



**Report on the Second Combined Working Group and Management Committee Meeting of EuroKUP (Urine and Kidney Proteomics Cost Action)**

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Keywords:	Kidney Diseases, Urine, Imaging Mass Spectrometry, Ontology, Clinical Proteomics



**REPORT****Report on the Second Combined Working Group and Management Committee Meeting of EuroKUP (Urine and Kidney Proteomics Cost Action)**

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For Peer Review

**Abbreviations**

EuroKUP: Urine and Kidney Proteomics COST Action

CKD: Chronic Kidney Disease

COST: European Cooperation in the field of Scientific and Technical Research

KUP Ontology: Kidney and Urine Proteomics Ontology

LCM: Laser Capture Microdissection

MC: Management Committee

WG: Working Group

For Peer Review

**Abstract**

EuroKUP (Urine and Kidney Proteomics; [www.eurokup.org](http://www.eurokup.org)) is a COST Action (European COoperation in the field of Scientific and Technical Research: [www.cost.esf.org](http://www.cost.esf.org)) fostering a multi-disciplinary network of investigators from 25 countries and focusing on facilitating translational proteomic research in kidney diseases. Four Working Groups (WG) focusing respectively on defining clinically important research questions in kidney diseases, kidney tissue proteomics, urine proteomics and bioinformatics have been generated. The EuroKUP members had their second combined WG and Management Committee (MC) meeting in Nafplio, Greece March 29-30 2009. This report summarizes the main presentations, discussions and agreed action points during this meeting. These refer to the design of collaborative projects and clinical center networks for specific kidney diseases; establishment of guidelines for kidney tissue proteomics analysis by laser-based imaging- and LCM-mass spectrometry; development and characterization of a “standard” urine specimen to be used for assessment of platform capability and data comparability in clinical proteomics applications; definition of statistical requirements in biomarker discovery studies; and development of a specialized kidney and urine ontology. Various training activities are planned involving training schools on LCM- and Imaging –MS, workshop on ontologies as well as short term travel grants for junior investigators.

**Keywords:** Kidney Diseases, Urine, Imaging mass spectrometry, Ontology, Clinical Proteomics

EuroKUP (Urine and Kidney Proteomics; [www.eurokup.org](http://www.eurokup.org)) is a COST Action (European COoperation in the field of Scientific and Technical Research: [www.cost.esf.org](http://www.cost.esf.org)) focusing on facilitating translational proteomic research in kidney diseases by bringing together and promoting interactions between basic scientists from different disciplines and clinicians working in the broader areas of kidney and urine proteomics. Currently the Action fosters a network of investigators from 21 European and 4 non-European countries [1].

The EuroKUP members held their second combined Working Group (WG) and Management Committee (MC) meeting in Nafplio, Greece from March 29 -30 2009.

The scientific program of the meeting included WG-specific sessions covering respective thematic areas: WG1 session included discussions on collaborative projects and establishment of clinical networks to address specific questions in kidney diseases; WG2 session addressed technical issues on tissue collection and processing for Laser Capture Microdissection (LCM)- and imaging mass spectrometry (MS); WG3 included discussions on the development of a standard urine specimen to be used as reference by urine proteomics investigators. In addition, it involved presentations and discussions on statistical requirements for biomarker discovery studies; and WG4 addressed issues related to the generation of a kidney specialized ontology. The detailed program, abstracts, and many of the presentations are posted on the web page of the Action ([www.eurokup.org](http://www.eurokup.org)).

The meeting opening lecture was given by Jorgen Frokiaer (Denmark) who provided an overview of the physiology and pathophysiology of renal aquaporins, as an

example of how a targeted proteomics approach can provide a better understanding of renal regulation of body water balance and with special focus on the pathophysiological role of aquaporins in clinical common conditions such as obstructive nephropathies.

This lecture was followed by the WG1 session on clinical relevance, chaired by Goce Spasovski (Former Yugoslav Republic of Macedonia) and Jesus Egido (Spain). Gordana Perunic-Pekovic (Serbia) presented data on the prevalence of chronic kidney disease (CKD) in patients with hypertension and aged > 60-years. Of 162 patients included in the study, 36% had pathological urine results and 26% had microalbuminuria. Forty four patients had chronic renal insufficiency with decrease glomerular filtration rate (GFR). These data, that are in –line with recent reports from other studies [2], clearly indicate a need for early diagnostic and prognostic parameters of renal insufficiency on primary care, to help decrease end stage renal disease, consequently save substantially in health care costs. Of note: the costs for dialysis are currently estimated to be in the range of 50.000 € per patient and year [3]. Jesus Egido (Spain) presented data on early biomarkers for cardiovascular disease by the analysis of human atherosclerotic plaques in culture. Focusing on the secreted proteins found in the tissue culture media, 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, sTWEAK concentrations negatively correlated with the carotid intima-media thickness. These studies suggest that sTWEAK may be novel biomarker of atherosclerosis.

In parallel, TOF-Imaging has been used to visualize and quantify metabolites directly from vascular tissues. An increase of non-esterified fatty acids (NEFA) on plaques from diabetic patients could be detected, that correlated with a higher extent of inflammation (higher NF- $\kappa$ B activation, MCP-1 expression and macrophage infiltration) in comparison with plaques from non-diabetic patients.

Alberto Ortiz (Spain) presented data supporting the involvement of CD74 and TRAIL in podocyte injury and diabetic nephropathy, with glomerular and tubular expression of CD74 found to be upregulated in clinical and experimental diabetic nephropathy. MIF, the CD74 ligand, induced expression of TRAIL and MCP-1 in podocytes and HK2 cells via CD74 in a p38-dependent manner. In diabetic glomeruli TRAIL was found upregulated and induced apoptosis in podocytes.

Fabiola Terzi (France) investigated urinary TGF- $\alpha$  in 198 patients with Autosomal Dominant Polycystic Kidney Disease (ADPKD) and 97 with non-cystic CKD by Western Blot analysis. Preliminary results show that TGF- $\alpha$  is excreted in urine exclusively of patients with moderate/severe renal failure. Further, TGF- $\alpha$  excretion seems to correlate with disease progression. Altogether, the results suggest that TGF- $\alpha$  may be a potential candidate biomarker for CKD progression. A perspective study is undergoing to confirm these findings.

The session included the presentation of several on-going and/or contemplated collaborative projects:

Patrick D'Haese (Belgium) gave an overview of the MAREMAR project targeting in the population of Morocco a) the estimation of prevalence of CKD, b) the identification of high risk populations for CKD and 3) the establishment of an intervention program for a 5 year follow-up period.

Goce Spasovski (MK) presented a proposal for a collaborative project addressing the development of biomarkers for CKD progression and elaborated on patient inclusion criteria for the study. Along the same lines, Ariela Benigni (Italy) discussed clinical study design for the analysis of diabetic nephropathy. As also outlined by other speakers, the extreme importance for non-invasive prognostic biomarkers in CKD has become evident. As a consequence, the forum agreed to initiate a prospective multi-center study aiming at validation of already known urinary biomarkers for diagnosis and prognosis for chronic renal disease, Goce Spasovski being the coordinator of the study. In parallel, financial support for this study will be sought for from the European Commission, the ERA/EDTA, and other funding bodies.

Joost Schanstra (France) and Jorgen Frokiaer (Denmark) presented a project addressing the development of urine and amniotic fluid biomarkers predicting the need for therapeutic intervention in urological malformations and reflux nephropathy. The WG1 session concluded with a presentation by Marta Sanchez-Carbayo (Spain) who provided an overview of different –omics approaches employed in her laboratory for the analysis of urological cancers.

The WG2 session on kidney proteomics was chaired by Guenter Allmaier (Austria) and Joost Schanstra (France) and involved extensive discussions on laser-based imaging- mass spectrometry and LCM combined with subsequent sophisticated mass spectrometric analysis. Main speakers for the session were Drs Isabelle Fournier (France) and Theo Luidier (The Netherlands). Dr Fournier, presented technical and methodological developments regarding MALDI-Imaging MS of formalin fixed and paraffin embedded tissues. In addition she presented targeted methodologies for tracking in tissues selected probes of interest. Dr Luidier presented various technical

developments in proteomics analysis of cell populations selected by LCM in combination to different types of mass spectrometric analyzers. Examples for the employment of the technique in the analysis of proteins as well as metabolites and lipids were provided. The session concluded with a general discussion on the pros and cons of the current status of imaging and LCM- MS for kidney tissues.

The WG3 session on urinary proteomics was chaired by Harald Mischak (Germany) and Hassan Dihazi (Germany). Antonia Vlahou (Greece) presented the development of two “standard” urine samples, corresponding respectively to normal male and female pools. 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. To this end, midstream urine from 7 male and 8 female apparently healthy volunteers (aged 20-50) was collected, combined, and frozen in 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 a 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 intra-lab and, in some cases, inter-lab reproducibility as well as similarity to published urinary data for each technology. These samples are available to all interested urine

proteomics researchers and thereby form the basis for the comprehensive compilation of the urine proteome.

Frank Molina (France) presented various biocomputing approaches for systematic 2D gel analysis, including tools for signal processing, quality assessment, alignment and data interpretation, as applied to urine proteomics analysis of Diabetic Nephropathy, aiming at the identification of early biomarkers for disease.

In a multi-center approach coordinated by Harald Mischak, the obstacles associated with the definition of biomarkers were highlighted, including mistakes frequently encountered in clinical proteomics publications. The consortium employed urinary proteome/peptidome data from apparently healthy male and female volunteers, aiming through their analysis, at demonstrating the effect of (erroneous) statistical analysis in clinical proteomic studies. Identification and validation of gender-specific urinary biomarkers was chosen based on the hypothesis that such biomarkers must exist, and that the “diagnosis” of gender is almost 100% correct. Harald Mischak presented the study design (**Figure 1A**) and application of classical statistics. In two additional presentations by Keith Harris (UK) and Alexandros Kalousis (Switzerland) different mathematical approaches were presented and their performance when using different sample sizes.

Up to 134 individual datasets were available in the training set, and 94 independent datasets served as blinded test set. Based on this large dataset, the group could highlight several critical issues in biomarker discovery:

- 1) The application of stringent statistical analysis is mandatory, especially adjustment for multiple testing. Avoidance of adjustment for multiple testing was clearly demonstrated to result in erroneous datasets.

- 2) Sample size estimation or use of correct methodology to perform error estimation is essential. This can be done with good confidence by resampling from a small initial pilot study.
- 3) In the absence of statistically valid biomarkers, combination of peptides/proteins to multidimensional models does generally not result in useful biomarker-models.
- 4) Combination of biomarkers in any multidimensional model generally outperforms single biomarkers or combination in linear models. PCA (Principal Component Analysis) and PLS (Partial Least Squares) performed poorly, in part due to the problem of missing values. Performance of different algorithms (Support Vector Machines, Ada Boost, Artificial Neural Networks, Bayesian networks) appears similar, with Bayesian algorithms having the possible advantage of all delivering a level-of-confidence associated with the results. However, most of the algorithms tend to overfit the data, hence evaluation/validation of the results in an independent blinded test set is mandatory.
- 5) Statistics, even when correctly adjusted for multiple testing, do not take into account the concordance of the biomarkers. This may be due to unknown underlying sampling bias, and will result in an overestimation of the quality of the biomarkers. This further highlights the importance of the mandatory validation in an independent test set.

The consortium concluded that almost all erroneous reports on proteomic biomarkers seem to be a result of poor data quality (in general undetected due to a lack of adequate platform validation) inappropriate application of statistics, and lack of result

confirmation in blinded test set of samples. It was emphasized that simple rules requiring addressing these three key issues when reporting biomarker discovery results will likely eliminate almost all of the invalid reports and should be agreed upon by the scientific community and adopted by journals.

The WG4 session on bioinformatics issues was chaired by Teresa Attwood (UK) and Erik Bongcam-Rudloff (Sweden). They provided an overview of the activities of WG4 dealing with the EuroKUP website ([www.eurokup.org](http://www.eurokup.org)) maintenance, the molecular methods database (MolMeth) and the results obtained with the questionnaires earlier distributed to the members dealing with usage of databases and web-based tools for proteomics data analysis. The main focus of the session was the delineation of actions necessary to develop a specialised KUP (Kidney and Urine Proteomics) ontology (**Figure 1B**, left-hand side). One of the driving forces for devising this ontology is to provide a robust framework for meaningful integration of analysis tools and databases relevant to KUP research. This approach builds on the Utopia (<http://utopia.cs.man.ac.uk/>; 4) philosophy, in which traditional bioinformatics resources are described using ontologies and integrated semantically: this yields responsive visualisation tools that, from a user's perspective, work together seamlessly (**Figure 1B**, right-hand side), while behind the scenes a sophisticated model hides the complexity of file formats, Web services, and so on. To set the scene, Steve Pettifer (UK) presented an introductory lecture on ontology basics, outlining what an ontology is and how it differs from a controlled vocabulary, the requirements and complexity of ontology building, the need for ontologies in the biomedical disciplines, and how they can be applied. This lecture was followed by a presentation of Julie Klein (France) presenting on behalf of the E-lico FP7 project (<http://www.e->

*lico.eu*) a rudimentary use-case ontology. Investigators interested in participating in the construction of these ontologies and databases were identified or are invited to contact Joost Schanstra.

The second day of the meeting included a general scientific session chaired by Aris Charonis (Greece) and Alexander Edelman (France). Various brief presentations dealing with sample pre-treatment for urine proteomics were given:

Hassan Dihazi (Germany) described techniques of depletion of high abundance proteins in urine by the use of commercial affinity columns as applied in the detection of urinary proteins associated with diabetic nephropathy. Using the Multiple Affinity Removal LC Column six interfering high-abundant proteins (Albumin, IgG, IgA, transferrin, haptoglobin and antitrypsin) were removed from urine samples. This approach led to the unmasking of more low copy number proteins and enabled detection of an increased number of proteins.

Alessia Farinazzo (Italy) presented the application of the combinatorial peptide-ligand libraries (CPLL, Proteome-miner) in combination with a variety of different elution systems for the detection of low-abundance urinary molecules. They specifically proposed three elution buffers including SDS, formic acid and cysteic acid respectively, that could be used as very highly effective single elution steps for the quantitative release of proteins from the CPLL beads.

Havard Loftheim (Norway) presented optimized approaches for urine sample processing as applied for the analysis of urine proteome of kidney transplanted patients. Specifically, their experimental pipeline involving sample desalting through ultrafiltration followed by albumin removal through an immuno-affinity column adapted for urine analysis and separation of tryptic peptides by two-dimensional

liquid chromatography (hydrophilic interaction followed by reverse phase chromatography), provided increased protein resolution in a highly reproducible manner. Nathalie Selevsek (Switzerland) presented sample preparation approaches for the detection and quantification of urinary N-glycosylated proteins as applied in bladder cancer research. Their methodology included tryptic digestion of urine proteins followed by periodate oxidation, capture on hydrazide beads and subsequent quantification of the glycosylated peptides by the SRM (Selection Reaction Monitoring) technique.

Additional presentations on biomarker candidates for various kidney diseases were also given by Alexander Edelman who presented their clinical and proteomic study design involving application of the Clinprot technology for the analysis of cystinuria; Anja Verhulst (Belgium) who presented data on the effect of statins on 2DE (DIGE) proteomic urinary profiles of treated healthy volunteers and Annalisa Vilasi (Italy) who presented differences observed when analyzing the urine soluble and exosomal proteome in patients with Gitelman's syndrome by a combination of 2DE-MS, 1DE followed by nano-LC-MS/MS or shot-gun LC-MS approaches. The session concluded with a commercial presentation by Dr McDowell from Waters on the recent developments on a novel ion mobility separation / MS<sup>n</sup> system optimized for top-down proteomics with a prior 2D (at basic and acid pH) LC separation.

Posters were presented by Luca Musante (Ireland) on the analysis of Streptozotocin (STZ) rat models of diabetes with emphasis on the analysis of urine protease profiles, exosomal and glycosylated proteins; Manousos Makridakis (Greece) presented studies on the analysis of secreted proteins in cell line models for aggressive bladder cancer and Jerome Zoidakis (Greece) presented protocols for the enrichment and comparative studies of metal binding proteins in urine.

Based on the WG sessions the following points of action were agreed on:

- 1) A training school on LCM-MS is taking place in Rotterdam (Erasmus MC; Organizer: Theo Luider), May 25-27, 2009. A second training school on imaging MS will take place in Helsinki (University of Helsinki; Organizer: Marc Baumann) during fall 2009 (more information may be found at the EuroKUP website)
- 2) Protocols for tissue collection and processing for LCM-MS and imaging MS will be posted at the EuroKUP website.
- 3) The EuroKUP website will also host the platform for data deposit for the “standard” urine sample.
- 4) A draft on statistical requirements for biomarker discovery studies will be prepared and distributed to the community with the objective to reach a consensus to be adopted by journals, funding agencies etc.
- 5) EuroKUP members were identified to work closely with e-LICO partners and together within the next few months to sketch out and ultimately elaborate a suitable KUP ontology. To this end, a joint hands-on ontology workshop is planned to take place alongside the next EuroKUP meeting, this coming October (described below).

The EuroKUP Management Committee assembled during the second day of the meeting to discuss administrative and budgetary issues related to the Action. A call for Short Term Scientific Missions (STSM) is open and young investigators wishing to perform EuroKUP-related collaborative research in a European laboratory are invited to apply. The next combined MC/WG meeting will be hosted by Meguid El Nahas, EuroKUP MC member, and will take place in Sheffield UK, October 30-November 1 2009.

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**Figure Legend**

A. Study design to demonstrate effects of statistical analysis in biomarker discovery studies. Identification and validation of gender-specific urinary biomarkers based on CE-MS profiles was conducted at different training set sample sizes (numbers in circles) and using a variety of statistical methods and learning algorithms (SVM, Ada

Boost, ANNs, Bayesian networks) for model generation. All results were subsequently evaluated in the blinded test set.

**B.** Generation of a specialized kidney and urine ontology. The left-hand diagram shows some of the concepts that must be encapsulated within the KUP ontology; the right-hand screen-shot illustrates the type of close integration of visualisation and analysis tools that such an ontology could underpin for KUP research.

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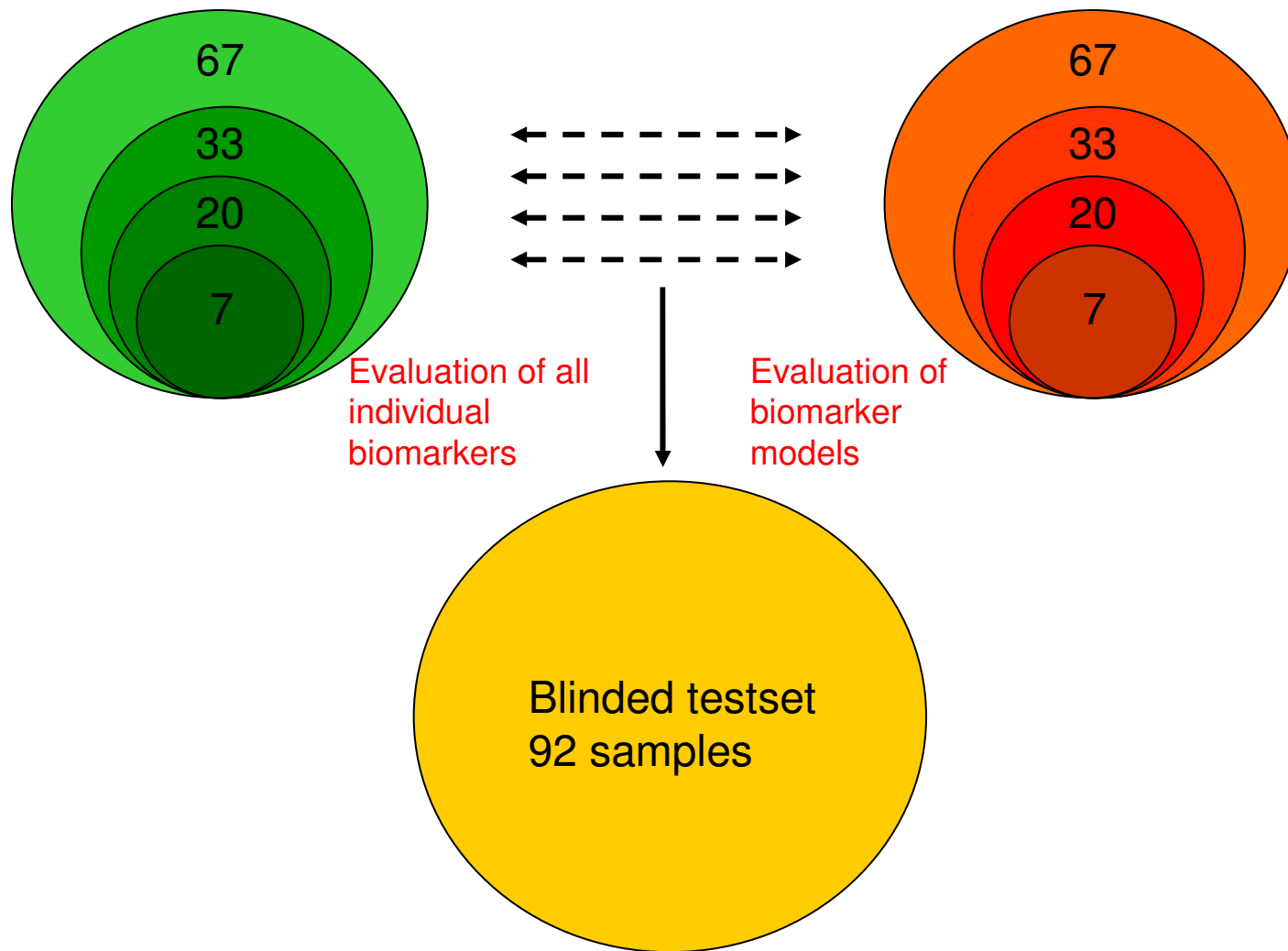
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\*: Member of EuroKUP MC; investigators interested in participating in the Action should contact the MC member of their country.

# Study Design

healthy female 19-40 years **CE-MS analysis** healthy male 19-40 years



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