

Scientific Program

**2nd Combined WG/MC Meeting of the COST Action
BM0702**

EuroKUP (Kidney and Urine Proteomics)



March 29-30, 2009

Amalia Hotel
Nafplio, Greece

Local Hosts: Antonia Vlahou Ph.D
Aris Charonis, M.D. Ph.D

Sunday March 29, 2009

- 11.00 – 11.45 **Introduction**
Opening lecture: Jorgen Frokiaer
- 11.50-13.30 **WG1 Session (Chairs: Spasovski, Egido)**
- 11.50-12.00 **Gordana Peruničić-Peković**
Early detection of chronic kidney disease in groups with risk factors
- 12.00-12.10 **Jesus Egido**
Vascular proteomics at the crossroad of chronic kidney disease
- 12.10-12.20 **A Ortiz**
Identification of new mediators of diabetic podocyte injury: CD74 and TRAIL
- 12.20-12.30 **Fabiola Terzi**
The EGF-R pathway: new potential biomarkers of chronic kidney disease progression
- 12.30-12.40 **Patrick D' Haese**
Chronic Kidney Disease (CKD) in Moroc. Early detection and intervention project - CKD screening program and 5-year follow-up of the at-risk population (MAREMAR)"
- 12.40-13.30 **Discussion on collaborative projects**
- 12.40-12.50 **Goce Spasovski**
Kidney proteomic analysis and progression of CKD
Report on the proposal of a collaborative project in CKD stage II-III patients with proteinuria, biopsied at baseline and after 2 years of follow up (Goce Spasovski, Meguid El Nahas, Ariela Begnini, Giovambattista Capasso)
- 12.50-13.00 **Ariela Begnini**
Kidney proteomic analysis in patients with diabetic nephropathy
Report on the proposal of a collaborative project in patients with diabetic nephropathy under treatment with various nephroprotective agents (Ariela Begnini, Giovambattista Capasso, Aristidis Charonis, Harry Holthoffer)
- 13.00 -13.10 **Joost Schanstra, Jorgen Frokiaer**
Kidney proteomic analysis in urological malformations and reflux nephropathy
Report on the proposal of a collaborative project in patients with urological

malformations and reflux nephropathy (Joost Schanstra, Jorgen Frokiaer, Gian-Marco Ghiggeri and Adrian Woolf)

13.10 -13.20 **Marta Sánchez-Carbayo**
Kidney proteomic analysis in urologic cancers
Report on the proposal of a collaborative project in patients with urological cancers.

13.20-13.30 **Harald Mischak**
Discussion related to the support of grant applications out of EuroKUP

Closing remarks for the session

13.30-14.30 **Light Lunch- Posters**

14.30 **WG2 session: Round Table Discussion on LCM (Chairs: Schanstra, Allmaier)**

14.30.14.50 **Isabelle Fournier**
MALDI-MSI: From Developments to Clinical Applications

14.50-15.10 **Theo Luider**

15.10-15.30 **Ferdinand von Eggeling**
Microdissected tissue – an underestimated source for proteomic biomarker discovery?

15.30-16.00 **Discussion**

16.00-16.30 Coffee Break

16.30-18.00 **WG3 session (Chairs: Mischak, Dihazi)**

16.30-16.40 **Antonia Vlahou**
Update on the development of the “standard urine sample”

16.40.17.30 **Definition of valid proteomic biomarkers: solutions to a currently unmet challenge**

Based on a simple classification problem, to distinguish male from female based on urinary proteomics, we want to highlight the essential steps in the definition of valid biomarkers, and in the establishment of multiparametric models.

16.40-16.50 **Harald Mischak**
Classical statistics: the requirement for adjustment for multiple testing

16.50-17.00 **Keith Harris**
Multivariate classification methods I

17.00-17.10 **Alexandros Kalousis**
Multivariate classification methods II

17.10-17.20 **Franck Molina**
BioComputing approaches to enhance biomarkers discovery in Diabetic nephropathy

17.20-17.30 **Harald Mischak**
Lessons to be learned from an inappropriate study or: how urinary proteomics must not be performed.
Highlighting the biggest and most severe mistakes that result in faulty reports, showcasing the recent paper by Quintana et al. in JASN as an example.

17.30-18.00 **Discussion**

18.00-19.30 **WG4 Session (Chairs: Attwood, Bongcam-Rudloff)**

18.00-18.30 **Terri Attwood and Erik Bongcam-Rudloff**
Status update for WG4

18.30-19.00 **Steve Pettifer**
Ontology Basics

19.00-19.30 **Ontology Discussion Session**

20.30 Dinner

Monday March 30

9.00-11.00 Scientific Presentations (Chairs: Charonis, Edelman)

9.00-9.10 Hassan Dihazi
The impact of protein depletion on the improvement of 2-DE analysis in case of diabetic nephropathy.

9.10-9.20 Alessia Farinazzo
Combinatorial peptide ligand libraries (CPLL) for low-abundance proteome analysis: investigation of different elution systems

9.20-9.30 Håvard Loftheim
Possible pitfalls in urinary proteomics: evaluating strengths and weaknesses in the sample pretreatment

9.30-9.40 Anja Verhulst

Investigating the effect of statins on the urinary proteome

9.40-9.50 Aleksander Edelman

Urinary proteome analysis for identifying biomarkers in cystinuria

9.50-10.00 Annalisa Vilasi

Urinary proteomics in Gitelman's syndrome

10.00- 10.10 Pavel Rehulka

Purification and separation of proteomic samples for MALDI-TOF/TOF MS analysis using simple gradient device and short methacrylate based capillary columns

10.10-10.20 Nathalie Selevsek

Screening for new bladder cancer biomarkers using selected reaction monitoring

10.20-10.40 Mark A. McDowall

A High Definition Proteomics Strategy...for Global Discovery, Hypothesis-Driven and Intact Protein Research

10.40-11.00 Coffee Break

11.00- 11.30 **Take-home messages from WG**

11.30- 13.30 **MC meeting**

EARLY DETECTION OF CHRONIC KIDNEY DISEASE IN GROUPS WITH RISK FACTORS

Gordana Peruničić-Peković¹, Zorica Rašić-Milutinović¹, Ljiljana Jandrić² Nadežda Zec¹ i Ljubica Djukanović³

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There is high prevalence of chronic kidney disease all over the world and it is one more a problem for all doctors and particularly for general practitioner. Those patients who have hypertension and diabetes mellitus frequently suffer from end stage renal disease. Today prevention of chronic kidney disease is very important and the main aim in their medical treatment. Slow and progressive damage of the kidney, over the years can last without symptoms and during that time we must look for parameters of kidney disease.

Aim of the study is to examine prevalence of kidney disease in patients with hypertension and those who are older than 60 years. The study which is followed in New Belgrade Dispensary there was two groups: the first group with hypertension and second group, with patients older than 60 years. We had anamnesis data, systolic and diastolic pressure and laboratory parameters. We examined 162 patients in both groups: 70% was older of 60 years and 93,8% had a hypertension more than 10 years and they had a therapy of ACE inhibitors. 36% of patients had pathological urine results and 26% had microalbuminuria. 44 patients had chronic renal insufficiency with decrease glomerular filtration rate (GFR).

Intensive treatment od comorbidity, risk factors and early diagnostic parameters of renal insufficiency on primary care, as well as including nephrologist in the treatment team can help in decrease of end stage renal disease patients and save the money for the health fund.

Key words: risk factors, hypertension, CKD, screening

VASCULAR PROTEOMICS AT THE CROSSROAD OF CHRONIC KIDNEY DISEASE

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Cardiovascular disease is the main cause of death in patients with chronic kidney disease. Therefore, it is of paramount importance an early identification of subjects at risk for CVD. We have searched novel sources of markers by analysing both the proteins released by human atherosclerotic plaques in culture and the metabolites present in human atherosclerotic plaques.

In the first approach, by focusing only on the secreted proteins found in the tissue culture media, there is an intended bias toward those molecules that would have a higher probability of later being found in the plasma. Among the differentially secreted proteins, decreased heat shock protein-27 (HSP27) was identified by 2DE and MS and tumor necrosis factor-like weak inducer of apoptosis (TWEAK) was identified by SELDI-TOF. In those studies, HSP27 and TWEAK release was drastically decreased in atherosclerotic plaques compared to healthy arteries and in plasma of patients with carotid atherosclerosis relative to healthy subjects. Furthermore, in a test population of 106 asymptomatic subjects, we showed that sTWEAK concentrations negatively correlated with the carotid intima-media thickness. These studies suggests that sTWEAK could be novel biomarkers of atherosclerosis.

In the second approach, TOF-Imaging has been used to visualize and quantify metabolites directly from vascular tissues. Interestingly, we found a significant increase of non-esterified fatty acids (NEFA) on plaques from diabetic patients, which correlated with a higher extent of inflammation (higher NF- κ B activation, MCP-1 expression and macrophage infiltration) in comparison with plaques from non-diabetic patients.

On the whole, proteomics, in combination with other complementary approaches like genomics, metabolomics and imaging techniques, is emerging as a very powerful tool that is expected to improve the diagnosis and treatment of patients at high risk of suffering a cardiovascular event, including patients with CKD.

Identification of new mediators of diabetic podocyte injury: CD74 and TRAIL

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Fundacion Jimenez Diaz, U. Autonoma de Madrid, Spain, U. of Helsinki, Finland. Dublin City University, Ireland. Universidad Austral, Valdivia, Chile. U. of Bristol, Bristol, U.K. St. Vincent's Hospital, University of Melbourne, VIC, Australia. U. of Michigan, Ann Arbor, MI. Childrens Hospital Los Angeles, Los Angeles, CA. National Institute of Diabetes and Digestive and Kidney Diseases, Phoenix, AZ. Fondazione D'Amico per la Ricerca sulle Malattie Renali, Milan, Italy.

Diabetic nephropathy is the main cause of end-stage renal disease requiring renal replacement therapy. A better understanding of secondary mediators of injury may lead to the design of new therapeutic strategies.

Expression profiling of patients with diabetic nephropathy identified CD74 as an overexpressed glomerular molecule. CD74 expression was determined by immunohistochemistry in human renal biopsies. Renal CD74 expression was also explored in a rat model of diabetes induced by streptozotocin. The regulation of CD74 and the consequences of CD74 ligation by migration inhibitory factor (MIF) were studied in cultured human podocytes and human proximal tubular HK2 cells.

Renal glomerular and tubulointerstitial CD74 mRNA expression was increased in Pima Indians with type 2 diabetes and diabetic nephropathy. Immunohistochemistry confirmed the increased glomerular and tubular expression of CD74 in clinical and experimental diabetic nephropathy and localized glomerular CD74 to podocytes. Cultured human podocytes expressed CD74 at the cell surface, and the expression was upregulated by high glucose concentrations and TNF α . High glucose also induced CD74 expression in HK2 cells. MIF induced ERK1/2 and p38 phosphorylation in cultured podocytes via the activation of CD74 and it induced the expression of TRAIL and MCP-1 in podocytes and HK2 cells in a p38-dependent manner. In diabetic glomeruli TRAIL was upregulated in podocytes. TRAIL expression was not upregulated by glucose in cultured podocytes. TRAIL induced podocyte apoptosis.

In summary kidney transcriptomics identified two novel mediators of podocyte injury in diabetic nephropathy: CD74 acts as a receptor for MIF in podocytes and promotes the expression of TRAIL, which, in turn, induced podocyte apoptosis.

The EGF-R pathway: new potential biomarkers of chronic kidney disease progression

Fabiola Terzi, Inserm U845, Team: Mechanisms and therapeutic strategies of chronic nephropathies, Hôpital Necker, Paris – France

By performing an experimental model of nephron reduction in different mouse strains, we have observed that the susceptibility to develop renal lesions is genetically determined in mice. In particular, whereas the C57Bl/6 strain undergoes compensatory growth alone, the FVB/N additionally develop severe and progressive renal lesions accompanied by hypertension and proteinuria. This model system provides a powerful tool for identifying biomarkers that distinguish beneficial processes from those that indicate progressive CKD.

By combining this model and genetically modified mice with genomic and molecular approaches, we have discovered that activation of epidermal growth factor-receptor (EGF-R) plays a critical role in the progression of CKD. In addition, we have discovered that the EGF-R transactivation mediates the deleterious effect of Angiotensin II, a potent mediator of renal deterioration. EGFR is activated by a family of growth factors, comprising of composed of EGF, Transforming growth factor alpha (TGF- α), heparin-binding EGF-like growth factor (HB-EGF), betacellulin, amphiregulin, epiregulin and epigen. Our ongoing project seems to suggest that these ligands may have different effect in renal diseases and suggest that TGF- α might have a major role. Interestingly, in 75% nephrectomized mice, TGF- α urinary excretion paralleled that of TGF- α expression in damaged kidney and seems to correlate with disease progression.

In order to evaluate whether this molecule might be a potential biomarker of CKD progression in humans, we have conducted a pilot retrospective study aiming at evaluating the correlation between urinary TGF- α excretion and the rate of progression to renal failure in patients with CKD. To date, 198 patients with Autosomal Dominant Polycystic Kidney Disease (ADPKD) and 97 with non-cystic CKD have been investigated. Urinary TGF- α has been evaluated by Western Blot analysis and renal function by MDRD. Preliminary results show that TGF- α is excreted in urine, but exclusively in patients with moderate/severe renal failure (MDRD < 60 ml/min/1.73 m²). More importantly, on a retrospective analysis, TGF- α excretion seems to correlate with faster progression. In addition, on few biopsies, we observed that TGF- α renal expression is increased in patients with CKD as compared to controls.

Altogether, the results suggest that TGF- α may be a potential candidate biomarker for CKD progression. A perspective study is undergoing to confirm these findings.

Chronic Kidney Disease (CKD) in Maroc. Early detection and intervention project - CKD screening program and 5-year follow-up of the at-risk population (MAREMAR)

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The main objectives of the MaReMar (Maladie Rénale Chronique au Maroc) study program are to (i) estimate the prevalence of CKD in the population of Maroc and (ii) identify subjects at risk to develop CKD and (iii) establish an intervention program for a follow-up period of 5 years

Hereto a baseline screening will be performed, using a limited number of clinical investigations and a short structured questionnaire. Based on the results of the baseline screening, subjects will be categorized in: Group I - CKD patients (receiving diagnostic work-up and appropriate treatment), Group II - patients with pathological findings at risk to develop CKD (receiving annual follow-up and treatment), Group III subjects with health related habits associated with an increased risk to develop CKD (receiving bi-annual follow-up) and group IV - subjects without signs or risk for renal failure. In group II, patients with the following profiles will be included: [II-1] subjects with isolated microalbuminuria, [II-2] hypertensive subjects with normoalbuminuria, [II-3] hypertensive subjects with microalbuminuria (to be randomized) and [II-4] pre-diabetes or diabetes with normoalbuminuria.

A total of 10.000 subjects will be screened and it is expected that out of these 200-800 CKD patients (2-8% of screened population) with signs of renal impairment; 2000-3000 patients (20-30% of screened population) at risk to develop CKD and 1000-2000 subjects (10-20% of screened population) with two or more health related habits increasing the risk will be recruited.

In view of the interesting set up of this study we would be very much interested to in addition also perform proteome analyses as the various populations under study offer an excellent opportunity to establish the urinary proteome of patients at risk for CKD and identify early markers. EuroKup offers a unique platform to participate herein and to further discuss issues of e.g. patient selection and establish procedures for sample storage, shipment and sample analysis as well as data processing.

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**Potential use of laser capture microdissection in
proteomic analyses and maldi-imaging in kidney disease:
*lessons from other organs and tissues***

EuroKUP WG2 - Round table - Nafplion, Greece –
Sunday March 29, 2009 - 14:30-16:00.

Although urine has shown to be an excellent tool for the discovery of biomarkers in kidney diseases, it will not allow to clearly define the pathological processes *in situ*. This is especially important for the kidney as it is made up of several functionally different structures composed of highly specialised cell types. Laser capture microdissection (LCM) followed by proteome analysis of the microdissected structures and MALDI imaging are potential tools to study these different kidney structures. EuroKUP partners, in response to the WG2 questionnaire (October 2008), have shown significant interest in LCM/proteomics and MALDI imaging of their samples (retrospectively or prospectively obtained). Many kidney biopsy samples of the EuroKUP partners are available as paraffin embedded. Published LCM/proteomics studies on kidney biopsies are absent, but LCM/proteomics and MALDI imaging has been successfully performed on other organs or tissues. Three experts in the field, Drs I Fournier, T Luider and F von Eggeling will during this round table each present their experience with LCM/proteomics and MALDI-imaging on other tissues, give their opinions on sample handling, and advice of how to store tissues for downstream LCM/proteomics and MALDI-imaging analysis. The main objective of this round table is, after the final discussion, to come up with clear guidelines how kidney samples for these types of analyses should be collected. A second objective of this round table is to motivate the EuroKUP community to participate in collaborative projects on LCM/proteomics and MALDI-imaging in kidney disease that holds a great promise in an organ as heterogeneous as the kidney.

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Elucidating changes at the proteome level for better understanding of cell signalisation pathways modifications in abnormal cells is a complex task that requires the development and use of dedicated and new tools. In this respect, MALDI Imaging has shown growing interest for proteomics applications in biology and clinical fields by allowing pathological biomarkers to be discovered and their distribution to be followed directly from tumour tissues. On tissue proteomics is a highly interesting approach for biomarkers hunting and has shown to avoid several of the problems classically encountered with fluids proteomic. However, even if the continuously growing number of publications of MALDI imaging applications to pathologies, methodology strategies still require to be improved.

A first part of developments concern MALDI imaging of Formalin-Fixed and Paraffin-Embedded (FFPE) Tissues¹. In fact, the biggest part of hospital tissue banks consists of FFPE blocks. Such samples are highly advantageous for pathologists since they do present a great stability in time. Although, such samples are a priori not well adapted for MALDI experiments. We have studied new methodological strategies for retrieving information and imaging old FFPE samples. *In situ* controlled enzymatic digestion of tissues has proved to be the easiest solution to image and get structural information on peptides and proteins from FFPE samples.

In situ chemical derivatization of peptides obtained after enzymatic digestions were also studied. Derivatizations have shown to be very helpful for peptides/proteins identification by either allowing *de novo* sequencing or leading to high increase in identification score after databanks interrogation.

On the other hand, we have also developed a new concept for both using MALDI imaging as a validation tool in clinical researches as well as broadening the range of analysable molecules by MALDI imaging to mRNA, higher mass proteins or sugars. This so-called specific imaging² is a targeted methodology for specifically tracking a probe of interest. We have developed new types of reporters adapted to MS detection that can be combined with different types of probes such as antibodies, oligonucleotides or lectin probes by constructing reporters including photoceavable moieties. By combining our specifically tagged probes with different hybridization techniques such as ISH or IHC, we can now image antigens, mRNA and glycoprotein's by imaging peptide reporter released during MALDI experiments by photocleavage under the laser irradiation. This makes MALDI Imaging becoming an interesting tool for biomarkers validation with possible correlation at the transcriptome/proteome level.

These developments were used in several applications including ovarian cancer³ and animal models for Parkinson⁴ for biomarkers hunting and validation. This proves that MALDI imaging has now found its way to be considered as an efficient tool for pathological proteomics.

Keywords

Imaging of FFPE tissues, *In situ* structural elucidation, Imaging of mRNA, Imaging antigens, Imaging of saccharides

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Microdissected tissue – an underestimated source for proteomic biomarker discovery?

Ferdinand von Eggeling, Günther Ernst, Christian Melle

Core Unit Chip Application (CUCA), Institute of Human Genetics and Anthropology, University Hospital Jena, Germany

Biomarkers, especially diagnostic useful ones, are needed for different diseases. This is in particular true for tumor marker. In nearly all neoplasias the early detection is of high importance for adequate therapies and therefore for the survival of the patients. Until now most markers are proteins, but also DNA was tested for its applicability as a marker. But most markers lack from sensitivity and specificity and/or that they are not able to detect tumours in early stages. Therefore all efforts have to be directed to find highly specific early biomarkers, which have also to be inexpensive that they can be applied in a screening or routine diagnostic.

In this talk we want to advance two hypotheses, which will be discussed from technical different point of views: (1) “biomarkers could be found more easily, where they arise” and (2) “If tissue is analysed it has to free of contaminant tissue”.

Pro and contra will be shown on own results gained with tissue microdissection and SEDLI technology. A short outlook will be given on other techniques allowing a spatial proteomic approach.

Update on the development of a “standard” urine specimen

A. Vlahou

The aim of this initiative is the establishment of a well characterized standard urine sample, that will be available to all laboratories working in the field of urinary proteomics to assess platform capability (assessment of pre-analytical steps, platform performance, normalisation).and also enable comparison of datasets. As a start in this direction, the group in Mosaiques Diagnostics collected and pooled urine from 7 male and 8 female apparently healthy volunteers (aged 20-50). All samples were midstream urine, collected between 8 and 10 am and frozen immediately at 1, 10 and 50ml aliquots. These two standards (male and female pools) were analysed in 9 different laboratories using several different proteomic platforms: 2DE-MS, CE-MS, and LC-MS, aiming at the generation of an initial comprehensive database of the urinary proteome as evidenced by the combinatorial application of these technologies. In addition, conventional analytical values from immunological assays and clinical laboratory testing were obtained. In all cases, participating laboratories applied their established protocols for sample preparation, analysis, and data processing. Data quality was assessed based on intralab and in some cases (CE-MS) inter-lab reproducibility of the data as well as their similarity to published urinary data for each technology. The results of this analysis support that while the samples have not been analyzed “to completion”, *i.e.*, not all of their compounds are currently known and quantified, they are the by far most extensively and comprehensively investigated urine samples reported on to date; more importantly, they are available to all urine proteomics researchers.

Definition of valid proteomic biomarkers: solutions to a currently unmet challenge

Sebastien Carpentier, Mohammed Dakna, Theodoros Damoulas, Keith Harris, Mark Girolami, Alexandros Kalousis, Walter Kolch, and Harald Mischak

The field of biomarker discovery or clinical proteomics is suffering from high hopes generated by reports on potential biomarkers, which in most cases subsequently could not be substantiated via validation. This development has resulted in large scepticism from both, clinicians and regulatory agencies, which will make the application of valid biomarkers even more of a challenge.

This circulus vitiosus has to be broken by pinpointing the major errors and highlighting key aspects which will enable definition of valid biomarkers with a much higher probability than currently observed. While some of the initial issues have already been dealt with, others are still unresolved.

It is now generally accepted that single biomarkers will not result in a major advancement: the complexity of disease is unlikely to be thoroughly displayed by a single marker; instead, a panel of such biomarkers must be employed. However, it is equally evident that such a panel must consist of clearly defined biomarkers, and not of an ill-defined signature that subsequently cannot be validated. This brings the issue of definition of a valid biomarker into focus. The main questions are:

- 1) Is the change observed in disease (frequency or abundance) of a certain molecule, based on data from a proteomics study, in fact a result of disease, or does it merely reflect an artefact due to technical variability in the pre-analytical steps, or in analysis. Additional good suspects for suggesting a (subsequently found erroneous) apparent association with disease are biological variability, or bias introduced in the

study (e.g. due to lifestyle, age, and gender). In fact, these two problems are likely responsible for the majority of erroneous biomarkers.

- 2) Which algorithms can be employed to combine biomarkers to a multi-marker model and how can the validity of a multi-marker model be assessed?

In an attempt to answer these questions, we have employed urinary proteome data from apparently healthy male and female volunteers (228 independent datasets total), aiming at determination of gender based on proteomics. Out of these datasets, we have examined different size training sets (up to 67 male and female samples each), and subsequently evaluated the results in an independent testset of 94 samples.

The most appropriate answer to the first question appears to be the application of stringent statistical analysis. In this report we demonstrate the importance of strict and correct use of statistics, especially adjustment for multiple testing. We demonstrate the deleterious results of inadequate statistics, and hope to eliminate several of the myths associated with this area.

Our result also show that different algorithms to combine multiparametric datasets perform similar (and very well) in establishing multi-marker models. However, it is equally evident that these only lead to meaningful results if the number of datasets employed is sufficient to allow definition of statistically valid biomarkers.

We hope that, as a result of this report, mandatory requirements for reporting a proteomic biomarker can be installed and will be adhered to, not only by scientists, but also by the leading journals and funding agencies.

BioComputing approaches to enhance biomarkers discovery in Diabetic nephropathy

Franck Molina

SysDiag CNRS/Bio-rad UMR3145, Complex system modelling and engineering for diagnosis

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Diabetic Nephropathy (DN) is a frequent complication of both types of diabetes. About 30% of patients with diabetes will be struck by DN. Diabetic nephropathy is thus the main cause of end stage renal failure in Western countries. From the clinical point of view, the disease is diagnosed when albumin begins to be excreted in urine (microalbuminuria state). However, at this stage the disease is probably already installed, although silent. We hypothesize that proteins that would precede albumin in the urine could be valuable early markers of the disease. The project is aimed at the identification of early DN biomarkers by using proteomic approaches. We plan to use comparative 2D gel electrophoresis of urine. This biomarker discovery process involves various steps where the original biocomputing approaches we proposed can contribute to guarantee relevant proteomic signal analyses, quality assessment and meaningful interpretation. In addition, we developed an original Bioinformatics approach based on network analyses to raise new hypothesis for proteomics biomarkers discovery.

On the validity of biomarkers and clinical proteomics

H Mischak

An array of insignificant manuscripts has resulted in a lack of credibility of (clinical) proteomics, especially in the eyes of clinicians and regulatory agencies. As these are the two groups that will finally decide on the fate of a biomarker, this is not a good development.

Unfortunately, the literature is still flooded with papers reporting on invalid biomarkers, and the lack of credibility is consequently further substantiated.

As an example I would like to take a critical look at data that were published recently by Quintana et al. in JASN, where the authors claim that they identified 14 protein ions that enabled discrimination of interstitial fibrosis and tubular atrophy from active rejection (in transplant patients) with 100 % accuracy.

Upon examination of the data we find:

- 1) The quality of the experimental data is pitiful. The resolution of the MS instrument seems to be in the range of 20 (based on supplementary table 1), about 1000-fold inferior to a state-of-the art MALDI-TOF mass spectrometer. This mass resolution will not enable to draw any meaningful conclusion, or allow identification of the claimed biomarkers.
- 2) The experimental data from most of the healthy controls do in fact represent background, most of these spectra do not contain any peptide-related signals, therefore controls can be distinguished from cases.
- 3) after evaluation of these data we must conclude that the conclusions drawn in the manuscript are not at all supported by the data. Neither the potential biomarkers, not the reported p- or q-values are correct.
- 4) the statistics applied is not appropriate.

Any attempt to reproduce these data (using the described biomarkers) must fail, so we can conclude that this report in the leading nephrological Journal once more proves clinical proteomics to be worthless.

This report, and in fact almost all of the invalid biomarkers can be eliminated by

- 1) using adequate controls, instrumentation and statistics and
- 2) validate the findings on an independent test set of appropriate size to prove significance.

The past experience has clearly shown that reports that are not in line with these simple requirements have an excellent chance of being irrelevant. Any report on "potential biomarkers that await further validation" can generally be seen as insignificant, the only substantial information from such a report is that the authors have access to an MS instrument. This should by no means suffice for publication.

To improve on the current state, we need clear rules on how to define and validate biomarkers. Only valid biomarkers should be reported on, and only research in this area that aims towards identification of valid biomarkers should be funded. If certain measures of validity would also in general be acceptable for the regulatory agencies, then this would also advance the field of biomarkers in pharmacological testing.

I suggest mandatory requirements for platform validation (repeatability, precision, limit of detection, resolution, mass accuracy), statistical evaluation (adjustment for multiple testing), and validation on a blinded set that is large enough to show statistical significance (e.g. p-value < 0,05 in ROC analysis).

While I assume it is rather naive to hope that these goals can be completely reached during one meeting, I also think it is necessary to start the process, and hope that it will lead towards useful results.

WG4 Session

Topics to be covered:

1) Status update for WG4 (review of the Website, update on results from the questionnaire, update on MolMeth, etc.) **Terri Attwood and E. Bongcam-Rudloff.**

2) **S.Pettifer** - the aim of this talk will be to cover such themes as, What is an 'ontology' (including different types of ontology; taxonomies, vocabularies, upper and lower ontologies; the different levels of sophistication and complexity of ontologies; and the various ways of representing these (SKOS, OWL, etc.)). It will discuss why ontologies are useful (e.g., for annotating biological concepts, for translation between concepts, for 'integration' and 'consensus', for formal reasoning, and [the particular speciality of the UTOPIA group] for database/tool integration and for driving user interface behaviour). Examples of problems that arise from the lack of ontologies will also be discussed (e.g., Uniprot name confusion, and when is a molecule not a molecule?). The talk will end by looking at where ontologies come from (i.e., whether community built or automated), and will discuss the issues that arise when creating ontologies.

3) **Ontology Discussion Session** - the aim of this discussion session is to identify the stakeholders in building the EuroKUP ontology, what are the community databases (internal and external) to be embraced, what are its tools, and so on. The discussion will pinpoint what the EuroKUP ontology is required for in this context (e.g., as a means of exchange/integration, as a means of describing the field in general, etc.) - as such, it will aim both to identify the scope of the proposed ontology, and to relate it to existing ontologies. Ultimately, the aim of the session will be to lay the foundations for how we plan, collectively, to build the EuroKUP ontology.

Combinatorial peptide ligand libraries (CPLL) for low-abundance proteome analysis: investigation of different elution systems

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Proteome treatments with CPLLs, in view of reducing high-abundance proteins and increasing the concentration of rare species, involve the adsorption on solid phase material. Subsequent elution of captured proteins may not be fully effective except when sequences of eluting agents are used. The standard way utilized up to the present has been a three- to four-step, sequential elution system consisting of various agents mixed together such as urea, thiourea, CHAPS, sodium chloride, citric or acetic acid and some polar solvents such as acetonitrile and isopropanol. These elution sequences produce several, distinct fractions (especially when using more than one library) adding to the burden of having to analyze all of them. We propose here three alternatives, highly effective, for implementing a single elution step, so as to reduce the workload on analysis of multiple eluted fractions. The first one consists in boiling the CPLL beads, after proteome capture, in 10% SDS added with 3% DTE (dithioerithrytol). The second one deals with elution in a mixture of TUC (7 M urea, 2 M thiourea, 3% CHAPS) and 40 mM formic acid. The third one consists in eluting in a mixture of TUC (7 M urea, 2 M thiourea, 3% CHAPS) and 30 mM cysteic acid. The SDS-treatment essentially releases quantitatively all the adsorbed material onto the CPLL beads, but, for further 2D map analyses, requires removal of excess SDS. The second and third ones ensure at least 95% recovery of proteomes from the CPLL beads. Both these last elution systems are fully compatible with subsequent 2D map analyses, and thus do not require prior removal of formic or cysteic acids. However, formic acid might modify amino acid residues on the eluted proteins, notably Ser and Thr residues via formylation (ester formation) and (although less likely at acidic pH values) via N-formylation of free amino groups (amido bond formation). In addition, formic acid tends to accumulate in the IPG strip around pH 3, whereas cysteic acid, in virtue of its very low pI value (1.90), is fully eliminated from the first dimension IPG strip. In the case of formic and cysteic acids elutions, it was demonstrated, via nanoLC-MS/MS, that the very few proteins left over by the treatment (and finally eluted in boiling SDS) do not represent new protein species not eluted by these eluants, but a left-over from the previous elutions, since they are all found in the TUC/acid eluates. E.g., in the case of salt-soluble proteins extracted from maize, only 23, high abundance proteins, are detected in the final SDS eluate, vs. >600 in the first TUC + cysteic acid eluate.

The impact of protein depletion on the improvement of 2-DE analysis in case of diabetic nephropathy.

Martina Gansz, Michael Koziolok, Gerhard A. Müller, Hassan Dihazi

Identification of kidney disease biomarkers has significantly increased the interest for study of the urine proteome. Unfortunately, kidney disease biomarkers for an earlier diagnostic often appear at low concentrations in urine. Their systematic detection is complicated by an extremely wide range of protein abundances. Depletion of high abundance proteins allows for visualization of proteins comigrating with, and masked by the high abundance proteins using methods such as 2-DE. Moreover, depletion allows for individual proteins in low abundance to be loaded at higher levels.

To improved visualization/detection of lower abundance proteins and identification of diabetic nephropathy related biomarkers in urine, we combined the depletion of high abundant proteins with two dimensional gel electrophoresis (2DE) and mass spectrometry. Samples from patients with type 2 diabetes (DM) without nephropathy and without microalbuminuria ($n = 7$), DM patients with microalbuminuria ($n = 5$), DM with macroalbuminuria ($n = 5$), patients with macroalbuminuria due to non-diabetic renal disease ($n = 5$), and patients with microalbuminuria due to non-diabetic renal disease ($n = 5$) were analysed. Using the Multiple Affinity Removal Removal LC Colum – Human 6 we removed chromatographically six interfering high-abundant proteins (Albumin, IgG, IgA, transferrin, haptoglobin and antitrypsin) from urine samples. Subsequently the proteins were chloroform/methanol precipitated and analysed with 2-DE and mass spectrometry. Removing six of the most abundant proteins from urine leads to the unmasking of more low copy number proteins and enables loading and detection of more proteins of interest. Moreover we could identify a high number of proteins, which were not visible in the non depleted urine. Comparative analyses of the different patients groups allowed the identification of proteins discriminating DM with microalbuminurea from the other groups. Our study highlights the advantage of reduction of urine proteome complexity for high protein coverage and identification of potential diseases markers.

URINARY PROTEOME ANALYSIS FOR IDENTIFYING BIOMARKERS IN CYSTINURIA

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Urinary protein measurement is a long-standing practice for the diagnosis and follow-up of renal disease. The recent standardisation of proteomic methods allows us to investigate urinary protein excretion in order to identify potential biomarkers of kidney diseases. Our preliminary study focuses in cystinuria, an hereditary kidney stone disease which frequency is 1-2% of nephrolithiasis in adults and 5-10% of those in children. Cystinuria is caused by a defect in reabsorption of cystine, a dibasic aminoacid, by the proximal renal tubule. Cystine is poorly soluble in urine especially at normal or acidic pH, and the increased levels of cystine in the renal tubule are at the origin of its crystallization. Cystine kidney stones are usually multiple, bilateral and very recurrent. However, the events which lead to chronic renal failure are not completely understood.

The goal of our study is to compare urinary protein patterns using a profiling proteomic approach, between cystinuric patients with no renal insufficiency (creatinine clearance estimated by the MDRD formula ≥ 60 ml/min/1,73 m²) and healthy individuals matched in age and sex with the patients. Cystinuric patients will be classified in two groups of 10 individuals, moderate and severe disease, as determined on the basis of clinical criteria including the number of stones and the occurrence of stone recurrence. Exclusion criteria are urinary infection, nephrotoxic treatment and tobacco smoking. The first morning urine samples will be collected as described in the standardized protocol (<http://intramural.niddk.nih.gov/research/UroProt/collection-storage.shtml>). Classical biochemistry analysis and cristalluria will be performed. Profiling analysis will be done for both soluble urinary proteins and exosomes using the ClinProt® (Bruker Daltonics) technology with weak cationic exchange magnetic beads (MB-WCX) and the ClinProTools® for the statistical analysis of the profiles.

Possible pitfalls in urinary proteomics: evaluating strengths and weaknesses in the sample pretreatment

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In the field of kidney transplantation, acute rejection is one of the major risk factors for developing chronic allograft nephropathy which again is an important cause of late graft loss. Suspicion of acute rejection episodes following renal transplantation is currently based on serum creatinine changes. This is not an ideal marker for acute rejection due to its lack of specificity and the fact that it consequently needs to be confirmed by a renal allograft biopsy. The objective of the present project is to identify a urine protein biomarker using differential proteomics allowing earlier detection as well as a non-invasive procedure for acute allograft rejection diagnosis

In this work, optimization of every step in a bottom-up urinary proteomics approach was studied with respect to maximize the protein recovery and making the downstream steps in the workflow fully compatible without compromising robustness. Sample enrichment and desalting using centrifugal filtration (5 kDa cut-off) yielded protein recoveries up to 97 % when 8 M urea was used. The TrisHCl/NaCl buffer was however considered a better choice due to better compatibility with the next step in the sample preparation even though the recovery was lower (88 %). The next step was depletion of human serum albumin (HSA), using an immuno-affinity column, which was successfully adapted for use in urine and lead to a 40 % reduction of total protein in a urine sample from a patient with proteinuria. Separation of the trypsin generated peptides in an off-line two-dimensional chromatographic system consisting of a Hydrophilic Interaction Chromatography (HILIC) column followed by a reversed phase chromatography (RP) column showed a high peak capacity and good repeatability of the chromatography. All operations were modified in order to keep sample handling between every step to a minimum, reducing the variability of each process. In order to test the suitability of the full method in an extensive proteomic experiment, a urine sample from a kidney transplanted patient was analyzed in parallel. The total variability of the method was satisfactory which is important to know in biomarker search. Eventually, we identified a total of 1668 peptides and 438 proteins from a single urine sample despite the use of low-resolution MS/MS equipment. The optimized and “streamlined” complex method was shown to have a great applicability and potential for future urinary proteomic studies.

Investigating the effect of statins on the urinary proteome

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The proximal tubular cells of the kidney are responsible for reabsorption of proteins from the tubular lumen. In studies using opossum kidney (OK) cells and primary human tubular kidney cells, receptor-mediated protein endocytosis was reduced by statins, inhibitors of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, which are widely used in the treatment of hypercholesterolemia. Three statins (simvastatin, pravastatin, and rosuvastatin) significantly inhibited the uptake of protein in a concentration-dependent way. This inhibitory effect of statins could be prevented by the co-addition of mevalonate, the product of HMG-CoA reductase. A reduced prenylation of some proteins critically involved in endocytosis has been put forward as the underlying mechanism.

These observations led to the suggestion that the occurrence of proteinuria in a minority of patients treated with high statin doses is the result of a reduced tubular reabsorption/endocytosis of normally filtered proteins. To further explore the clinical relevance of such a mechanism, the composition of the urinary proteome under statin treatment was investigated in normal healthy volunteers by two-dimensional gel electrophoresis based proteomics analysis.

Midstream morning urine (pooled samples of 3 days) was collected of 6 healthy volunteers. Three of them started receiving rosuvastatin during 5 days followed by a 2 week wash out and subsequent 5 days treatment with pravastatin. The other three volunteers first received pravastatin followed by wash out and rosuvastatin. The statin treatment period was preceded and followed by a 3 and 5 days off-statin treatment period respectively. In total 36 (pre-treatment, during treatment and post-treatment samples of two statin treatment periods of 6 volunteers) pooled urine samples of 3 consecutive days were collected. Samples were analysed by two independent laboratories. Different methods for sample preparation were optimized in both laboratories in order to obtain complementary data. Method 1 includes filtering (over 5 μm filter), binding to a pre-wetted SPE column, elution with 70% acetonitrile/0.1% TFA, freezing, freeze drying and resuspending. Method 2 includes centrifugation, dialysis (molecular weight cut of 3500 Da) and clean up using 2D-DIGE clean up kit. After sample preparation, 2D-DIGE analysis was performed. Gel Analysis using DeCyder software is ongoing. For each statin differential statistical analysis will be performed comparing the pre-, during- and post-treatment urinary proteome. Furthermore the “during rosuvastatin and pravastatin treatment” urinary proteomes will also be compared to each other. Finally inter- and intra-volunteer variability will be studied to allow accurate interpretation of possible differences.

URINARY PROTEOMICS IN GITELMAN'S SYNDROME

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Introduction and aims: Gitelman's syndrome is an autosomal recessive renal tubular disorder characterized by hypokalemic metabolic alkalosis, hypomagnesemia, hypocalciuria and polyurea. This disorder is caused by mutations in the solute carrier family 12, SLC12A3, which encodes the thiazide-sensitive NaCl cotransporter (NCC). NCC is expressed at the apical membrane of the cells lining the distal convoluted tubule (DCT) and loss of NCC function leads to decreased Na⁺ and Cl⁻ reabsorption in the DCT. Decreased Na⁺ reabsorption in the DCT leads to increased solute delivery to the collecting tubule and to an alteration of urinary concentrating and diluting mechanism, mediated from specific plasma membrane proteins: receptors, transporters and ion channel. In order to increase our understanding into the disruptive molecular mechanism under cell dysfunction leading to Gitelman's syndrome phenotype, three different and complementary proteomic methodologies have been applied. These approaches were targeted to urinary low molecular weight proteins, freely filtered from the glomerulus (soluble fraction) and to membrane associated proteins excreted in urine in low density membrane fractions, called exosomes.

Methods In a first set of experiment we compared the urinary protein soluble fraction of Gitelman's syndrome patients versus normal subjects, by two different analytical proteomic strategies: a) 2-dimensional gel electrophoresis (2DE) followed by MALDI-TOF mass spectrometry analysis; b) 1-dimensional gel electrophoresis (1D-SDS-PAGE) followed by tandem mass spectrometry (nano-ESI-LC-MS/MS). In a second set of experiment, the extracted exosomal urine fraction from both Gitelman's syndrome patients and normal subjects was analyzed by c) 1D-SDS-PAGE followed by nano-ESI-LC-MS/MS; d) shotgun proteomics: in solution digested protein mixture followed by nano-ESI-LC-MS/MS.

Results and Conclusions The proteomic analyses on the soluble fraction showed a significative up regulation, in Gitelman's syndrome affected patients, of several of the S100 family of calcium binding proteins involved in intracellular and extracellular regulatory activities: S100A7; S100A8; S100A9; S100A11. On the contrary, the analyses on the urinary exosomal fractions showed a sensible reduction, in Gitelman's syndrome patients, in any of the peripheral membrane protein belonging to the Rab-GTPase family: RAB1A; RAB1B; RAB3A; RAB3D; RAB 3D; RAB7A and RAB10. These small GTPase proteins regulates many mechanism of proteins membrane trafficking, including vesicles formation, vesicles movement and membrane fusion. In addition, a consistent reduction of the water channel proteins AQP2, and an altered expression of other different transporters, has been detected. Taken together, our findings showed tangible differences in the urinary polypeptide pattern characterizing Gitelman's syndrome patients, that could be cause specific and may explain the phenotype of this disorder, characterized by a defective tubular reabsorption of several transporter proteins and ion channels.

Purification and separation of proteomic samples for MALDI-TOF/TOF MS analysis using simple gradient device and short methacrylate based capillary columns

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Preparation of samples in proteomics is an important step especially in the case when MS analysis follows. An application of a suitable purification step is necessary before MS acquisition. This is often done during LC separation procedure that is coupled either on-line (ESI) or off-line (MALDI) to mass spectrometer. The presented work uses a simple gradient forming device in combination with short monolithic capillary column for purification and separation of protein digests that were then analyzed in off-line mode using MALDI-TOF/TOF mass spectrometry. The quality of obtained separation was good and most of peptide signals were present only in one or two MALDI fractions. The comparison of results both for model (in-solution digest of bovine serum albumin) and real (in-gel digests of water soluble barley grain proteins) protein digests show a clear improvement in the overall obtained information about the analyzed sample.

Acknowledgement

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Screening for new bladder cancer biomarkers using selected reaction monitoring

Nathalie Selevsek, Mariette Matondo, Ruedi Aebersold, Marta Sánchez-Carbayo & Bruno Domon

Reliable biomarkers detectable in urine have imminent relevance in the follow-up for recurrence of bladder cancer (BCa). A hypothesis-driven strategy combining quantitative mass spectrometry together with reduction of sample complexity was used in this study. It is based on the targeted analysis using selected reaction monitoring (SRM) of *N*-linked glycopeptides isolated from urine allowing the detection and quantification of low abundant proteins, which are buried in the strong biochemical background. The strategy was applied to evaluate biomarkers for bladder cancer in human urine.

Proteins (isolated from human urine) were submitted to trypsin digestion and subsequent capture of the glycosylated peptides using periodate oxidation and solid phase capture on hydrazide beads were performed. Urine samples were collected from patients with T1G3 non invasive BCa (n=10) and control patients without BCa (n=10), and were screened for the presence of selected proteins using *N*-glycosite peptide surrogates by SRM.

In order to validate the SRM workflow, a list of 50 *N*-linked glycoproteins were selected from literature data mining, which were already associated with bladder cancer. The corresponding proteotypic peptides (i.e. sequences that are unique to a single protein) and their fragment ions were derived from MS/MS spectra stored in public data repositories such as PeptideAtlas in order to build the SRM assays. Six to eight SRM transitions (i.e. precursor ion/ fragment ion pairs) for each peptide were used to identify the selected peptides/proteins in human urine.

The SRM analysis allowed the measurement of more than 90 urinary *N*-glycosite peptides (i.e 600-800 SRM transitions) in one single LC-MS/MS run. Several of the selected peptides were detected successfully in the *N*-glycosite urine samples and represent the basis for systematic quantitative measurements in large series of human urine samples.

A High Definition Proteomics Strategy

...for Global Discovery, Hypothesis-Driven and Intact Protein Research

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Systems Biology and Proteomics

Genomics, Transcriptomics, Proteomics, Interactomics and Metabo(n/l)omics provide an interconnected series of windows through which to observe and potentially comprehend biological systems on a molecular level. Proteomics, in addition to simply identifying and quantifying proteins, strives to characterize protein structure and function, protein-protein/nucleic acid/lipid complexes, enzyme-substrate interactions, post-translational modifications, protein processing, protein folding, protein activation, cellular and sub-cellular localization, protein turnover/synthesis and protein isoforms caused by differential splicing. The ability to capture and compare all of this information between two (or more) cellular states is desirable to fully understand cellular responses to genetic or environmental stimuli. However, the numerous practical and analytical challenges involved in the realization of this grand vision of “systems biology” are enormous!

This presentation summarizes recent advances in liquid chromatography, mass spectrometry, ion mobility separation and informatics designed to address some of the analytical challenges inherent in realizing the grand vision for proteomics - within a systems biology context.

High Definition Global Discovery Proteomics

In global discovery proteomics high protein sequence coverage is critical for confident protein identification. High sequence coverage is frustrated by “under sampling of the proteome” with conventional data dependent LC/MS/MS. We propose a new data independent UPLC/MS^E methodology enabling all (peptide related) ions to be monitored in parallel with high selectivity. This results in the acquisition of a “Global Expression Dataset” that provides a comprehensive record of all peptides that are detectable by electrospray ionisation. These data sets can be mined exhaustively for both qualitative and quantitative information (e.g. identify up-regulated and down-regulated proteins). In addition our UPLC/MS^E approach enables all proteins identified to be quantified (relative and absolute) without derivatization or chemical modification.

High Definition Targeted / Hypothesis-Driven Proteomics

Increasingly systems biology (proteomics) research is hypothesis-driven - where a mechanistic model is tested with hypotheses involving perturbation of the system of interest with subsequent collection of quantitative data (for targeted proteins) that describe the model under investigation.

We have developed a novel informatics strategy to enable rapid method development for targeted proteomics experiments that expertly selects proteotypic peptides for quantitative protein assays with optimum Multiple Reaction Monitoring (MRM) parameters. Our approach uniquely develops targeted UPLC/MRM methods by interrogating archived UPLC/MS^E (discovery) data in contrast to commonly used *in silico* projections of proteotypic peptide suitability.

Intact Protein Analysis

Analysis of intact proteins and protein complexes is essential to understand their interactions and functions. Electrospray TOF MS is an established method for the characterization of large biomolecules. Ion Mobility Separation (IMS) in combination with high resolution MS provides higher definition in such studies. In addition IMS can provide rapid measurement of a protein's collision cross-section (*i.e.* size) that can expedite computational modeling of that protein's structure. We will summarize recent developments of a novel high efficiency IMS/MSⁿ system optimized for intact protein characterization and “Top-Down” proteomics research.

Streptozotocin (STZ) model of diabetes. Use of rat urine as source for early biomarkers

Luca Musante, Alessandra Ravidà', Ilkka Miinalainen, Louise Sewell Marjut Kreivi, Michaela Bowden, Pirkko Muhonen and Harry Holthöfer

Background

The constant increase of diabetes unavoidably leads to end-organ complications with unsatisfactory treatment options and associated high costs. Predictive diagnostics and biomarkers are urgently needed to identify most vulnerable patient cohorts and to allow earlier start of targeted therapies. Proteome research of the urine offers a promising source for novel markers because it is easily accessible in large quantities without the use of invasive procedures.

Normal urinary proteins include soluble proteins and protein components of “solid phase”: exosome, lysosomal secretory vesicles plus other uncharacterized components called generally “cellular debris”. The improvement of proteomic technologies has revealed an enormous complexity of normal urine proteome. It contains more than 1500 different gene protein products and even higher if protein fragmentation into respective peptides of larger proteins is taken into account.

Disruption of the glomerular barrier together with tubular injury results in increase of plasma proteins in the urine thus overwhelming a diagnostic “rare” proteome (proteins and/or peptide represented in very low concentration). High abundance of serum proteins in the urine easily masks and prevents reliable differential analysis unless rigorous preanalytical fractionation protocols are used.

The stz- induced diabetes of the rat reduces the amount of confounding genetic, environmental, exercise and dietary background factors to a minimum. This information, then, can be extrapolated into analytics of human kidney disease patients for the final goal to achieve mechanistic understanding and useful markers of disease activity.

Results

Our protocol partitions the STZ rat urines into fractions for protein profiling and

simultaneously enriches proteins of very low concentration. The critical parameters are to

1. Avoid denaturing conditions of electrophoresis methods and *in vitro* assays to reveal the ***urinary proteases profile***.
2. Analyse the ***urinary exosome proteins*** and different subpopulations of exosomes during the progression of DN.
3. Analyse simultaneously the urinary glycoproteome for specific glycosignatures of diagnostic value.

The research protocol and markers yielded will be reported and discussed.

Proteome Analysis to Study Failure of the Glomerular Filtration Barrier

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Podocyte damage and loss is central to the development of chronic kidney disease (CKD). Damage of podocytes leads to foot process effacement and alterations of the slit diaphragm with subsequent proteinuria. In various glomerular diseases, damaged podocytes eventually detach from the glomerular basement membrane and can be found in the urine. Thus, proteome analysis of podocytes and urine may yield important insight into podocyte failure and may provide relevant biomarkers of CKD.

To identify the molecular changes in podocytes that are primarily caused by high glucose concentrations in diabetes, we investigated the protein expression profiles in a podocyte cell line under long-term high glucose exposure (30 mM vs. 10 mM for 2 weeks). Proteins were separated by 2-DE, and we identified several differentially expressed proteins that were up- or downregulated more than 2-fold by high glucose concentrations using MALDI-TOF MS and Mascot software. These proteins belong to several protein classes, including cytoskeletal proteins and specific annexins (annexin III and VI). Downregulation of annexin III and VI by high glucose concentrations was confirmed by qRT-PCR, Western blot, and immunostaining, and was also observed in glomeruli of kidney biopsies from patients with diabetic nephropathy.

In the future, these studies of podocyte cell lines will be complemented by analyses of the urine proteome of participants of the Study of Health in Pomerania (SHIP). SHIP comprises more than 4,300 participants, which were extensively examined for clinical and subclinical phenotypes. Recently, the genome wide SNP patterns have been determined in all available samples using the Affymetrix SNP 6.0 array, in addition to several already determined laboratory analytes, including renal parameters. Available blood and urine samples will now be used for a comprehensive gel-based and gel-free proteome analysis of selected subgroups of the SHIP cohort to identify proteins displaying CKD associated changes in intensity. These candidates will then be validated in a second phase in larger groups of SHIP using technologies such as multiple reaction monitoring (MRM). The final aim of this study will be the generation of complementary genomics, metabolomics and proteomics datasets, which will be subjected to an integrated analysis to reveal associations with CKD.

Our data demonstrate that molecular alterations in glomerular disease can be identified by proteome analysis of cultured podocytes. Urine proteome analyses in the population based SHIP cohort will further assist in the elucidation of the molecular mechanisms involved in failure of the glomerular filtration barrier.