboration with Dr. Remond Fijneman).

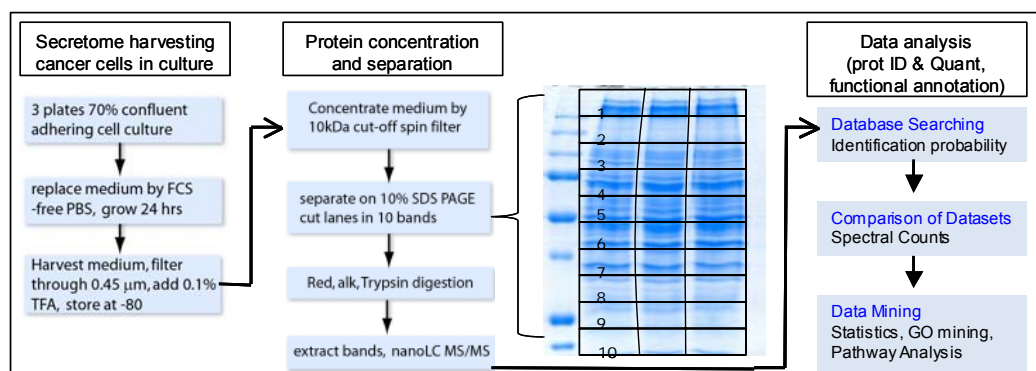


Dr. Sander Piersma joined the OPL as a research associate at the start in April 2006. He studied Chemistry at the University of Amsterdam (1993), obtained his PhD in the Dept. of Enzymology at the Technical University of Delft (1998), and was a post-doc at the RIKEN Institute in Tokyo and at TNO. He worked as project leader at the Institute for Atomic and Molecular physics on development of imaging mass spectrometry. His research interest is development of innovative mass spectrometry-based proteomics for cancer research and biomarker discovery. Email: s.piersma@vumc.nl

METHOD EVALUATION In the past year, Dr. Sander Piersma of the OPL has evaluated 3 different proteomics workflows for secretome analysis in terms of sensitivity and reproducibility. To this end, conditioned media of non-small cell lung cancer cells (provided by Ing. Simone Span and Dr. Frank Kruyt) were analyzed by: 1. protein separation in TC2 cartridges, followed by protein digestion of the fractions and nanoLC-MS/MS 2. Protein separation by 1D gel electrophoresis followed by in-gel digestion of gel bands and nanoLC-MS/MS and 3. Digestion and peptide separation by strong cation exchange chromatography followed nanoLC-MS/MS.

The workflow outlined in Figure 9, combining 1D gel-based protein separation followed by nanoLC-MS/MS of the in-gel digested peptides clearly identified the largest number of proteins in the media with good reproducibility of biological replicates. For more details see boxed abstract of the study. We were encouraged by the detection of low abundant protein such as growth factors in the secretome dataset.

Figure 9. Flow chart depicting the optimized, best performing work flow for cancer cell secretome analysis



OUTLOOK We envision that targeted approaches that balance sensitivity and throughput will ultimately prove to be most productive for finding novel and robust biomarkers in biofluids. The focus of the OPL on information-rich subproteomes such as cell/tissue secretomes combined with GeLC-MS/MS, provides a sensitive and semi-HTP discovery platform.

Abstract:

Comparison of label-free proteomics methods for the quantitative analysis of cancer cell secretomes

Sander Piersma¹, Simone Span², Frank Kruyt², Connie R. Jimenez¹

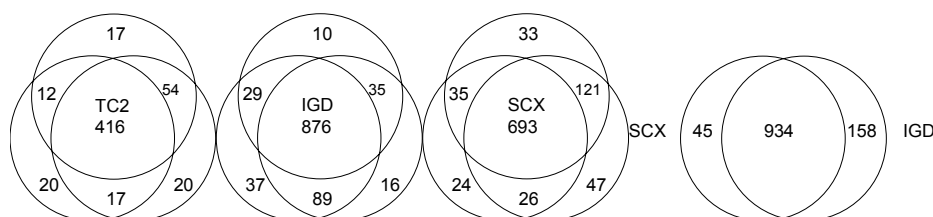
¹OPL, ²Dept. Medical Oncology, VU University Medical Center, Amsterdam

Introduction Biomarker discovery in plasma is hampered by the plasma proteome complexity as well as its dynamic range. We focus on a sub-proteome consisting of the proteins secreted by tumor cells; the secretome. The secretome has a reduced complexity compared to plasma, is enriched for cancer-cell specific proteins and moreover, secreted proteins are likely to be detected in blood. The reduced complexity allows for coverage of a higher dynamic range as well as detection of lower abundance proteins. Here we describe a comparison of ID-based LC-MS/MS workflows for secreted proteins.

Methods Adhering H460 non small-cell lung cancer cells were grown, and 24 hours prior to harvesting cells were washed and cultured on serum-free medium. Medium was collected and concentrated by ultrafiltration (10 kDa cut-off). Three methods for secretome analysis were compared: 1. protein hydrophobic interaction (C2 resin) followed by tryptic digestion, 2. 10% SDS-PAGE or 4-12% SDS-PAGE followed by in-gel digestion and 3. solution digestion followed by off-line SCX chromatography. The final step for all 3 methods was nanoLC-MS/MS using a LTQ-FT instrument. Data was searched against IPI-human using Sequest (min 2 peptides, <10 ppm MMD) and ID's were validated by applying the Peptide prophet (P>95%) and Protein prophet (P>99%) algorithms using Scaffold.

Results One of the key points in obtaining a good secretome fraction is replacing the medium by serum-free medium, thus minimizing interference by bovine serum-derived proteins. However, serum-free incubation time has to be optimized to minimize apoptosis and cell lysis. For H460 NSCLC cells we found 24 hours to be optimal with respect to protein yield and cell viability.

We compared 3 workflows with biological triplicate samples. Intact proteins separation by C2 hydrophobic interaction resin method yielded 580 proteins (N=3 experiments) with 416 proteins in all 3 experiments. Conversely, the 10% SDS PAGE IGD analysis yielded 895 proteins (N=3) with 699 proteins identified in all 3 experiments. Comparing this with 4-12% gradient gel and off-line SCX gave 1092 and 979 identified proteins with 876 and 693 proteins in all 3 experiments, respectively. The H460 secretome was enriched for secreted proteins compared to H460 total cell lysate (1726 proteins identified in a 10 gel band IGD experiment) as found by Signal P, Secretome P and GO analysis. As compared to literature (1) this data set comprises the largest Secretome to date. The 4-12% SDS-PAGE LC-MS/MS analysis was superior over the other workflows.



Innovative aspects

1. The best performing, optimized workflow based on GeLC-MS/MS for secreted protein analysis yields several-fold more protein identifications than reported in literature,
2. The secretome represents a promising sub-proteome enriched for candidate biomarkers
3. The optimized workflow has been successfully applied to the differential analysis of tumor tissue secretomes in a colon tumor mouse model

SECRETOME PROTEOMICS OF MOUSE COLON TUMORS

In collaboration with Dr. Remond Fijneman, we have applied the optimized, best-performing GeLC-MS/MS workflow to the differential analysis of the secretomes of colon tumors and normal colons isolated from a mouse model for colorectal cancer (APC conditional truncation, courtesy Prof. Riccardo Fodde).

To this end, masters student Maral Pourghiasian collected secretomes from 3 independent freshly dissected mouse colon tumors (Fig. 10A) and 3 control pieces of colon that were both incubated for 1 hr in medium. We were pleasantly surprised by the rich information harbored in the colon tumor secretome GeLC-MS/MS dataset. First of all, we identified over 2000 proteins of which a large proportion (~ 30%) was significantly regulated. Further underscoring the power of the approach: among the regulated proteins, many proteins with a known role in colon cancer biology were identified. Importantly, the majority of the up-regulated proteins in the mouse tumor secretome dataset were also detected in human CRC tissue (236/321; Fig. 10B). A subset of 49 candidate biomarkers was also differential at the mRNA level in human adenomas versus carcinomas ($p < 0.05$). Proteins in the overlap between datasets provide high priority candidate protein biomarkers for follow-up studies. Indeed, the top-49 colon tumor secretome candidates include 4 known (mouse and human, blood and stool) CRC biomarkers, validating our approach.

OUTLOOK The potential presence of cancer-related secretome proteins in plasma or faeces of human CRC patients is currently being explored using western blotting. Ultimately we wish to develop selected, validated candidate biomarkers for early stage CRC into a prototype non-invasive antibody-based test.



Dr. Remond J.A. Fijneman studied biochemistry at the RU Leiden (1991), and performed his PhD research on "identification of susceptibility genes for non-familial cancer" at the Netherlands Cancer Institute in Amsterdam (1997). He studied the link between inflammation and cancer at Harvard Medical School (Boston, USA) and the Netherlands Institute for Developmental Biology (Utrecht). In 2000, he came to the VU University Medical Center where he investigated the role of macrophages in tumor development. Since 2004 he is appointed at the Tumor Profiling Unit (head: G.A. Meijer). His current activities focus on the effects of stromal cells on colon tumor development, and on identification of biomarkers for molecular imaging of colorectal cancer.
Email: rja.fijneman@vumc.nl

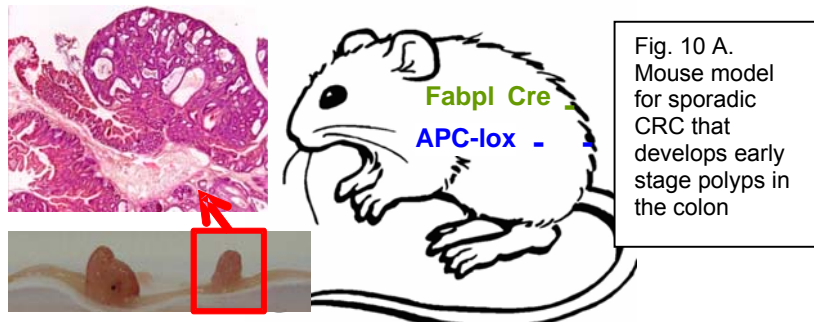
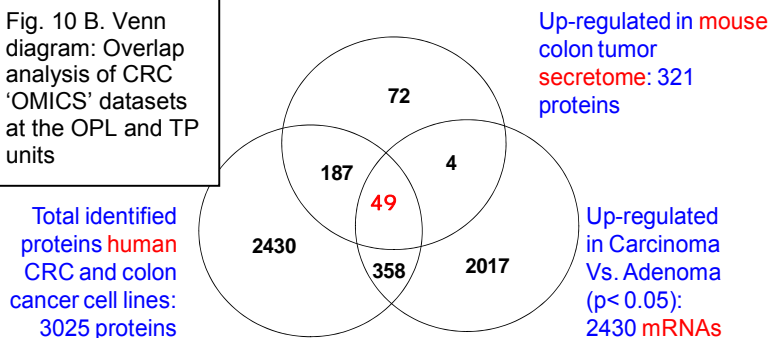


Fig. 10 A. Mouse model for sporadic CRC that develops early stage polyps in the colon

Fig. 10 B. Venn diagram: Overlap analysis of CRC 'OMICS' datasets at the OPL and TP units



Why mouse colon tumor secretome proteomics for biomarker discovery?

1. Tumor secretome contains high 'undiluted' concentrations of tumor-secreted/shed proteins
 - No genetic heterogeneity between samples
 - Mouse tumors represent early stages of carcinogenesis
2. Secreted proteins are more likely to be found in plasma
3. Sub-proteome, thus reduced complexity
 - Increased dynamic range of detection
 - Low abundant proteins accessible

Abstract:
**Identification of proteins enriched in mouse colon tumor secretome
as candidate biomarkers for blood/stool-based screening of colorectal cancer**

RJA. Fijneman¹, M. de Wit², M. Pourghiasian³, S. Piersma³, P.M. van Diemen², V.W.H. van Hinsbergh^{1,4}, R. Smits⁴, R. Fodde⁵, GA. Meijer² and CR. Jimenez³

Depts. of 1Medical Oncology, 2Pathology, 3OncoProteomics Laboratory, 4Physiology, VU University Medical Center, Amsterdam, 5Dept. of Pathology, JNl, EMC, 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 for reducing the high number of CRC deaths. The Faecal Occult Blood Test (FOBT) lacks sensitivity and specificity, while colonoscopy is a burdensome procedure.

AIM The aim of this study is to identify biomarkers that can be used to develop a blood-based test for early diagnosis of CRC. The tumor secretome 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 blood-based biomarkers.

APPROACH. Using a mouse model for sporadic CRC (conditional APC-truncation), colon tumors and healthy control tissues were excised, washed briefly, and incubated in a small volume of PBS at 37°C for 1 hour. Tissues, cells, debris, and insoluble proteins were removed by centrifugation steps. The remaining soluble fraction, the “secretome”, was analyzed by in-depth (GeLC-MS/MS) proteomics.

RESULTS Mouse colon tumor secretomes (n=3) were compared to secretomes of healthy colon tissues (n=3) from age- and gender-matched mice. This procedure revealed 2174 different proteins, 146 of which exhibited more spectral counts in each tumor sample than in any control sample. Of these, 80 proteins exhibited an average of 4-fold more spectral counts in tumor samples than in controls (semi-quantitative measurement), including several proteins which were previously reported as potential plasma markers for mouse APC truncation tumors, thereby confirming the validity of our approach. Importantly, numerous proteins were identified with a role in human CRC.

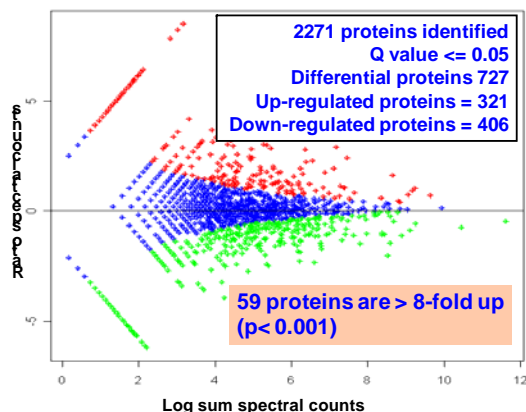


Figure. G-test statistics shows that a large proportion (~30%) of the proteins identified in the mouse colon and colon tumor secretomes is regulated. Analysis courtesy Dr. Marc Warmoes.

OUTLOOK We are currently examining by Western blotting which of these putative secretome biomarkers can be found at increased levels in blood of tumor-bearing mice, and may serve as promising candidate biomarkers for further validation in human blood from CRC patients and control subjects.

This research was supported by the 1st AEGON International Scholarship in Oncology and by the VUmc Cancer Center Amsterdam

Research highlight III

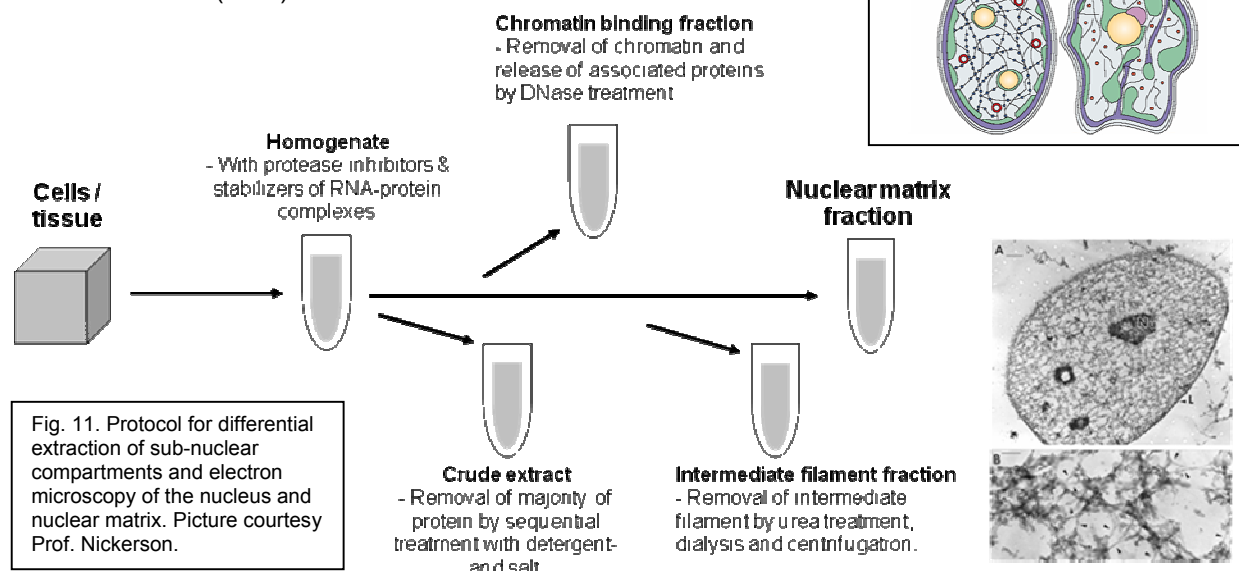
Sub-nuclear proteomics of human tumor tissue

Important morphological changes in tumor cells are alterations of nuclear structure, such as nuclear size and shape, numbers and sizes of nucleoli and chromatin texture; however, little is currently known about the molecular mechanisms behind the structures of cancer cells observed by the pathologist under the microscope. In this respect, proteomics of the nuclear matrix, and other sub-nuclear fractions could yield protein insight into the missing link between the present diagnostic paradigm in cancer, based primarily on pathological investigation of tissue biopsies, and cancer genetics. Moreover, the nuclear matrix proteome turns out to be a valuable source of protein biomarkers for cancer as is evidenced by the FDA-approved Bladdercheck test that measures urine levels of the nuclear matrix protein NUMA/ NMP22

Since 2006 human colorectal tumor tissue is being collected for proteomics by people of the Tumor Profiling Unit, headed by Prof. Gerrit Meijer. In the past year, we have started to exploit this high quality material and to investigate the potential of sub-nuclear proteomics for molecular insight into cancer pathways. To this end, we investigated the protein composition in sub-nuclear compartments using human colorectal cancer (CRC) as a model.

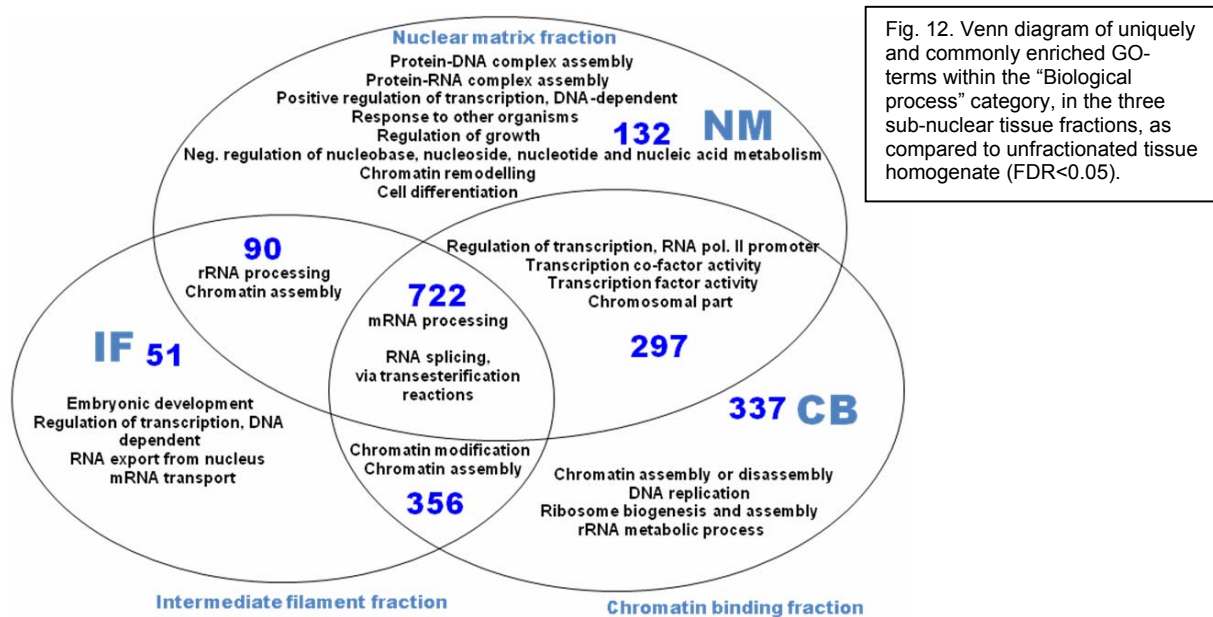


Jakob Albrethsen got his masters in protein biochemistry at the University of Copenhagen, Denmark in 2001. He got his PhD in 2006 at the same university in collaboration with the Glostrup University Hospital, Denmark, on proteomics of colorectal cancer tumors and serum. His research into clinical proteomics was continued in 2007 by a one-year project at the OPL dedicated to examination of the nuclear matrix of colorectal cancer by the use of state-of-the-art proteomics equipment at the OPL.
Email: j_albrethsen@hotmail.com



As a first step, Dr. Jaco Knol established at the OPL the Fey and Penman protocol outlined in Figure 11 for differential extraction of sub-nuclear compartments, yielding a chromatin-binding fraction, a intermediate filament fraction and the nuclear matrix fraction. Subsequently, Dr. Jakob Albrethsen evaluated the reproducibility of nuclear matrix isolation from colorectal tumor tissue and established the protein parts list of the 3 sub- nuclear compartments of a pooled colorectal tumor sample and performed all the data mining.

The analysis of the 3 sub-nuclear CRC fractions, yielded a rich dataset of a total of 2020 non-redundant proteins, including nuclear matrix proteins implicated in CRC as well as several of the candidate genes identified by genomics. Gene ontology mining of this dataset revealed insight into nuclear subdomain functions (Figure 12 and abstract in box). Furthermore, mining subdomain-enriched proteins for protein-protein interactions shows that specific protein complexes are present in the different sub-nuclear fractions. Importantly, the entire workflow from biochemical isolation to protein identification was reproducible and is therefore applicable to profiling multiple patient samples for differential analysis.



OUTLOOK Sub-nuclear proteomics of tumor tissue is a feasible and promising avenue for exploring oncogenesis and identification of targets for treatment. The nuclear matrix fraction contains ~50% of the known cancer-related nuclear proteins and is therefore a relevant target for discovery. We anticipate that differential sub-nuclear proteomics will identify nuclear proteins that are associated with chromosomal instability and colorectal adenoma-to-carcinoma progression. Subsequently, we will explore their biomarker potential for early detection.

Abstract:

SUBNUCLEAR PROTEOMICS THROUGH DIFFERENTIAL EXTRACTION OF COLORECTAL CANCER TISSUE

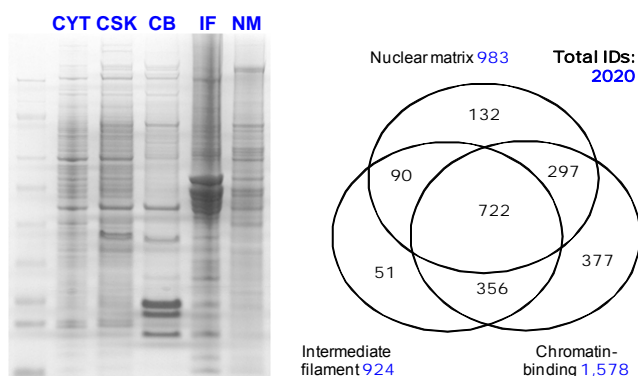
Jakob Albrethsen¹, Jaco C Knol¹, Sander Piersma¹, Meike de Wit², Sandra Mongera², Beatriz Carvalho², Gerrit A Meijer², and Connie R Jimenez¹

1OPL, 2Tumor Profiling Unit, Dept. Pathology, VU University Medical Center, Amsterdam.

Introduction Abnormalities in nuclear shape and chromosome structure are key features of the cancer cell. An important determinant of nuclear structure is the nuclear matrix, a dynamic, filamentous protein-RNA structure thought to have a central role in chromosome organization and function. As a first step towards the study of normal and aberrant NM function, we have utilized differential extraction of tumor tissue cells, yielding cytosolic-, cytoskeletal-, chromatin-binding-, intermediate filament-, and nuclear matrix-enriched fractions. Here, we investigate the reproducibility of the entire workflow, and report on the proteins enriched in the subnuclear fractions as well as the gene ontologies associated with these proteins.

Methods Colorectal carcinoma tissue was differentially extracted essentially as published [Fey EG and Penman S (1988) PNAS 85: 121]. Following potter-homogenization in CSK buffer (0.5% Triton X-100, 300 mM sucrose, salts, + inhibitors), filtering through gauze, and a 5-min incubation, the homogenate was spun (5 min at 750xg, sup='cytosol'). The detergent-insoluble pellet was then extracted with CSK, and processed as above (sup='cytoskeleton'). The detergent- and salt-resistant pellet was incubated for 30 min with RNase-free DNase I, extracted for 5 min with ammonium sulfate-containing CSK, and spun (5 min, 1000xg, sup='chromatin-binding (CB) fraction'). The detergent-, salt-, and DNase-resistant pellet was solubilized in denaturing buffer containing 8 M urea and dialyzed O/N against filament-renaturing buffer. Renatured 'intermediate filaments (IF)' were spun down for 90 min at 217,000xg, and the supernatant was collected as the 'nuclear matrix fraction'. The latter three fractions were subjected to SDS-PAGE, lanes were cut in 10 sections, subjected to in-gel digestion, and analysed on a nanoLC system equipped with a C18 reversed phase column and coupled on-line to an LTQ-FTICR mass spectrometer. The top 5 peptide signals from full scans in the FTICR segment were subjected to MS/MS in the linear ion-trap. After searching the human IPI database with Sequest software, results were analyzed with Scaffold. For each protein identified, the number of unique peptides as well as the spectral counts were exported to an Excel spreadsheet.

Results Through nanoLC-MS/MS analysis, we have established the parts list of the CB-, IF-, and NM-enriched fractions. In all three fractions combined, we identified a total of 2,020 non-redundant proteins (identified by two peptides at > 95% confidence). Gene ontology mining indicated differential extraction of subnuclear domains. For example, the CB fraction was significantly enriched for proteins associated with chromatin assembly and disassembly, the IF fraction for proteins involved in RNA export, and the NM fraction for proteins implicated in protein-DNA complex assembly. In a triplicate analysis of a single NM fraction, 888 of 1,047 proteins (85%) were reproducibly identified, and the average CV of the number of unique identified peptides per protein was 28% for proteins found in all replicates. This indicates a good reproducibility of the entire workflow from biochemical isolation to MS analysis. Together, the results show the feasibility of subnuclear proteomics of tumor tissue, which provides a promising avenue for exploring a putative NM role in oncogenesis.



Innovative aspects

- Differential analysis of subnuclear fractions of cancer tissue by label-free GeLC-MS/MS reveals insight into potential compartment-specific functions and uncovers many cancer-related proteins.
- Evaluation of the complete workflow of nuclear matrix isolation in terms of reproducibility.
- The optimized workflow is currently being applied to the differential analysis of colorectal adenomas and microsatellite instable and chromosomal instable carcinomas

EDUCATION

In 2007 several lectures (5 in total) were given to bachelors, masters and PhD students. The classes taught resulted in one stage-student that will start an internship in 2008 on secretome/exosome proteomics. In addition, three 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.

Academic teaching	
Course	Class
Dr. CR. Jimenez and Dr. S. Piersma	
Collaborator training- FEB07	3-day proteomics lab course
Collaborator training- JUNE07	3-day proteomics lab course
Collaborator training- OCT07	3-day proteomics lab course
Dr. CR. Jimenez	
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
Masters Oncology, Therapeutics	Proteomics in cancer therapy
Master class Genomics Momentum	Clinical proteomics
Dr. Thang Pham	
Nov07 Bachelors, bioinformatics, FEW-VU	Protein quantification and statistical strategies

Received training

Besides teaching, some OPL members received training courses that are listed in the table below.

Courses/ training of OPL people	
4x2 days	Academic Leadership Course (Jimenez)
Sept	Advanced training LTQ-FTMS (Piersma)
Jan-May	STEP B, a basic statistics course (Jimenez)

SCIENTIFIC OUTPUT 2007

Peer-reviewed Publications

1. **Jiménez, CR.** (2007) Mass spectrometry-based proteomics: trends in tools and strategies. Eur. Pharm. Revs. Dec. p24-25.
2. **Jiménez, CR., S. Piersma, TV. Pham** (2007) High-throughput and targeted in-depth mass spectrometry-based approaches for biofluid profiling and biomarker discovery. Biomarkers in Medicine. 1(4), 541-565.
3. **Jiménez, CR.,** Koel-Simmelink, M., **Pham, TV.,** Van der Voort, L., Teunissen, CE. (2007) Endogeneous peptide profiling of cerebrospinal fluid by MALDI-TOF mass spectrometry: Optimization of magnetic bead-based peptide capture and analysis of preanalytical variables. Prot. Clin. Applic. 1(11), 1385-1392.
4. **Jiménez, CR, El Filali, Z, JC. Knol,** Li KW, Hoekman, K, FAE Kruij, 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.
5. Codrea, MC., **Jiménez, CR.,** Heringa, J., Marchiori, E. (2007). Tools for computational processing of LC-MS datasets: a user's perspective. Computer Methods & Programs in Biomedicine, 86(3), 281-290.
6. Codrea, MC., **Jiménez, CR., Piersma, S.,** Heringa, J., Marchiori, E. (2007). Robust peak detection and alignment of nanoLC-FT mass spectrometry data. Proceedings of the 5th European Conference on Evolutionary Computation, Machine Learning & Data Mining in Bioinformatics, Springer LNCS 4447, p35-46.

Thesis chapters:

1. Time-course MALDI-TOF-MS serum peptide profiling of non-small cell lung cancer patients treated with bortezomib, cisplatin and gemcitabine. Jens Voortman, Thang V. Pham, Jaco C. Knol, Giuseppe Giaccone, Connie R. Jimenez
2. Influence of variations in sample handling on SELDI-TOF MS serum protein profiles for colorectal cancer. 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

Other

Gillooly, D., Breivold, E. & **Jiménez**, CR. (2007) Body Fluid Peptide Profiling Using Magnetic Bead Technology. *Quest*, 4(2), 17-21.

In press manuscripts 2008

1. 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).
2. Li, KW and Jiménez, C.R (2008) Synapse proteomics current status and quantitative applications *Expert Revs* (in press)
3. 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.* (in press)

INDICATORS of ESTEEM

Obtained grants

- 2007 CenE/ VanLanschoot (230K). PhD student for breast cancer proteomics
- 2007 Womens Club of The Hague (40K). Project to finance a research nurse to collect nipple aspirate fluid for proteomics in the context of breast cancer

Invited Lectures Jimenez

- Fall meeting, Danish Proteomics Society. Proteomics targeted to sub-cellular compartments and secretomes for cancer biomarker discovery
- 3rd BioMS meeting, London. Pre-analytical variables in CSF sample handling
- 6th Dutch Endo-Neuro-Psycho Meeting 2007 (ENP2007). Doorwerth. Native peptide profiling of cerebrospinal fluid by MALDI-TOF mass spectrometry
- EU kp6 Angiotargeting consortium meeting, Prague. Glioblastoma membrane proteomics to explore the angiogenic switch in a xenograft model
- HUPO 2008 (Human Proteome Organisation). Seoul. South Korea. Glioblastoma membrane proteomics in a rat xenograft model
- 4th Munster Single Cell Meeting. Munster. From mass spectrometric peptide profiling of single neurons towards imaging mass spectrometry
- Genomics Researchers Event (NGI) Amsterdam. Proteomics: from biological discovery to candidate biomarker
- 7th Fall meeting of the Netherlands Proteomics Platform, Amsterdam. Subnuclear proteomics for cancer biomarker discovery
- Farewell symposium Giaccone, Amsterdam. New Tools: Proteomics

Other academic activities

Participation and function in scientific societies

Function	Organisation
Dr. Connie Jimenez	
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	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	
Member	Dutch Society for Pattern Recognition and Image Processing
Member	Netherlands Proteomics Platform

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- 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)

National

- Dr. Monique Slijper, Dr. J. Krijgsveld, University of Utrecht, Netherlands Proteomics Center
- Prof. Dr. A.M. Deelder, Biomolecular mass spectrometry resource, LUMC, Leiden
- Dr. Rene Houtman, Dr. Rob Ruitenbeek, PamGene, Den Bosch
- Prof. Jos Beijnen, Prof. Hans Bonfrer, Dr. Olaf van Tellingen, Dr. Jos Jonkers, NKI, Amsterdam

Local

- Dr. Elena Marchiori, Dept. Computer Science, FEW, VU, Amsterdam
- Dr. Aad van der Vaart, Dept. Mathematics, FEW, VU, Amsterdam
- Dr. Jaap Herringa, Dept. Bioinformatics, FEW, VU, Amsterdam
- Dr. Ka Wan Li, Prof. dr. Guus Smit, Dept. Molecular & Cellular Neurobiology, VU

APPENDIX: PROJECT SUMMARIES

Automated serum peptide profiling using novel magnetic C18 beads off-line coupled to MALDI-TOF mass spectrometry

Zineb El Filali¹, Jaco C. Knol¹, Klaas Hoekman², Frank A.E. Kruijt², G. Giaccone², August B. Smit³, Ka Wan Li³, Connie R. Jimenez¹

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Aim: To develop a kit-free automated protocol for magnetic-bead-assisted serum peptide profiling using MALDI-TOF mass spectrometry

Serum peptide profiling by mass spectrometry is an emerging approach for disease diagnosis and biomarker discovery. A magnetic bead-based method for off-line serum peptide capture coupled to MALDI-TOF mass spectrometry has been introduced recently. However the reagents are not available to the general scientific community. Here, we developed a protocol for serum peptide capture using novel magnetic DynaBead-RPC18 beads and automated the procedure on a high-throughput magnetic particle processor. We investigated bead equilibration, peptide binding and peptide elution conditions. The method is evaluated in terms of peaks counts and reproducibility of ion intensities in control serum (1hr clotting). Overall, the serum sample processing protocol reported here is reproducible, robust and allows for the detection of ~200 serum peptides at m/z 800-4000. The average intra-experiment %CV of normalized ion intensities for crude serum and 0.5% TFA/0.15% n-octyl glucoside -treated serum, respectively, are 12% (2-38%) and 10% (3-21%) and the inter-experiment %CVs 24% (10-53%) and 31% (10-59%). Importantly, this method can be used for serum peptide profiling by anyone in possession of a MALDI-TOF-MS. In conjunction with the KingFisher®96, the whole serum peptide capture procedure is high-throughput (~20 min per isolation of 96 samples in parallel), thereby facilitating large-scale disease profiling studies.

This research was supported by VUmc Cancer Center Amsterdam and by the Center of Medical Systems Biology

References

- Jiménez, CR, El Filali, Z, JC. Knol, Li KW, Hoekman, K, FAE Kruijt, 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.
- Gillooly, D., Breivold, E. & Jiménez, CR. (2007) Body Fluid Peptide Profiling Using Magnetic Bead Technology. Quest, 4(2), 17-21.

*I. High-throughput body fluid peptide profiling by MALDI mass spectrometry for pattern diagnostics***Endogeneous peptide profiling of cerebrospinal fluid by MALDI-TOF-MS:
Optimization of magnetic bead-based peptide capture and analysis of pre-analytical variables**

Marleen Koel-Simmelinck^{1,2}, Thang V. Pham¹, Jaco Knol¹, Laura van der Voort³, Charlotte E. Teunissen², Connie R. Jimenez¹

¹OncoProteomics Laboratory, Department of Medical Oncology, ²Department of Molecular and Cellular Biology and Immunology, ³Department of Neurology, VU University Medical Center, Amsterdam, The Netherlands.

Aim: To optimize high-throughput peptide profiling using MALDI-TOF mass spectrometry for cerebrospinal fluid (CSF) analysis and to investigate pre-analytical variables in CSF sample handling.

CSF perfuses the brain and spinal cord. CSF contains peptides and proteins important for brain physiology and potentially also relevant to brain pathology. High-throughput endogeneous peptide profiling by mass spectrometry is an emerging approach for disease diagnosis and biomarker discovery. A magnetic bead-based method for off-line serum peptide capture coupled to MALDI-TOF mass spectrometry has been introduced recently.

In this study, we optimized the peptide capture method for profiling of CSF and investigated the effect of a number of pre-analytical variables. The CSF profiles contain ~100 reliably detected peptides at m/z 800-4000 with reproducible ion intensities (average 7% CV). The investigated pre-analytical variables include: time at RT before storage, storage temperature, freeze-thawing cycles, and blood contamination. The CSF peptidome (<20 kD) is relatively stable and can withstand a few hours at RT and several freeze-thaw cycles. Several peptides sensitive to storage at -200C, including Cystatin C, were assigned based on mass or identified by MS/MS. Hemoglobin α and β chains were detected in blood contaminated samples, at levels invisible to the eye (0.01%). These peptides may be used for quality control in a MALDI-TOF-MS screening strategy to select high quality samples for in-depth proteomics analysis in disease studies. Currently, we are applying the method to the analysis of CSF samples of patients with different multiple sclerosis sub-types.

This research was supported by VUmc Cancer Center Amsterdam and by MS Research

Reference

- Jiménez, CR., Koel-Simmelinck, M., Pham, TV., Van der Voort, L., Teunissen, CE. (2007) Endogeneous peptide profiling of cerebrospinal fluid by MALDI-TOF mass spectrometry: Optimization of magnetic bead-based peptide capture and analysis of preanalytical variables. *Prot. Clin. Applic.* 1(11), 1385-1392.

CSF and serum peptidomics to identify differential peptides and signatures for clinical subtypes in Multiple Sclerosis

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¹Dept. of Molecular and Cellular Biology and Immunology. ²OncoProteomics Laboratory, Dept. of Medical Oncology, ³Dept. of Neurology, VU University Medical Center, Amsterdam, The Netherlands.

BACKGROUND Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system. Axonal damage is the cause of disease progression in MS and cerebrospinal fluid (CSF) biomarkers, to detect the earlier stage of axonal damage, are of current interest. For complex diseases such as MS, the old paradigm of a single causal factor and diagnosis based on a single marker is no longer valid. It is becoming increasingly clear that novel discovery-based technologies, such as proteomics, that profile large numbers of peptides and proteins simultaneously hold great potential for finding panels of diagnostic and prognostic biomarkers.

AIM. The aim of the present study is to identify differential peptides and signatures for clinical subtypes in Multiple Sclerosis. The long-term aim is to develop candidate biomarkers for disease progression and axonal loss in MS.

APPROACH We determined the native peptide patterns in body fluids that were processed by magnetic-based assisted peptide capture and MALDI-TOF mass spectrometry. Included in the analysis were samples derived from a large patient cohort study, whereby CSF and serum samples of 83 MS patients with different clinical subtypes (41 relapsing remitting, 29 secondary progressive and 13 primary progressive) and 39 neurological controls (13 with inflammatory neurological diseases and 26 with other neurological diseases) were collected in a standardized manner.

RESULTS. Statistical analysis of the CSF and serum mass spectra is on-going. The peptide ion intensities are being analyzed by univariate statistics and using pattern recognition algorithms. The data will be correlated to clinical scores and MRI markers for atrophy, the surrogate marker for axonal loss in MS. Preliminary data show, a number of statistically significant mass peaks discriminating clinical subtypes of MS or discriminating MS patients from controls.

OUTLOOK. Differential peptides or peptide mass signatures in CSF and serum that can discriminate MS subtypes may aid in patient stratification. Identification of these peptides may allow for alternative assay development.

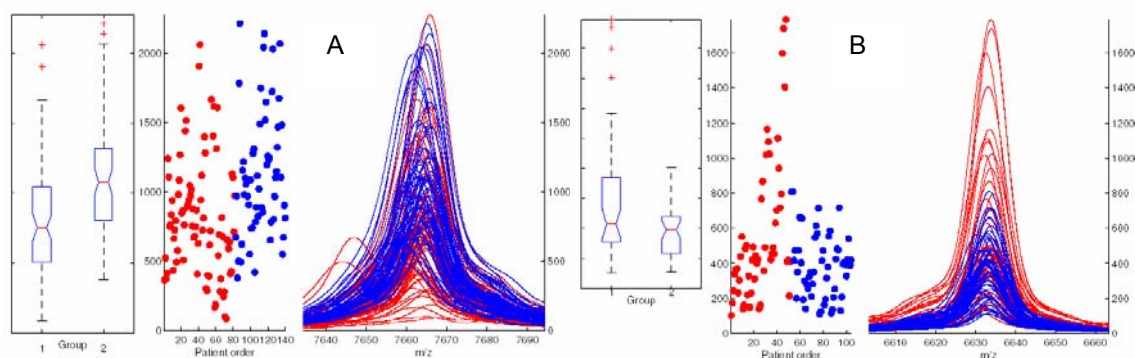


Figure. Examples of discriminating protein mass peaks in CSF (A) and serum (B) of MS patients (red) and neurological controls (blue).

This research was supported by VUmc Cancer Center Amsterdam and by MS Research.

Proteomics of meningiomas in correlation with growth rate

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¹Dept. Neurosurgery, ²OncoProteomics Laboratory, VUmc-Cancer Center Amsterdam, VU University Medical Center,

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 μ l SDS sample buffer. The tissue lysates were subsequently subjected to SDS-PAGE (40 μ g 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, slow 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, slow growth and WHO II samples. Finally, 20 proteins were unique for WHOII samples, including several mitochondrial proteins and enzymes.

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 International Meningioma meeting 2008*

Evaluation of in-depth, multi-dimensional nanoLC-MS/MS-based proteomics for biomarker discovery in CSF

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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.

Goal. 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).

Approach. Here we evaluate two fractionation 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) from different amounts of starting material and robustness/speed of analysis:

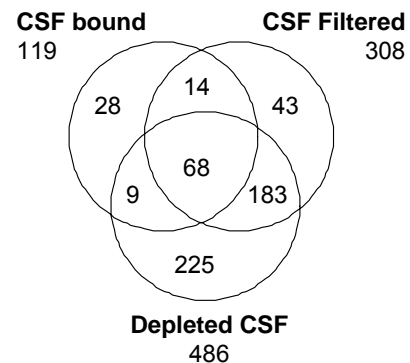
1. Filtration of CSF to fractionate CSF into a peptide fraction (< 10 kD) and a protein fraction (> 10 kD) and
2. Immuno-depletion of the top 14 abundant proteins to create a CSF fraction enriched for more low abundant proteins.

Methods. Proteomics was performed using 100 and 400 μ l CSF as starting amounts. CSF fractionation was performed using Amicon MWCO filters (10 kD, Millipore) or Seppro-IgY Supermix 1 Column (IgY14, Genway). IgY depleted CSF needs to be concentrated further using amicon filters prior to loading on a 1D gel. In GeLC-MS/MS proteins are separated by conventional SDS-PAGE, whole lanes are cut in 10-equal bands and each band is in-gel digested with trypsin. Extracted tryptic peptides are identified by reversed phase nano-liquid chromatography on-line coupled to MS (nanoLC-MS/MS).

Results. In a small-scale pilot depletion experiment using 100 μ l CSF in one cycle of immunodepletion and filtration, 359 different CSF proteins were identified at high confidence (minimum 2 peptides per protein at > 95% CI.). In a second experiment with 400 μ l of CSF as input, we compared sample preparation using CSF filtration alone to CSF immunodepletion followed by filtration. The total dataset contained 570 proteins of which 308 proteins in filtered CSF and 486 proteins in immuno-depleted CSF (see Figure). Immuno-depletion was not perfect: still albumin was detected by 47 peptides as opposed to 177 peptides in the filtered CSF, a reduction of ~74%. Moreover, in the bound fraction that is supposed to contain only 14 proteins, 119 proteins were identified.

Conclusions.

- *Scaling up* CSF sample input for IgY14 depletion by 4x (100 to 400 μ l CSF) has increased the number of identified proteins by 26% at the price of throughput
- In IgY14 immunodepleted CSF, 37% more proteins are identified as compared to filtered CSF



Acknowledgements: This research is supported by the European project: cNeuPRO

Membrane proteomics to reveal invasion and angiogenesis-related tumor-host interactions in a rat xenograft model of glioblastoma

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¹ Norlux Neuro-oncology, CRP-Santé; 84, Val Fleuri, L-1526 Luxembourg, Luxembourg and ⁴ Department of Biomedicine, University of Bergen, Bergen, Norway; ² OncoProteomics Laboratory, VUmc-Cancer Center Amsterdam, VU University Medical Center, Amsterdam, The Netherlands ³Dept. of Molecular and Cellular Neurobiology, Vrije Universiteit Amsterdam, The Netherlands

A xenograft animal model of human glioblastoma multiforme (GBM) was developed by implantation of human GBM spheroids into the brain of nude rats. Four generations of animals were developed by serial transplantation of resulting tumors. The phenotype of the first generation tumors shows infiltrative growth with no apparent sign of angiogenesis. In contrast, tumors in the last generation animals show highly angiogenic and aggressive tumor growth.

The **objective** of our study was to explain the molecular background of the phenotypic change – the angiogenic switch – at the protein level. For this purpose we used high throughput protein profiling technology based on isobaric peptide labeling (iTRAQ method) and quantitative mass spectrometry on MALDI TOF/TOF on membrane enriched fractions of the tumors. Multiplex iTRAQ reagents allowed differential analysis of four tumor samples in a single experiment. With the use of specially designed software we performed isoform specific quantitation and reliably distinguished between rat/host and human/tumor proteins. We identified 850 non redundant proteins in 'lighter' and 918 proteins (CI ≥ 95%) in 'heavier' membrane fractions respectively. Of these, 413 (207 up- and 206 downregulated) and 375 (190 up- and 185 downregulated) isoform and species specific proteins were regulated in last vs. first generations in 'lighter' and 'heavier' membrane fractions. Our preliminary results indicate that several are involved in tumor angiogenesis, invasion and progression. Differential expression of some of these proteins is being validated by alternative methods, including Western blotting, immunohistochemistry and qPCR.

This research was supported by the EU Angiotargeting project

Secretome proteomics of stimulated platelets

Sander R. Piersma¹, Henk J. Broxterman², Muhammed Kapci¹, Richard R. de Haas², Klaas Hoekman²,
Henk M.W. Verheul², Connie R. Jiménez¹

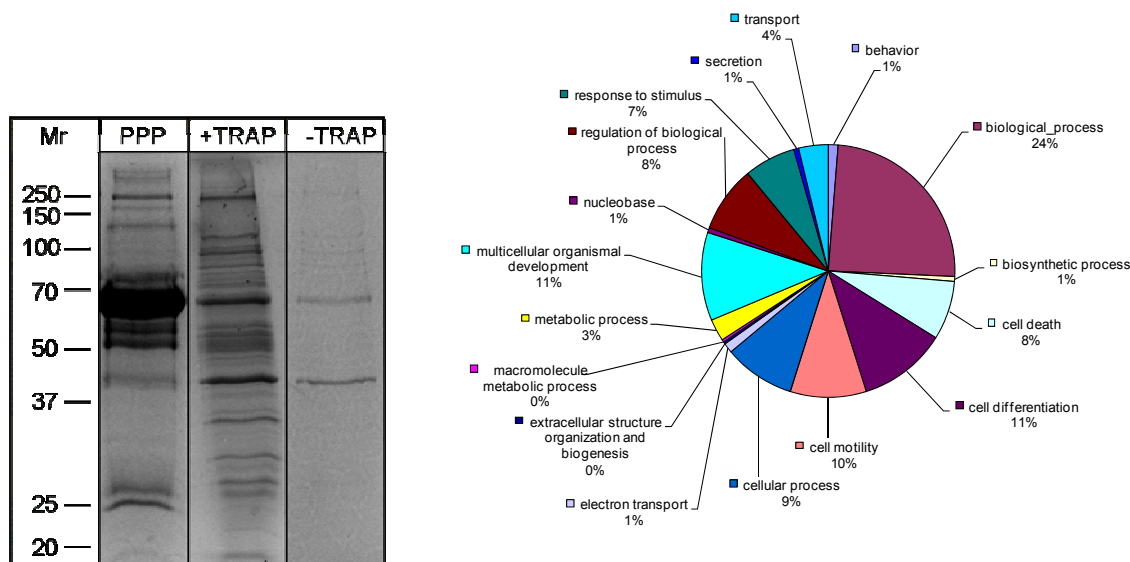
¹OPL, ²Dept. Medical Oncology, VU University Medical Center

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.



This research was supported by the VUmc Cancer Center Amsterdam
Submitted to the Journal of Proteomics

Differential proteomics of multicellular colorectal adenocarcinoma spheroids and primary tissue

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¹NorLux Oncology, Luxembourg, NorLux Oncology Norway and ²OncoProteomics Laboratory, VUmc

Tumor spheroids initiated from primary biopsy specimen provide a valuable three-dimensional cell culture system sharing several biological features with tumors in situ. As solid tumors are three-dimensional structures, multicellular spheroids mimic tumor growth more realistically than monolayer cultures. Cell-cell contact, composition of the extracellular matrix and hypoxic conditions in the spheroid recapitulate some important aspects of the tumor microenvironment. Spheroids are therefore a widely-used experimental model to investigate metabolism, distribution and efficacy of anti-neoplastic drugs. A variety of human malignancies are capable of forming spheroids in culture including primary colorectal adenocarcinoma. The objective of our study was to define the spheroids at the proteome level and compare them to the primary tissue from which they were derived. Differential protein expression of colorectal tumor spheroids vs. primary biopsies from the same patient was assessed by label-free one-dimensional gel electrophoresis linked to nano scale liquid chromatography Fourier Transform MS (geLC FT MS) and spectral counting. More than 700 proteins were identified with 95% confidence interval (CI) for peptide and 99% CI for protein thresholds and ≥ 2 peptides sequenced. Of these, the difference in expression of over 500 proteins was quantifiable by spectral counting and over 100 showed a differential expression of ≥ 2.5 fold. Data analysis is currently on-going to link the differential proteins to biological processes and pathways. A detailed characterization of the spheroids at the molecular level will give insight into the advantages and limitations of this model in the study of tumor biology and in particular in anti-neoplastic drug testing.

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

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

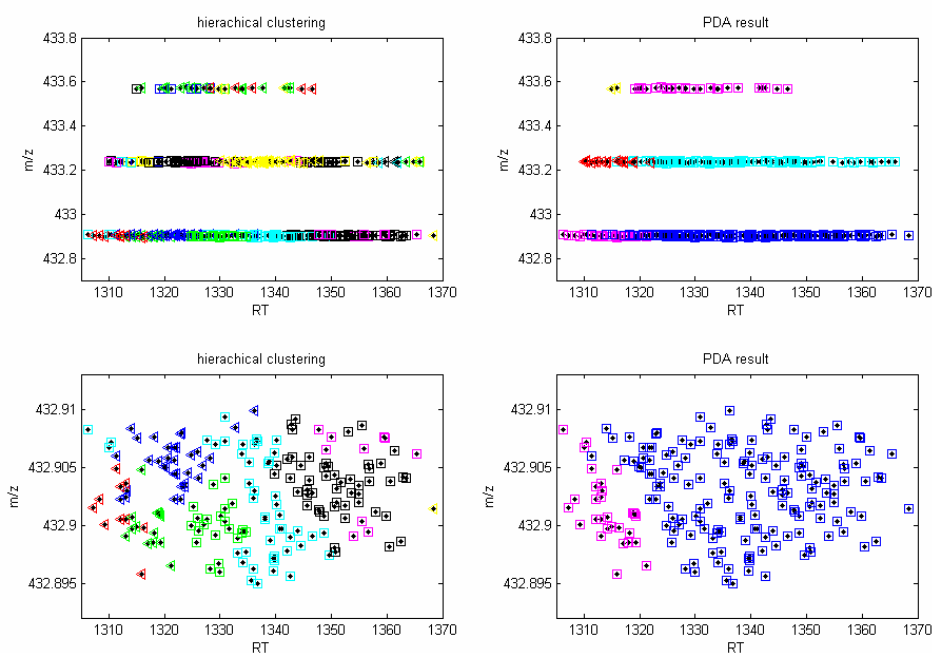
Robust Peak Detection and Alignment of high dimensional nanoLC-FT Mass Spectrometry Data

Marius C. Codrea¹, Connie R. Jimenez², Sander Piersma², Jaap Heringa¹, and Elena Marchiori¹

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In liquid chromatography-mass spectrometry (LC-MS) based expression proteomics, samples from different groups are analyzed comparatively in order to detect differences that can possibly be caused by the disease under study (potential biomarker detection). To this end, advanced computational techniques are needed. Peak alignment and detection are two key steps in the analysis process of LC-MS datasets. In this paper we propose an algorithm for LC-MS peak detection and alignment.

The goal of the algorithm is to group together peaks generated by the same peptide but detected in different samples. It employs clustering with a new weighted similarity measure and automatic selection of the number of clusters. Moreover, it supports parallelization by acting on blocks. Finally, it allows incorporation of available domain knowledge for constraining and refining the search for aligned peaks. Application of the algorithm to a LC-MS dataset generated by a spike-in experiment substantiates the effectiveness of the proposed technique.



References

- Codrea, MC., Jiménez, CR., Heringa, J., Marchiori, E. (2007). Tools for computational processing of LC-MS datasets: a user's perspective. *Computer Methods & Programs in Biomedicine*, 86(3), 281-290.
- Codrea, MC., Jiménez, CR., Piersma, S., Heringa, J., Marchiori, E. (2007). Robust peak detection and alignment of nanoLC-FT mass spectrometry data. *Proceedings of the 5th European Conference on Evolutionary Computation, Machine Learning & Data Mining in Bioinformatics*, Springer LNCS 4447, p35-46.

This research was supported by NWO-Horizon breakthrough grant (Marchiori and Jimenez)

APPENDIX PROJECT SUMMARIES continued:
ABSTRACTS OF MISCELLANEOUS COLLABORATIVE PROJECTS

Exploration of imaging potential of genomics candidates by cell-surface proteomics

M. de Wit¹, R.J.A Fijneman², B. Carvalho¹, S. Piersma³, C.R. Jimenez³ and G.A. Meijer¹

¹Dept. Pathology, VU University Medical Center, ²Dept. Medical Oncology, VU University Medical Center

³OncoProteomics Laboratory, VUmc-Cancer Center Amsterdam.

BACKGROUND. Molecular imaging by MRI requires accessible targets and ligands (antibodies) that recognize (tumor) cell surface molecules. Based on microarray expression analysis, we previously obtained a list of genes whose expression is significantly increased in high-risk colon adenomas and carcinomas compared to low-risk adenomas. From this list, genes encoding proteins predicted extracellular domains have the best potential to function as diagnostic biomarkers. However, gene expression levels are not always correlate with protein expression and prediction of cell surface location is not straightforward. Therefore, the challenge is to select the right ones for validation as development of antibody-based assays is expensive and time-consuming.

AIM. The aim of this study is to identify biomarkers associated with high-risk colon adenomas and carcinomas that can be applied for molecular imaging.

APPROACH To narrow down the list of genomics candidates to a subset with high potential for use in imaging applications, we set out to identify cell surface proteins on colon cancer cell lines. Cells were grown until 70-80% confluency. Upon treatment with sulfo-NHS-SS-Biotin, biotinylated proteins were isolated from the whole cell lysate. Western blotting was applied to confirm that samples were indeed enriched for cell surface proteins. Affinity purified proteins were fractionated using 1D SDS-PAGE and further processed for in-depth proteomics analysis by liquid-chromatography followed by tandem mass spectrometry (LC-MS/MS).

RESULTS. A total of 1046 proteins were identified upon analysis of colo 205 cells (three biological replicates), of which 563 proteins were reproducibly detected in all three samples. The isolated protein fractions were clearly enriched in membrane proteins when compared to the whole lysate after isolation of the biotinylated proteins. Integration of the cell-surface proteomics data with the microarray expression data showed that 98 of the 1046 proteins were found to be overexpressed in carcinomas compared to adenomas ($P < 0.05$).

OUTLOOK. These preliminary data indicate that this strategy resulted in the successful identification of cell surface proteins. Currently we are extending our experiments using to several other CRC cell lines, including RKO, HCT116 and HT29.

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

Fishing for new Fanconi genes by proteomics of protein complexes

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

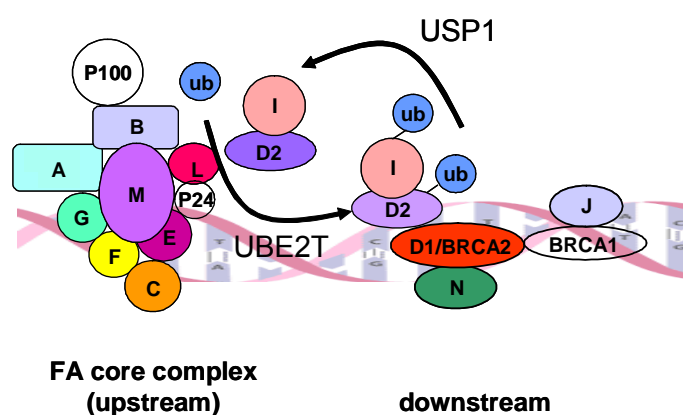


Figure. Current model of the FA/BRCA pathway, showing the 13 known FA genes (in colour) and p24, p100, and BRCA1 (uncoloured). The latter 3 proteins formally do not qualify as genuine FA genes, since so far no FA patients have been identified with mutations in these genes. Proteins p24 and p100 are essential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

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

APPROACH Our collaboration with o.a. Dr. David Livingston (Harvard Medical School, Boston) has demonstrated that protein complex purification is a valuable tool for identifying new players in the FA/BRCA pathway. FANCI protein complexes were isolated from human HeLa cells via co-immunoprecipitation (co-IP) with a purified rabbit polyclonal antibody against FANCI. Co-IPs with an unrelated antibody of the same type served as a negative control. Proteins present in the precipitates were identified by mass-spectrometry. Specificity was demonstrated by the fact that 25 different peptides were detected for FANCI itself, in contrast to 1 FANCI peptide in the negative control.

RESULTS A total of 15 putative interacting factors in the size range of ~120 ~260 kDa were detected. We selected one of the low ranking FANCI-#14 interactors (2 different peptides) for validation. The interaction could be detected by co-precipitation with an independent FANCI antibody. Our approach thus seems to identify specific FANCI-interactors. We then determined, whether the identified proteins could represent novel or unexpected biological processes and/or functions. Possible roles in centrosome function and cytokinesis were new, thus providing interesting leads for follow-up research.

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 other 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 immun-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 Spectrometry (MS). Purified viral proteins and exosomes lysates were subjected to a tryptic digest separated by SDS-Page and analyzed by LC-LTQ-FT MS. Peptides were queried against an extended database containing EBV peptide 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 171 exosomal proteins including class I and II molecules and the exosomal marker proteins HSP70, flotillin-1 and alix which were confirmed by western blotting. We found a near analogous protein profile as published previously using a MALDI-TOF mass spectrometer. Querying against a database containing viral peptide sequences thus far we have not been able to detect viral proteins, while western blotting clearly showed the presence of viral LMP1 in our exosomes prep. Several exosomal proteins such as the tetraspanin CD63 and viral LMP1 were detected on western but were not yet identified by MS analysis. In a previous report it was suggested that exosomal tetraspanins may not be detected by MS due to the poor resolution characteristics by SDS-PAGE or low abundance. We offer an alternate interpretation and anticipate that the tryptic digest may not yield a peptide profile suitable for detection by MS. LMP1 for instance contains a limited amount of trypsinization sites that only yield three peptide sequences that may be identified by MS. In this case, digestion with alternative enzymes may provide a solution. Our studies using purified viral proteins may answer the questions whether EBV proteins can be identified by MS or not.

Conclusions: Our pilot experiments indicate that our exosomal purification method as well as the MS analysis is accurate and reproducible.

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

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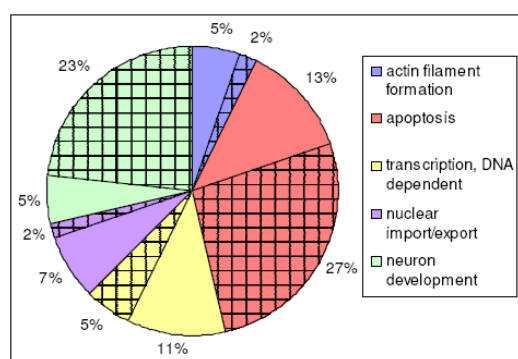
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BACKGROUND. In recent years, the 14-3-3 protein family has emerged as a key regulator of a wide range of cellular processes in human cells. Seven mammalian 14-3-3 genes have been identified (β , η , ϵ , γ , σ , θ and ζ) and their solved crystal structure 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 to 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.

Identification of novel signaling partners of the HCMV encoded chemokine receptor 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 to promote tumor growth in mice [Maussang et al., PNAS, 103(35):13068-73, 2006].

AIM & APPROACH In order to elucidate the oncogenic signalling pathways activated by US28, signalling partners are currently being identified in immuno-precipitated US28-protein complexes using mass spectrometry.

RESULTS In total 69 proteins were identified of which 36 proteins were found to specifically co-precipitate with US28. Several of these co-precipitating proteins are involved in cell-cell adhesion. We are currently investigating these proteins using classical biochemistry and molecular biology experiments.

OUTLOOK Future experiments will include comparisons of complex compositions between different US28 mutants. Furthermore, using membrane fractionation techniques, US28 complex compositions in the different cell compartments will be analyzed by mass spectrometry. These analysis will give more insight into vGPCR signalling and yield new candidate drug targets.

Proteomics van tuberculose

Bij de afdeling Medische Microbiologie en Infectiepreventie (MMI) wordt fundamenteel onderzoek gedaan naar tuberculose. Een groot probleem in het tuberculose onderzoek is dat er nog niet voldoende bekend is over hoe deze bacterie in staat is in de gastheer te overleven en het immuunsysteem van de gastheer te manipuleren. In dit proces spelen gesecreteerde eiwitten waarschijnlijk een belangrijke rol. Daarom zijn we op zoek gegaan naar eiwit secretiesystemen in deze bacterie, om vervolgens te bepalen welke eiwitten via dit secretiesysteem naar buiten gaan. Onlangs hebben we in dit onderzoek een nieuw secretiesysteem ontdekt dat ESX-5 genoemd is (Abdallah et al., 2006). Dit systeem is belangrijk voor de virulentie van *Mycobacterium marinum* (een nauwe verwant van de tuberculose bacterie en de veroorzaker van dissimilatie tuberculose). Met behulp van Proteomics wilden we vervolgens bepalen welke eiwitten wel uitgescheiden worden via ESX-5.

Het supernatant van zowel wild-type *M. marinum* bacterie als de ESX-5 secretie mutant is geïsoleerd en geconcentreerd. Daarna zijn de eiwitten gescheiden met behulp van 2D gel electroforese. Na vergelijking van deze patronen zijn een aantal spots uitgekozen die wel in het supernatant van de wild-type bacteriën maar niet in die van de secretiemutant zijn aangetroffen. Deze eiwitten zijn geanalyseerd met behulp van MALDI-TOF/TOF massa spectrometrie (4800 MALDI-TOF/TOF; Applied Biosystems). Hieruit is naar voren gekomen dat ESX-5 verantwoordelijk is voor de secretie van 2 belangrijke groepen eiwitten, de PE_PGRS en de PPE eiwitten. Additionele experimenten met antilichamen gericht tegen de PE_PGRS eiwitten heeft laten zien dat alle gesecreteerde PE_PGRS eiwitten afwezig zijn bij de mutant.

ESX-5 is een belangrijk secretie systeem is voor deze bacterie dat verantwoordelijk is voor de secretie van PE_PGRS en PPE eiwitten; bij elkaar worden er mogelijk meer dan 100 eiwitten via ESX-5 gesecreteerd. Het mycobacterium onderzoek bij de afdeling MMI heeft door deze data een grote voortgang kunnen maken en er zijn verschillende nieuwe medewerkers op dit project aangenomen. De volgende stappen zullen ook gebruik maken van MS analyse; we willen namelijk nu hetzelfde doen voor de verwekker van tuberculose, *i.e. Mycobacterium tuberculosis*, en ook verschillende condities bestuderen, bijvoorbeeld secretie in gastheer cellen, en analyseren of we dan veranderingen in het secretiepatroon kunnen waarnemen.

Betrokken personen:

Wilbert Bitter	UHD, hoofdonderzoeker
Theo Verboom	Analist, contactpersoon MS analyse
Abdallah Abdallah	AIO (VUmc) op dit project, onlangs gepromoveerd
Maria Daleke	OIO (ALW), onlangs begonnen op dit project
Edith Houben	Post-doc (VENI), onlangs begonnen op dit project

Publicaties:

- AM Abdallah, T Verboom, F Hannes, M Safi, M Strong, D Eisenberg, RJP Musters, CMJE Vandenbroucke-Grauls, BJ Appelmelk, J Luirink, and W Bitter (2006) A specific secretion system mediates PPE41 transport in pathogenic Mycobacteria. *Mol. Microbiol.* 62: 667-679.
- AM Abdallah, T Verboom, NC Gey van Pittius, PW Mahasha, C Jiménez, M Parra, N Cadieux, MJ Brennan, BJ Appelmelk and W Bitter (2008) Mycobacterial PPE and PE_PGRS proteins are transported via a type VII secretion system, submitted

