Editorial

Renal and Urinary Proteomics

The study of the renal and urinary proteome with the goal to discover biomarkers of (renal) diseases is a promising and rapidly expanding area of research. The field has produced a wealth of data and information over the last few years and has reached a point where the initial discovery phase needs to be translated into clinically tangible results. The ultimate clinical utility of these approaches largely depends, as in many cases, on appropriate *study design*, which includes a clinically relevant question that can potentially be answered by studying the proteome, reproducible proteomic platforms, appropriate statistics and independent validation of potential biomarkers [1]. Despite the vast research efforts on biomarker discovery during the past decade, proteomic biomarkers have not yet achieved the desired clinical impact. We have therefore proposed ''Renal and Urinary Proteomics'' as a topic for a Special Issue of *Proteomics – Clinical Applications* to gather viewpoint and review articles to facilitate research on clinical proteomics. In addition, a number of original research contributions adhering to good practice for biomarker discovery are also included in this Special Issue.

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Two associations have been particularly active to promote this field of research and propose guidelines on clinical proteomics: The European Kidney and Urine Proteomics (EuroKUP, www.eurokup.org) COST Action and the Human Kidney and Urine Proteome Project (HKUPP, www.hkupp.org), one of the HUPO Initiatives. We are delighted to see that many contributions in this Special Issue originate from members of these two associations.

As aforementioned, transformation of basic proteomic research to clinically useful data and applications is a key issue and the evident main focus of clinical proteomics. An important issue, often overlooked, is the clinical question in the study. Working on human samples does not necessarily make a study clinically relevant. That is why we have invited a number of physicians to express their views and expectations on the use of clinical proteomics. Delles et al. lay out their ideal roadmap for biomarker discovery in vascular disease, and Spasovski et al. discuss the use of proteomics in chronic kidney disease (CKD) and list the needs and expected answers of clinical nephrologists. John Ioannidis proposes a roadmap for successful application of clinical proteomics, while Efthymios Manolis from the European Medicines Agency describes a new pathway for the qualification of novel methodologies. These contributions collectively should help to guide researchers of the field of clinical proteomics to focus their works towards the clinical applications and validation by the regulatory agencies.

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Owing to its availability, ease of collection and correlation with pathophysiology of diseases, urine has been the sample source of choice in most of clinical proteomics studies, particularly on renal diseases. Zürbig and colleagues review published studies of urinary proteomics in kidney and urogenital diseases that have the high chance to end up as clinical tools. Sigdel et al. and Blanco-Colio et al. review these issues on two more focused topics: renal transplantation and vascular disease, respectively.

Determining the origin of urinary biomarkers is at this point a matter of educated guessing. For improved understanding of the underlying pathophysiology it is therefore important to determine the site of biomarker production in situ. Charonis et al. discuss in a viewpoint contribution at the beginning of the issue whether technology and renal tissue collection have advanced enough to perform parallel kidney tissue and urinary proteomic studies, and what would be the added value of this parallel analysis. This viewpoint paper is accompanied by a dataset brief contribution by Blutke and colleagues (placed at the end of this issue) that shows common pathological mechanisms in two mouse models of glomerular hypertrophy by analysis of the glomerular proteome, followed by validation on human biopsies of different CKD.

A number of original research manuscripts in this Special Issue describe the identification and initial validation of biomarkers of a variety of renal and non-renal diseases. Husi and colleagues identified, using SELDI-TOF MS, a number of potential biomarkers of gastrointestinal cancer that were subsequently validated in an independent cohort using immunoblotting. Chen et al. identified potential biomarkers of pre-eclampsia, a hypertensive disorder of pregnancy, using iTRAQ coupled to two-dimensional LC-MS/MS. Some of these potential biomarkers were subsequently validated using ELISA. Two original research contributions on the identification of urinary biomarkers of renal allograft rejection are also included in this Special Issue. Srivastava et al. identified several urinary biomarkers of long-term allograft loss using antibody arrays that were subsequently validated using reverse-capture protein microarrays. Urinary biomarkers of acute T-cell-mediated allograft rejection were identified using CE-MS by Metzger et al. and validated using the same technology on a blinded cohort. Some of the biomarkers suggested involvement of MMP8, which was subsequently validated on acute rejection biopsy material. Non-invasive detection/surveillance of (renal) diseases is particularly appreciated in the paediatric population. Piyaphanee and colleagues described the SELDI-TOF-based identification of a fragment of α-1B-glycoprotein as a marker to differentiate paediatric steroid resistant from steroid-sensitive nephrotic syndrome. Finally, an original study using a combination of SDS-PAGE, 2D-PAGE, Western blotting and protein identification by HPLC–MS/MS carried out by Castagna et al. identified changes in female urinary proteome along the menstrual cycle with and without contraception. Several potential biomarkers were found differentially expressed and the results were confirmed by immunoblotting. Pending further confirmation, some of these proteins may potentially represent new contraceptive targets.

Facing the ever increasing wealth of information produced by -omics studies, tools are under development to extract the data from multiple -omics sources. Mayer and colleagues present an example of the extraction of relevant pathophysiological information on diabetic nephropathy from public domain sources. Interestingly, while direct feature comparison showed little overlap, expansion of the features to pathways identified modification of complement and coagulation pathways, PPAR signalling and the renin angiotensin system in diabetic nephropathy. This mining example also highlights the importance of publicly accessible databases of (urinary) biomarkers of (renal) diseases. In this context, Siwy et al. present an update of the biomarkers identified by CE-MS for 47 pathophysiological conditions. All CE-MS data are now accessible in one database that will facilitate the comparison of biomarkers of different pathologies.

All these articles demonstrate the substantial potential of urinary proteomic biomarkers. At the same time, they also highlight a shortcoming that must be addressed now: numerous studies describe different potential biomarkers for a variety of diseases, but

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their utility and performance in the actual clinical setting has generally not been addressed (with very few exceptions). It appears high time now to concentrate on qualifying/validating these biomarkers in large, multicentric efforts (best if done in a combined effort that allows assessment of several different biomarkers in the same setting). Only such an approach, best with advice from the regulatory agencies, will enable subsequent clinical utilization. If the vast number of potential biomarkers described by now cannot be translated into clinical practice or cannot even be properly validated, then the entire approach has failed in the end. As such, it is now urgent to combine efforts, seek funding for such multicentric, international validation studies of the described potential biomarkers, and then perform these studies. In parallel, biomarker discovery efforts should focus on addressing well-defined hypothesis-driven questions, which target a better understanding of tissue pathophysiology and as such have the potential to unravel new biology-driven biomarkers and therapeutic targets.

Altogether we hope that this Special Issue on Renal and Urinary Proteomics will be very useful for all the readers and researchers in this area, and that it helps to move forward this exciting field of research and catalyse the transformation of these data into concrete clinical tools.

Reference

[1] Mischak, H., Allmaier, G., Apweiler, R., Attwood, T. et al., Recommendations for biomarker identification and qualification in clinical proteomics. *Sci. Transl. Med.* 2010, *2*, 46ps42.

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REVIEW

Urine proteomics in kidney and urogenital diseases: Moving towards clinical applications

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To date, multiple biomarker discovery studies in urine have been conducted. Nevertheless, the rate of progression of these biomarkers to qualification and even more clinical application is extremely low. The scope of this article is to provide an overview of main clinically relevant proteomic findings from urine focusing on kidney diseases, bladder and prostate cancers. In addition, approaches for promoting the use of urine in clinical proteomics including potential means to facilitate the validation of existing promising findings (biomarker candidates identified from previous studies) and to increase the chances for success for the identification of new biomarkers are discussed.

Received: November 3, 2010
Revised: March 4, 2011
Accepted: March 9, 2011

Keywords:
Kidney / Urine

1 Urine proteome mining

The analysis of urine is an important task in clinical diagnostics. The proteome of human urine has been investigated comprehensively to analyze disease processes regarding the kidney and the urogenital tract [1, 2]. Alterations in the urinary proteome may reflect defects of tissues, whenever the function of them is affected. Urinary proteins/peptides are generated from glomerular filtration, as well as derive from shed epithelial cells, tubular secretion, semen, and secreted exosomes. Hence, urine is considered a goldmine for biomarkers for urogenital as well as systemic diseases [3, 4]. This is reflected by the fact that urine proteins have long been used as adjuncts to current diagnostic/prognostic means and clinical decision making, e.g. albuminuria, FDA approved bladder cancer (BCa) tests such as NMP22 Bladder-Check, BTA stat, etc. [5], just to name few examples. Nevertheless, it is clear that proteomics has not yet realized their full potential, as defined by providing biomarkers that will make clinical decision-making ''easier, better, faster, cheaper'' (quoted from [6]).

On the other hand, highly encouraging results have started becoming available (summarized below [7, 8]); these along with our accumulated knowledge by now on erroneous practices of the past along the clinical proteomics workflow (ranging from faulty study design to inappropriate data analysis and inadequate data reporting and design of verification studies [6, 9–11]) provide solid reasons to believe that urine proteomics will soon achieve their long anticipated clinical impact.

Regardless the clinical question, large-scale studies involving the qualitative and quantitative analysis of the human urine proteome are essential. Common proteomic technologies include a variety of methodological approaches,

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Abbreviations: AGE, advanced glycation end products; **BCa,** bladder cancer; **CAD,** coronary artery disease; **CKD,** chronic kidney disease; **DN,** diabetic nephropathy; **ECM,** extracellular matrix; **GFR,** glomerular filtration rate; **PCa,** prostate cancer

*Members of the European Kidney and Urine Proteomics COST Action (BM0702).

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e.g. 2-DE MS, LC-MS, SELDI-MS, and CE-MS (reviewed in [12, 13]).

In brief, some of the main findings from the analysis of soluble urine proteins may be summarized in Table 1 (for exosomal proteins readers may check on Gonzales et al. [14] for a recent review). It should be noted that most proteome mining studies by now included the analysis of pooled urine samples; occasionally, results were confirmed on individual samples (e.g. [15]). Even though sample pooling diminishes individual variability, it is preferred during proteome mining for technical (protein enrichment) reasons.

Furthermore, urine contains many naturally occurring peptides deriving mainly from target-orientated digestion of specific proteins. These peptides can only be investigated using the so-called 'bottom-up' proteomic technologies (for more details, see [16]). One of these approaches is the CE-MS [17]. Currently, 953 urinary peptides have been identified at the sequence level (Siwy et al., accompanying paper).

Comparison of the published data in an effort to compile a comprehensive view of the urine proteome is very difficult and attempts are scarce [18], which is mainly due to differences in protein annotation, reporting systems described in different studies as well as lack of sufficient data on protein identifiers as defined by different platforms (e.g. experimental/observed mass, p*I* and other physicochemical characteristics), so as to enable inter-platform data comparison and distinction of protein isoforms.

In an effort to overcome this problem, Mischak et al. [19] reported the generation of urine samples (male and female pools), available in large quantities to support standardization purposes in urinary proteomic investigations. These 'standard' samples were characterized comprehensively by proteomic and peptidomic approaches (1-D gel-nano-LC MS/MS (GeLC-MS), 2-DE-MS, LC-MS/MS and CE-MS). For the urinary peptidome, the sequences of 361 peptides (including post-translational modifications), corresponding to 31 different protein precursors, are provided. Fifty-six percent of these peptides were identified by both LC-MS/MS and CE-MS/MS. In the case of proteome analysis, 329 different proteins are reported with most of them being identified by GeLC-MS. The combinatorial use of proteomic/peptidomic approaches allowed to cover a wide mass range and revealed their complementarities. These standard samples are available upon request to meet the needs for reliable data comparison during biomarker discovery and verification/validation as well as facilitate the detailed compilation of the urinary proteome.

Collectively, the existing proteome mining data clearly demonstrate that urine contains a wealth of information; known protein families are well represented in multiple forms (proteolytic products, modified isoforms, etc). Even though we lack the understanding of how exactly these urinary proteins and peptides relate to tissue physiology, the available data have been helpful for the design of clinical proteomic analyses targeting the identification of disease-specific proteins and peptides in urine.

A review of main clinically relevant proteomic findings from urine in the case of urogenital diseases, mainly kidney diseases, bladder and urogenital cancers, is provided below. An overview of the reported biomarkers is also provided in the Supporting Information Table.

2 Chronic kidney disease (CKD)/diabetic nephropathy (DN)

Renal damage and kidney diseases are often described by progressive loss of renal function over a period of months or years and can eventually lead to end-stage renal disease (ESRD). For survival, patients with ESRD require renal replacement therapy (dialysis and/or kidney transplantation). DN, glomerulonephritis, and hypertension are the most common causes of CKD in North America, Europe, and Japan [20]; notably, DN develops in up to 40% of all diabetic patients [21]. Currently, increased urinary albumin excretion in the range of 30–300 mg/day (microalbuminuria) is the best prognostic indicator of DN. Poor

Table 1. Summary of representative urine (soluble) proteome mining studies

Year of discovery	Authors	Number of proteins/ peptides identified	Method	References
2001	Spahr et al.	124	LC-MS/MS	[129]
2002	Pang et al.	103	1-DE and 2-DE-MS, LC-MS	[130]
2004	Pieper et al.	150	2-DE-MS	[131]
2004	Oh et al.	113	2-DE-MS	[132]
2002	Thongboonkerd et al.	47	2-DE-MS (acetone precipitation)	[133]
2006	Zerefos et al	141	2-DE-MS (preparative electrophoresis)	[134]
2005	Smith et al.	48	2-DE-MS	[135]
2005	Castagna et al.	383	2DE-MS (beads coated with hexameric peptide ligand libraries)	[136]
2006	Adachi et al.	1543	1-DE, LC-MS/MS	[137]
2009	Kentsis et al.	2362	Protein LC, 1-DE, LC-MS/MS (analysis of both exosomal and soluble factions)	[15]

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glycemic control and increased arterial blood pressure are other well-known risk factors to develop DN. However, these clinical signs do not fully reflect the risk of developing DN. An indicator of elevated blood glucose levels similar to those observed in diabetic patients seems to be the increased non-enzymatic glycation of proteins. The levels of advanced glycation end products (AGE) reflect a balance between their formation and catabolism [22, 23] and a large body of evidence indicates that oxidative stress is the common denominator link for the major pathways involved in the development and progression of diabetic micro- as well as macro-vascular complications of diabetes.

Proteolysis of AGE results in the formation of AGE peptides excreted normally in urine. In diabetes patients regardless the co-existence of renal disease, AGE peptides additionally accrue in plasma. It has been suggested that these compounds may be involved in the onset and further development of late diabetic complications [24] due to their high reactivity. Uncertainty is increased by the fact that in some instances renal function is already reduced even at the very first signs of microalbuminuria and additionally not all microalbuminuric patients will progress to DN [25]. Glomerular filtration rate (GFR) is the current gold standard for the estimation of renal function; it requires infusion of external substances (e.g. inulin, iothalamat, Cr-EDTA), is expensive and time consuming, hence prohibiting regular use in clinical trials or extensive epidemiologic studies [26]. GFR may also be estimated using equations involving serum creatinine measurements (modification of diet in renal disease (GFRMDRD) equations or Cockcroft–Gault (GFRCG) equations) which have also imperfections [27, 28]. Therefore, the identification of reliable DN risk markers is a very important clinical need.

A detailed review on DN biomarkers was recently published by Ameur et al. [29]. Herein, we briefly summarize studies involving urine proteomics analysis and which we consider as meriting further attention based on either findings verification through follow-up studies and/or contribution to insight to disease pathophysiology. In general, a renal biopsy is the gold standard to identify renal diseases, but due to ethical objections a renal biopsy is nearly never taken from diabetic patients. Furthermore, several studies have indicated that the presence of retinopathy being present is a good alternative for discrimination between DN and non-diabetic glomerulopathy in type 2 diabetic patients with albuminuria [30–32]. Jain et al. [33] identified immunglobulin G, α-1-acid glycoprotein, α-1-microglobulin, and α-2-glycoprotein as biomarkers specific for microalbuminuric diabetes patients in a study involving 100 diabetic (type 2) patients. They also analyzed samples from non-diabetic patients, which were found to lack these biomarkers. These findings are in agreement with more recent results, as described below. To analyze the time of appearance of the biomarkers in correlation with albumin, the authors also tested diabetic patients over a longer period to monitor the microalbuminuria negative to positive alteration. The precedence of the biomarkers to albumin in the investigated cases signified their potential use as earlier markers of DN. Rao et al. [34] recently identified proteomic differences in the urine of diabetic subjects with and without macroalbuminuria (> 200 mg/g albumin/creatinine ratio) using 2-DE MS. The authors used immunodepletion of abundant proteins (e.g. IgG, IgA, albumin, antitrypsin, haptoglobin, transferrin), which facilitated the detection of increased levels of defense-response proteins, several glycoproteins, and vitamin D-binding protein in type 2 diabetic patients with macroalbuminuria. This result is in agreement with an earlier report by Thongboonkerd et al. [35], which similarly revealed an increase in vitamin-D-dependent calcium-binding protein (calbindin D28k) levels in animal models (transgenic mice model of type 1 diabetes). Collectively, these results suggest an involvement of Ca- and/or vitamin-D-binding proteins in the development of DN and, if further verified, may provide a stepping stone towards our improved understanding of the disease underlying pathophysiology. Using 2-DE MS, Varghese et al. [36] identified various plasma proteins in urine allowing differential diagnosis between DN and other types of chronic renal disease (e.g. membranous glomerulonephritis, focal segmental glomerulosclerosis, and lupus nephritis). These findings were in agreement with a large extent with earlier potential DN biomarkers identified by Rao et al. [34] (see also Supporting Information Table). Summarizing, it seems that most of the now described DN biomarkers correspond or originate from highly abundant blood proteins. Their detection in urine may be attributed to 'leakage' associated with the pathological state. However, if these changes can in fact also mark the onset of DN progression remains to be defined.

Various studies involving the analysis of the urine peptidome by CE-MS for the investigation of CKD and DN have been conducted [37–41]. Of special note are the work of Rossing et al. who analyzed urinary biomarker profiles in four groups of type 2 diabetic patients [40] as well as in diabetic type 1 patients with normoalbuminuria and macroalbuminuria [41]. Recently, a multi-center prospective blinded study by the PREDICTIONS network (http://www.rzuser.uni-heidelberg.de/~jb5/aboutus_index.htm) was performed to verify the pattern described in Rossing et al. [41]; In brief, using a set of 148 subjects, discrimination of diabetes mellitus type 2 patients with versus without DN was provided with an area under the curve (AUC) value of 0.957 after unblinding [42].

Additionally, Good et al. [37] established a CKD-specific biomarker pattern in a study involving the analysis of urine samples by CE-MS from 340 patients with biopsy-proven various types of CKD (DN, IgA nephropathy, focal segmental glomerulosclerosis, membranous glomerulonephritis, minimal change disease, vasculitis, and systemic lupus erythematosus) and 552 controls (healthy individuals

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and patients with other diseases and no indication for CKD). This pattern consisted of 273 peptides and in a subsequent and independent heterogeneous cohort (34 controls and 110 cases) analyzed blindly, CKD was detected with sensitivity of 85.5% and specificity of 97.8%.

Many of the DN and all of the CKD biomarkers have been sequenced (Supporting Information Table); among the discriminatory peptides are albumin and uromodulin fragments, but there is also a disease-related marked and intriguing decline of specific collagen peptides. Collagen fragments, especially fragments of collagen α-1 (I) chain, appear to be the major constituents of urinary peptides [17]. These peptides likely reflect normal physiological turnover of the extracellular matrix (ECM) [43]. Specifically, urinary collagen fragments appear at reduced levels in diabetes, and even more, in DN or CKD. It is thereby plausible that decreased concentrations of collagen peptides in urine might be associated with a reduction in elastase activity [44, 45] with concomitant increase in ECM deposition. In addition to DN/CKD, collagen fragments are also the source of identified biomarkers for the diagnosis of coronary artery disease (CAD) [46, 47]. The main difference of the biomarkers for the diagnosis of CAD versus CKD is the direction of their regulation. Whereas most of the collagen-derived biomarkers for CAD showed increased urinary excretion, the collagen fragments indicated CKD by their relative paucity. The difference in their regulation may arise from different activity of collagenases. High levels of circulating collagenases have been identified in patients with stable angiographic coronary atherosclerosis [48] or peripheral arterial disease [49]. Elevated MMP-9 activity has been found in unstable plaques, suggesting a crucial role in plaque rupture [50]. In contrast, in CKD patients a decreased activity of collagenases was observed [51]. Regardless of the primary etiology, severe CKD is characterized by interstitial fibrosis, tubular atrophy, and glomerulosclerosis. Therefore, it has been assumed that the accumulation of proteins in the ECM and collagens that represent the fibrotic kidney may be the result of reduced activity of matrix metalloproteases [52]. Furthermore, accumulation of ECM was recently shown to be associated with decreased urinary excretion of several specific collagen fragments in patients with DN [41].

Several studies have also addressed the discovery of early diagnostic markers for CKD/DN. It should be noted that in these cases, the cross-sectional study design frequently used for discovery of diagnostic biomarkers is no longer applicable; a longitudinal study design is instead recommended. A first pilot study was performed by Otu et al. [53]. In this study urine samples of normalbuminuric type 2 diabetic Pima Indians were analyzed with SELDI-MS at baseline and after 10 years. In this study, patients who developed DN within this time (progressors) were compared with age-, gender-, and diabetes duration-matched controls, which had not developed DN (non-progressors). Several discriminatory peptides were found, providing a first hint that early predictive markers for the development of DN may exist in urine.

Along the same lines, Merchant et al. [54] performed a longitudinal study to investigate the urine profiles of microalbuminuric diabetic type 1 patients in relation to renal function. Specifically, the renal function of these patients was investigated over 10–12 years based on which patients were divided to non-progressors ($n = 40$) and progressors ($n = 21$), in a retrospective study. By the use of LC-MALDI-MS, peptides of tenascin-X, collagen α 1 (IV) and α 1 (V), zona occludens 3, FAT tumor suppressor 2, and inositol pentacisphosphate 2-kinase were detected as potential early biomarkers for renal function decline in microalbuminuric patients. The differential expression of inositol pentacisphosphate 2-kinase was further confirmed by immunohistochemical analysis of kidney biopsies. Both Otu et al. and Mechant et al. provided the proof of principle, e.g. showed the potential of urinary proteome/peptidome analysis for the prediction of DN. Along the same lines, in Rossing et al. [40] the validated DN biomarkers enabled pinpointing microalbuminuric diabetic type 1 patients that showed over a period of 3 years progression of disease, with a sensitivity of 100%. Further ongoing studies support that early diagnosis of DN development with high accuracy rates is possible for both normalbuminuric and microalbuminuric patients over a period of up to 6 years (Rossing et al., unpublished work).

Urine peptidomics has also been used for the evaluation of benefit of therapeutic intervention. Rossing et al. [40] analyzed urine samples from type 2 diabetes patients with CE-MS and generated a DN pattern of 113 biomarkers to distinguish between diabetes patients with and without DN. Macroalbuminuric patients were treated with different doses of candesartan (angiotensin II receptor blocker), which resulted in significant changes of 15 of the 113 urinary markers in the DN pattern to levels comparable with those of normoalbuminuric patients. These pattern changes were not strictly dose-dependent, nevertheless, changes of individual peptides (e.g. constituents of the pattern) correlated with alterations in urinary albumin concentrations at each candesartan dose. Furthermore, the CKD biomarkers described in Good et al. [37] were evaluated in a subgroup of hypertensive diabetic type 2 patients with microalbuminuria from a 2-year irbesartan versus placebo treatment trial (IRMA2 substudy) [55]. The group demonstrated that daily administration of 300 mg irbesartan over a period of 2 years induced specific changes at the expression levels of urinary peptides. The latter included the CKD biomarkers [37], as well as several additional urinary peptides which were not, however, altered in the placebo-treated individuals. Of high biological relevance are considered the changes of urinary collagen fragments associated with early diagnosis of DN. Thereby collectively these existing data support that urine proteomics has great potential in the development of novel diagnostic, but also prognostic as well as tools to assess the potential benefit of therapeutic intervention for DN/CKD [7].

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3 Acute kidney injury

AKI is a common complication in hospitalized patients; a recent meta-analysis highlighted the increased mortality associated with AKI, independent of other factors. Over the last decade, the incidence of AKI has increased from 60 to 500 events/100 000 population [56].

Currently, no effective therapy of AKI is available. To improve the associated serious prognosis, efforts are focused on the primary and especially secondary prevention, rendering the early detection of AKI a clinical and research priority. Recent definitions of AKI, namely the Risk, Injury, Failure, Loss of renal function, and End-stage kidney disease (RIFLE) classification or the Acute Kidney Injury Network criteria, incorporate serum creatinine and urine output as the principal markers to define and detect AKI [57]. However, these and other clinically available and widely used markers (blood urea nitrogen, tubular enzymuria or proteinuria, elevated serum creatinine or oliguria) are less than optimal and have substantial further limitations [58]. Therefore, a fast, specific and reliable biomarker assay that enables monitoring of patients in a clinical setting is of great clinical interest.

In the last 5 years, numerous new markers such as neutrophil-gelatinase-associated lipocalin (NGAL), interleukin-18 (IL-18), cystatin C, and kidney injury molecule 1 in the urine and/or serum have been studied and proposed as early detection markers of AKI [59–62]. Persistently, these markers performed well in initial pilot trials. However, these promising results could often not be confirmed in later, larger multi-center trials and limitations of these biomarkers in the early diagnosis of renal injury were demonstrated [63–66].

A promising finding was described by Zhou et al. [67], who analyzed urinary exosomes from a rat model of cisplatin-induced AKI, by difference gel electrophoresis (2-D DIGE). Among the different regulated proteins, fetuin-A was found upregulated in the AKI animals, compared with animals with prerenal azotemia. Urinary fetuin-A was detected 2 days before the increase of creatinine in serum. This finding was further validated in animals with bilateral renal inschemia and reperfusion injury as well as in ICU patients with AKI.

Metzger et al. [68] applied CE-MS for the detection of urinary biomarkers – predictors for AKI. They used a training set of 87 urine samples, collected from ICU patients at different days grouped according to later development of AKI or maintenance of normal kidney function. Based on this analysis, an AKI-specific peptide profile (20 urinary polypeptides) was developed. The biomarkers were specific peptides derived from six different proteins. Fragments of α-1-antitrypsin, albumin, and β-2-microglobulin were upregulated; peptides derived from collagen type I and III and fibrinogen α were downregulated in AKI. Comparison of the peptides comprising the AKI and CKD biomarker profiles [37] demonstrated that two of five collagen 1 α (I) fragments of the former and one of three fibrinogen fragments in the latter (AKI) as well as, all albumin-derived fragments, one α 1-antitrypsin fragment, and one β-2-microglobulin fragment, overlapped. The AKI panel provided good diagnostic accuracy with an AUC value of 0.84 and 0.90, during validation in two blinded sets of prospectively, longitudinal collected samples from 20 ICU patients, and from 30 patients after hematopoietic stem cell transplantation, respectively. Additionally, 28 healthy controls all scored negative (100% specificity). Comparison of the AKI marker panel with levels of creatinine, cystatin C, KIM-1, IL-18, and NGAL supported that the proteomic profile could detect AKI, at least 4–5 days earlier than serum creatinine; further validation of these promising results is underway.

4 Acute renal allograft rejection

Acute rejection is an important factor that determines long-term function and survival of renal allografts. Approximately 15–30% of the transplanted patients suffer from one or multiple acute rejections which occur mainly in the first year of transplantation [69, 70].

Timely detection and sufficient anti-rejection therapy of acute rejection episodes is important to conserve allograft function. However, detection of acute rejection in an early stage is challenging [71]. Regular monitoring for increases in serum creatinine or decrease in creatinine clearance, with subsequent indicated biopsy, implies that the rejection is detected in an advanced stage where the graft is already impaired by the rejection process.

Protocol biopsies have been used to detect acute rejection in an earlier, subclinical stage, where functional impairment is not present yet [72]. An inherent limitation of this approach is that even with multiple biopsies it is impossible to capture every rejection episode. Therefore, many attempts have been made to develop non-invasive tests in blood or urine which are able to detect acute rejection [73–75]. Most of these approaches used single markers or combinations of a few markers. Mostly due to lacking specificity and sensitivity of these markers, none of these tests is established in the clinical routine and post-transplant care of the patients. Additionally, several groups have reported successful use of mass spectrometry of urinary samples to detect acute rejection using a panel of marker peptides [76–80]. Overall good performance was reported in theses studies with AUC values ranging from 0.85 to 0.97. Verification of these results on larger patient cohorts is pending.

Acute tubulointerstitial rejection is often associated with acute tubular injury (ATI) [81]. Therefore, an important clinical question is whether proteomic markers identified in acute rejection are indicative for the rejection process itself or if they are non-specifically reflect tubular injury. Schaub et al. described cleaved urinary β-2-microglobulin as a potential biomarker for acute tubular damage in renal allografts [82]. However, fragments of β-2-microglobulin corre-

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late with ATI in samples with and without rejection, but not with rejection itself. In this line, β-2-microglobulin fragments were recently described as markers for acute kidney injury in non-transplanted patients [68].

Interestingly, various recent studies [79, 80, 83] (see Supporting Information Table) support that the levels of diverse collagen fragments are altered in samples with acute T-cell-mediated tubulointerstitial rejection. Aligning these fragments to other, commonly present urinary collagen α-1 (I) peptides, showed that those collagen fragments associated with rejection posses a characteristic sequence motif at their c-terminus which is indicative of increased ECM degradation by metalloproteinases. By immunohistological staining of biopsy sections, a significant number of MMP+ polymorphonuclear cells close to the endothelium were detected as early event of the rejection process. Therefore, metalloproteinase expression at peritubular capillaries may be an indicator for trans-endothelial migration of infiltrating cells into the interstitium and into the tubules [84, 85].

5 Urological malignancies

Several applications of proteomics technologies for cancer biomarker discovery have been reported. A brief summary of few representative studies on prostate and BCas involving follow-up validation of findings is provided below.

5.1 Prostate cancer

Prostate cancer (PCa) is third in incidence among other male malignancies [86]. Prostate-specific antigen (PSA) screening has largely facilitated PCa detection [87]; nevertheless, PSA has low specificity [88, 89] resulting in up to 700 000 unnecessary prostate biopsies per year in the USA. Additionally, currently it is not possible to differentiate between aggressive and more indolent prostate tumors; the latter have no major impact on mortality for 10–15 years [90], whereas in contrast the former may become metastatic within approximately 2–3 years [90], underscoring the need for novel prognostic makers.

Discovery of diagnostic markers for PCa has been extensively addressed [13, 91, 92]. It should be noted that even though there is ample literature evidence describing PCa biomarker discovery in plasma/serum and tissue, data on urine analysis are still scarce. First, urinary proteomic analyses were performed by Rehman et al. using 2-DE MS [93] and by M'Koma et al. using reverse phase chromatographic separation and MALDI-MS analysis [94], but verification of these findings and identification of the discriminatory peptides is lacking. Potential biomarkers revealed by these studies are listed in the Supporting Information Table.

Identification of CE-MS urinary profiles diagnostic for PCa was recently described [95]. In a prospective study, Theodorescu et al. described a panel of 12 peptide biomarkers (Supporting Information Table) which in a blinded test set detected PCa with a sensitivity of 83% and specificity of 97%. Of special note, the PCa-specific biomarkers were detectable solely in first void urine, indicating that these peptides may originate from prostatic fluids. There is by now increasing evidence in support of this hypothesis [96, 97] also emphasizing on the importance of urine sampling methodology in PCa biomarker discovery.

5.2 Bladder cancer

BCa is the second in incidence and mortality cancer of the genitourinary system accounting for an estimated 380 000 cases annually worldwide and 70 530 incidents and 14 680 deaths in US in 2010 (http://www.cancer.gov/cancertopics/types/bladder). BCa consists of a spectrum of malignancies categorized into three main groups of distinct clinical behavior, prognosis, and primary management: superficial (Ta-Tis-T1), locally invasive (T2-4), and metastatic cancer (N+/M+) [98, 99]. At initial diagnosis, most patients (70%) present superficial disease being restricted to the urothelium (Tis, Ta) and up to muscularis mucosa (T1); 20–30% have muscle-invasive tumor with approximately 5% exhibiting clinically evident distant metastases. Notably, BCa recurrence rate is about 20% with an associated 15% progression rate to higher grade and/or invasive lesions; this imposes frequent follow-up of patients by cystoscopy – an invasive and highly costly procedure – rendering BCa one of the most costly types of cancer in terms of management. Additionally, invasiveness results in very poor prognosis: more than 50% of cases succumb to their disease within 5 years [99]. Collectively, these facts define the clinical needs and research questions for BCa: these include development of early diagnostic markers for invasive cancers and definition of markers for recurrence and progression superior in accuracy to the current non-invasive golden standard test of urine cytology [100]; deciphering the molecular mechanisms associated with pathophysiology of the invasive phenotype to enable development of novel therapeutic approaches (reviewed in [6, 101]).

Multiple studies have been conducted towards discovering BCa protein biomarkers. Among the potential biomarkers that attracted a lot of interest are bladder tumor antigen, nuclear matrix protein 22, BLCA-4, hyaluronidase, cytokeratins (8, 18, 19), tissue polypeptide antigen, and tissue polypeptide-specific antigen, soluble Fas and survivin; nevertheless, their exact potential use in the clinic is still to be defined (reviewed in [6, 100, 102–103]). Some of these biomarkers were initially discovered following the application of classical proteomics technologies with the characteristic example of BCa antigen (BLCA-1,-4, -6). Using 2-DE analysis of urothelial tissue, the association of BLCA proteins with BCa was observed [104–107]. Detection of BLCA-4 in urine by the use of specific immunoassays

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provided BCa detection with a specificity of 95% and a sensitivity of 89% [106]. Validation of these results in clinical trials is in progress. Multiple additional studies using 2-DE approaches have also been conducted through the years (reviewed in [108]; some additional representative findings [109, 110] are also shown in the Supporting Information Table, indicating various potential biomarkers; nevertheless, large-scale validation of the results and identification of specific use are still pending.

Recently, Orenes-Pinero et al. [111] identified differentially expressed proteins between patients with bladder tumors and controls using 2-D DIGE coupled to MS. These biomarkers (e.g. regenerating protein 1 (Reg-1; current name: Lithostathine-1-α), cytokeratin 10, T-cell surface glycoprotein CD5) were confirmed to be associated with BCa progression on BCa cell lines by immunoblotting and on bladder tumors by immunohistochemistry. Moreover, the association of Reg-1 with tumor staging and clinical outcome was confirmed by tissue microarrays. Notably, Reg-1 was validated using an independent test set ($n = 80$) resulting in an AUC of 0.88. These results suggest that the involvement of Reg1 in BCa progression merits further investigation, particularly in view of its association with progression of other types of tumors [112, 113].

SELDI technology has also been employed in the investigation of urine proteome for BCa detection by multiple investigators. The performance of SELDI profiles in detecting BCa ranges from 72 to 93% sensitivity and 63 to 87% specificity in blinded sets of samples from the different studies [114–119]. However, due to differences in chip surfaces and sample preparation methods employed in individual studies as well as lack of discriminatory peptide identifications, the data sets are not comparable.

Vlahou et al. [115] identified one of the transitional cell carcinoma (TCC) peptide markers detected by SELDI as α-defensins; this peptide was further found to induce BCa cell motility in vitro [120]. Along the same lines, Munro et al. [117] identified urinary α-defensins as a TCC biomarker in a study involving SELDI analysis of a total of 227 subjects. In a separate study, immunohistochemical analysis further confirmed the overexpression of α-defensins in invasive BCa [120]. Despite this evidence, the involvement of α-defensins in inflammatory processes raises concerns regarding the ultimate specificity of these proteins as urinary biomarkers for BCa detection; On the other hand, further investigation of the mechanism of α-defensin expression by bladder epithelial cells and its means of action (autocrine–paracrine) in relation to tumor cell motility and invasiveness may provide valuable insights for therapeutic intervention.

As reviewed in [13], Theodorescu et al. [121] analyzed urine samples with CE-MS of a total of 445 subjects including BCa patients and subjects with other malignant or benign urogenital diseases or no evidence of disease. Twenty-two BCa-specific markers were revealed and further validated in a blinded study involving a prospective cohort of 180 subjects (31 BCa patients, 138 patients with benign urological diseases, and 11 healthy individuals); the obtained sensitivity for BCa detection was 100% with specificity between 86 and 100% depending on the control type.

Recently, a CE-MS biomarker profile consisting of fragments of collagen α 1 (I) and (III), membrane-associated progesterone receptor component 1, and uromodulin was found to be predictive of muscle invasive BCa [13, 122], and as a component of nomogram also including tumor grade, it significantly improved sensitivity (92%) and specificity (68%) in detection of muscle invasive disease.

5.3 Outlook

There has been a remarkable expansion in the employment of urine in biomarker discovery the last few years. Characteristically, using the keywords 'urine' and 'protein biomarker' a PubMed search retrieves almost 5000 articles published in the last 10 years. If the keywords 'renal' or 'kidney' are further added to the search then >1300 articles are retrieved. The respective numbers for the previous two decades are significantly lower with ca. 2610 and 570 articles on urine protein biomarker (584 and 135 after adding the term 'renal') for the decades 1990–2000 and 1980–1990, respectively (Fig. 1). This significant increase in the last 10 years can be attributed in part to advancements in proteomics technologies which allowed for a better and more in depth exploration of the urine proteome; this fact along with the well-known advantages of urine (e.g. non-invasive collection, availability in large quantities, ability to reflect systemic diseases, etc.) established urine as a 'gold-mine' in biomarker research. Coverage of all available recent information on biomarker discovery for renal/urogenital diseases would be out of the realm of this review; we instead opted to emphasize on data from proteomic studies that have progressed to some extent in the 'biomarker validation' pipeline and which thereby may hold greater promise in achieving clinical impact in the near future.

Despite the existing promising findings, considering the extensive research efforts, resources, and ample literature on the subject, it would be expected that the impact of urinary proteomics on clinical practice would have already been

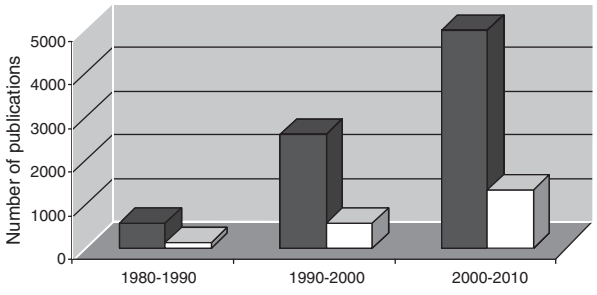

Figure 1. Number of publications on 'urine and protein biomarker' (gray bar) and 'urine and protein biomarker and kidney (or renal)' (white bar) in the last decades.

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realized. The observed delay underscores the need for a thorough evaluation of steps involved in the biomarker pipeline – from initial discovery up to clinical assay development – targeting the identification of erroneous practices and bottlenecks and potential solutions to overcome them [9, 10, 123]. Along these lines, in the recent report by Mischak et al. [11] a group of ca. 50 investigators provide guidelines for identifying and qualifying proteomic biomarkers that could also be used as reporting requirements in biomarker discovery studies. Adherence to these recommendations is expected to result in a decrease in the number of false positives. e.g. pool of biomarkers entering the validation pipeline. This would mark a significant improvement in biomarker research, yet it is not sufficient to reach the ultimate goal of bringing proteomics findings to the clinical practice faster. Large-scale validation of biomarkers through rapid and inexpensive assays is an additional important bottleneck in the whole process. The technological needs (e.g. appropriate ELISA, high-throughput MS-based platforms) have been described [124, 125] and vigorous research efforts have been undertaken to optimize available platforms. An equally important factor that is frequently overlooked is the need for high numbers of high quality, clinically annotated urine samples to support such studies. As a first step, the EuroKUP (European Network for Kidney and Urine Proteomics) and HKUPP (Human Kidney and Urine Proteome Project) consortia have recently developed a standard protocol for urine collection (Tables 2 and 3) based on the accrued knowledge in the field and targeting a balance between perfection and feasibility at the clinical setting. This standard protocol may be seen as one little piece of a more extensive project addressing the development of common data elements (CDE) and a unified biobanking system for kidney (or other) diseases. At present, clinical samples for specific research questions even if available are disparate and of frequently questionable quality. Development and adoption of CDE will support the formation of a unified system for urine specimen collection and increase cross talk across institutes, clinical centers but also different studies and findings thereof. Such a task is not trivial and requires a coordinated effort from multiple disciplines. Experience from existing such initiatives [126, 127] can be recruited and aid significantly in this direction.

Collectively, urine proteomics has the potential to impact clinical decision-making in the diagnosis, prognosis as well as therapeutic intervention of kidney (and other) diseases. This statement can by now be supported by the existing experimental evidence. Undoubtedly, increase in understanding the disease pathophysiology and implementation of novel integrative (e.g. 'systems biology') approaches in biomarker research will gear up the discovery of successful biomarkers and therapeutic targets as supported in various recent reports [1, 128]. In parallel, however, to this effort, establishment of the needed infrastructure to ensure cross-talk between available and future resources, particularly of biospecimens, and importantly a more organized framework encompassing biomarker discovery and verification with a clear focus on clinical implementation and impact on specific clinical needs are urgently needed so as to prevent the loss of valuable biologic material and fulfill faster the

Table 2. Standard protocol for urine collection for proteome analysis developed by HKUPP and EuroKUP[a)]

(i) Type of urine sample Mid-stream of second morning urine (preferably) or morning random-catch urine
(ii) Container Sterile (preferably) or clean urine collectors
(iii) Pre-treatment Centrifuge at 1000 × *g* for 10 min to remove cell debris and casts
(iv) Storage Aliquot supernatant without disturbing the pellets and overfilling tubes Usual working volumes: 1.5, 10, or 50 mL Store at −80°C (preferably) or −20°C Record time until freezing (it should be no longer than 3 h; if longer, addition of preservatives is needed)
(v) Avoid freeze–thaw cycles. Always keep a record of this event

a) For detailed information and variations of standard protocol, please visit http://www.eurokup.org/node/137 and http://www.hkupp.org/Urine%20collectiion%20Documents.htm.

Table 3. Sample minimal identifiers recommended by HKUPP and EuroKUP

A. Storage information
1. Unique sample code
2. Storage temperature (−20/−80)
3. Institution
4. Date – time of collection
5. Time until freezing
6. Aliquot (volume) and number of aliquots
B. Case Information
7. Unique patient code
8. Clinical diagnosis
9. Age
10. Gender
11. Height
12. Weight
C. Recommended laboratory information
14. Urinary protein amount
15. Urine creatinine
16. Hematuria
17. Serum creatinine[a)]
18. Urine pH[a)]
19. Serum protein (serum albumin)[a)]
20. Serum cholesterol[a)]

a) Not absolutely needed (but usually helpful) information. In general, needed clinical information depends on the disease under investigation.

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(well justified) high expectations from urine clinical proteomics.

The authors acknowledge funding supports as follows: EuroKUP COST Action (to P.Z., H.D., V.T., and A.V.), DECanBio FP7 (201333 to A.V.), and SysKID FP7 (241544 to P.Z.). The Thailand Research Fund RTA5380005, Office of the Higher Education Commission, and Mahidol University under the National Research Universities Initiative (to V.T.).

The authors have declared the following conflict of interest: Petra Zürbig and Jochen Metzger are employees of Mosaiques Diagnostics GmBH which developed CE-MS for clinical proteomics applications.

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Biochemical and Biophysical Research Communications 424 (2012) 629–634

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Isolation and characterizations of oxalate-binding proteins in the kidney

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ARTICLE INFO

Article history:
Received 4 July 2012
Available online 14 July 2012

Keywords:
Kidney stone
Nephrolithiasis
Oxalate
Oxalate-binding
Proteins
Proteomics

ABSTRACT

Oxalate-binding proteins are thought to serve as potential modulators of kidney stone formation. However, only few oxalate-binding proteins have been identified from previous studies. Our present study, therefore, aimed for large-scale identification of oxalate-binding proteins in porcine kidney using an oxalate-affinity column containing oxalate-conjugated EAH Sepharose 4B beads for purification followed by two-dimensional gel electrophoresis (2-DE) to resolve the recovered proteins. Comparing with those obtained from the controlled column containing uncoupled EAH-Sepharose 4B (to subtract the background of non-specific bindings), a total of 38 protein spots were defined as oxalate-binding proteins. These protein spots were successfully identified by quadrupole time-of-flight mass spectrometry (MS) and/or tandem MS (MS/MS) as 26 unique proteins, including several nuclear proteins, mitochondrial proteins, oxidative stress regulatory proteins, metabolic enzymes and others. Identification of oxalate-binding domain using the PRATT tool revealed "*L-x(3,5)-R-x(2)-[AGILPV]*" as a functional domain responsible for oxalate-binding in 25 of 26 (96%) unique identified proteins. We report herein, for the first time, large-scale identification and characterizations of oxalate-binding proteins in the kidney. The presence of positively charged arginine residue in the middle of this functional domain suggested its significance for binding to the negatively charged oxalate. These data will enhance future stone research, particularly on stone modulators.

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1. Introduction

Recently, kidney stone disease has been investigated extensively at the molecular level. Among all stone types, calcium oxalate (CaOx) is the most common inorganic matrix found in kidney stones [1]. In addition to inorganic matrix, organic compounds, particularly proteins, are also found in kidney stones [2]. Many previous studies have focused on identification of CaOx kidney stone modulators and proposed that some of the calcium- and oxalate-binding proteins may serve as the stone modulators [3–6]. From these efforts, some of calcium-binding proteins that have inhibitory activity against CaOx crystal growth and aggregation have been successfully identified [7–12]. On the other hand, a much fewer number of previous studies have attempted to characterize oxalate-binding proteins, which also have potential role in kidney stone disease [6]. A number of oxalate-binding proteins have been isolated from kidney homogenate and stone matrix [6]. Among these, only histone has been successfully identified and characterized [13]. This under-investigation is probably due to a lack of simple method for isolation of oxalate-binding proteins in the past.

We have recently developed an oxalate-affinity chromatographic column for highly efficient and simplified isolation/purification of oxalate-binding proteins [14]. In the present study, our oxalate-affinity chromatographic column containing oxalate-conjugated EAH Sepharose 4B beads was applied to purify oxalate-binding proteins from porcine kidney. The recovered proteins were resolved by 2-DE compared to those recovered from the controlled column containing unconjugated EAH-Sepharose 4B (to subtract the background of non-specific bindings). The oxalate-binding proteins were then identified by Q-TOF MS and/or MS/MS analyses. Finally, the identified proteins were subjected to identification of functional domain responsible for oxalate-binding.

2. Materials and methods

2.1. Preparation of oxalate-affinity chromatographic column

The oxalate-affinity chromatographic column was prepared as we described previously [14]. In principle, oxalic acid was conjugated (through carboxylic groups) to EAH Sepharose 4B (via primary amine groups). Coupling efficacy was determined quantitatively

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.07.015

by measuring the remaining primary amine groups on EAH-Sepharose 4B beads using a competitive nindydrin assay [14]. For the controlled column, EAH Sepharose 4B beads were treated and processed with similar procedures as for the oxalate-affinity column, but without oxalic acid conjugation.

2.2. Isolation of oxalate-binding proteins from porcine kidney

A porcine kidney was bought from a local fresh poultry market. The tissue was then dissected into thin slices, washed with phosphate buffered saline (PBS), snap frozen in liquid nitrogen and ground into powder using pre-chilled mortar and pestle. Tissue powder was then resuspended in Tris–HCl buffer (pH 6.5). The kidney sample was then centrifuged at 12,000*g*, 4 °C for 5 min to remove the particulate matters. The supernatant was saved and protein concentration was measured by the Bradford method. To isolate oxalate-binding proteins, 3 mg of the recovered porcine kidney proteins was passed through the affinity column with a flow rate of 1 mL/min. Thereafter, the column was first eluted with 20 mL of a binding buffer containing 10 mM 2-morpholinoethanesulfonic acid (MES) in NaOH (pH 6.5), 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 5 mM KCl to remove non-specific binding proteins. Oxalate-binding proteins were then eluted with 10 mL of an elution buffer containing 10 mM MES in NaOH (pH 6.5), 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM KCl, 1 M NaCl and 1 mM oxalic acid with 10 fractions (1 mL/fraction). In parallel, 3 mg of the recovered porcine kidney proteins was passed through the controlled column with a flow rate of 1 mL/min. The subsequent steps were exactly the same as for the oxalate-affinity column.

2.3. 2-DE

The eluted protein fractions obtained from the previous step were pooled, desalted by dialysis against 18 MΩ cm (dI) water, and concentrated by lyophilization. The concentrated proteins were then resuspended in 150 μl of rehydration buffer containing 7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 120 mM dithiothreitol (DTT), 2% ampholytes (pH 3–10), 40 mM Tris–HCl, and a trace amount of bromophenol blue. The protein solution was then rehydrated overnight in an Immobiline™ DryStrip, non-linear pH 3–10 (GE Healthcare Bio-Sciences, Uppsala, Sweden). Thereafter, the isoelectric focusing (IEF) was run in Ettan IPGphor III IEF System (GE Healthcare Bio-Sciences) at 20 °C, using a stepwise mode to reach 9083 V h with a current limit of 50 μA/strip. After the IEF completion, proteins on the strip were equilibrated in a buffer containing 6 M urea, 130 mM DTT, 30% glycerol, 112 mM Tris-base, 4% SDS and 0.002% bromophenol blue for 15 min, and then with another buffer containing 6 M urea, 135 mM iodoacetamide, 30% glycerol, 112 mM Tris-base, 4% SDS and 0.002% bromophenol blue for 10 min. For the second dimension, the proteins on the IPG strip were resolved further in 12% polyacrylamide slab gel (8×9.5 cm) using the SE260 Mini-Vertical Electrophoresis Unit (GE Healthcare Bio-Sciences) with a constant voltage of 150 V for 2 h. The resolved protein spots were stained with SYPRO Ruby fluorescence dye (Invitrogen-Molecular Probes; Eugene, OR) and visualized with Typhoon laser scanner (GE Healthcare).

2.4. Spot matching and quantitative intensity analysis

Image Master 2D Platinum (GE Healthcare) software was used for matching and analysis of protein spots in 2-D gels. Parameters used for spot detection were (i) minimal area = 10 pixels; (ii) smooth factor = 2.0; and (iii) saliency = 2.0. A reference gel used for matching the corresponding protein spots among different gels was created from an artificial gel by combining all of the spots presenting in different gels into one image. Background subtraction was performed and the intensity volume of each spot was normalized with total intensity volume (summation of the intensity volumes obtained from all spots within the same 2-D gel). Differentially expressed protein spots were defined as those, which were present only in the sample derived from oxalate-affinity chromatographic column or had intensity levels > 3-fold as compared to those obtained from the controlled column. These differentially expressed protein spots were excised and subjected to in-gel tryptic digestion and identification by Q-TOF MS and MS/MS analyses.

2.5. In-gel tryptic digestion

The excised protein spots were washed twice with 200 μL of 50% acetonitrile (ACN)/25 mM NH_4HCO_3 buffer (pH 8.0) at room temperature for 15 min, and then washed once with 200 μL of 100% ACN. After washing, the solvent was removed, and the gel pieces were dried by a SpeedVac concentrator (Savant; Holbrook, NY) and rehydrated with 10 μL of 1% (w/v) trypsin (Promega; Madison, WI) in 25 mM NH_4HCO_3. After rehydration, the gel pieces were crushed and incubated at 37 °C for at least 16 h. Peptides were subsequently extracted twice with 50 μL of 50% ACN/5% trifluoroacetic acid (TFA); the extracted solutions were then combined and dried with the SpeedVac concentrator. The peptide pellets were resuspended with 10 μL of 0.1% TFA and purified using $ZipTip_{C18}$ (Millipore; Bedford, MA). The peptide solution was drawn up and down in the $ZipTip_{C18}$ 10 times and then washed with 10 μL of 0.1% formic acid by drawing up and expelling the washing solution three times. The peptides were finally eluted with 5 μL of 75% ACN/0.1% formic acid.

2.6. Protein identification by Q-TOF MS and MS/MS analyses

The trypsinized samples were premixed 1:1 with the matrix solution containing 5 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN, 0.1% (v/v) TFA and 2% (w/v) ammonium citrate, and deposited onto the 96-well MALDI target plate. The samples were analyzed by Q-TOF Ultima™ mass spectrometer (Micromass; Manchester, UK), which was fully automated with predefined probe motion pattern and the peak intensity threshold for switching over from MS survey scanning to MS/MS, and from one MS/MS to another. Within each sample well, parent ions that met the predefined criteria (any peak within the *m/z* 800–3000 range with intensity above 10 count ± include/exclude list) were selected for CID MS/MS using argon as the collision gas and a mass dependent ± 5 V rolling collision energy until the end of the probe pattern was reached. The MS and MS/MS data were extracted and outputted as the searchable *.txt* and *.pkl* files, respectively, for independent searches using the MASCOT search engine (http://www.matrixscience.com) to query to the NCBI mammalian protein database, assuming that peptides were monoisotopic. Fixed modification was carbamidomethylation at cysteine residues, whereas variable modification was oxidation at methionine residues. Only one missed trypsin cleavage was allowed, and peptide mass tolerances of 100 and 50 ppm were allowed for peptide mass fingerprinting and MS/MS ions search, respectively.

2.7. Identification of oxalate-binding domain

The PRATT tool (http://www.ebi.ac.uk/Tools/pratt/) [15,16], provided by the European Bioinformatics Institute (EBI) was used for characterization of the functional domain in the identified oxalate-binding proteins. All the proteins identified by Q-TOF MS and/or MS/MS analyses (in FASTA format) were altogether aligned and

subjected to the unbiased opened search (without any restrictions or predefined pattern) to define the functional domain responsible for oxalate-binding. After obtaining oxalate-binding domain, sequences of some of the known kidney proteins, which are not the oxalate-binding proteins (see their identities in *Supplementary* Table S1), were submitted to search for such functional domain (to demonstrate the specificity of the identification of the oxalate-binding domain).

3. Results & discussion

The efficacy and specificity of the oxalate-affinity chromatographic column were confirmed in our previous study [14] using the known oxalate-binding protein p62 [17] as the positive control and carbonic anhydrase as the negative control. The extracted porcine kidney proteins were then passed through the oxalate-affinity column to isolate oxalate-binding proteins. In parallel, the proteins were passed through the controlled column to subtract the background of non-specific bindings. The eluate fractions from each column were pooled, and the recovered proteins were resolved by 2-DE and visualized by SYPRO Ruby staining. Using the Image Master 2D Platinum software to match and quantify protein spots across different gels, a total of 38 protein spots were defined as differentially expressed spots between the two groups (Fig. 1). All of these potential oxalate-binding proteins were successfully identified by Q-TOF MS and/or MS/MS analyses as 26 unique proteins. Note that some of them had many isoforms and were thus identified as one unique protein. Details of their identities, identification numbers, identification scores, percentage of sequence coverage (%Cov), numbers of matched peptides, isoelectric points (pI) and molecular weights (MW) are summarized in Table 1.

All the identified proteins were subjected to characterization of oxalate-binding domain using the PRATT tool (http://www.ebi.ac.uk/Tools/pratt/) [15,16], provided by the European Bioinformatics Institute (EBI). The data revealed a domain/pattern "L-x(3,5)-R-x(2)-[AGILPV]" in 25 of 26 (approximately 96%) unique proteins identified by Q-TOF MS and/or MS/MS analyses (Fig. 2). Among these 25 proteins with oxalate-binding domain, 15 proteins had more than one oxalate-binding domain in their sequences, and the greatest number of this domain in each protein was 19, which was found in the sequence of transformation/transcription domain-associated protein isoform 2 (spot #38). Interestingly, the presence of positively charged arginine residue in the middle of this functional domain suggested its significance for binding to the negatively charged oxalate. To demonstrate the specificity of this oxalate-binding domain, sequences of some of the known kidney proteins, which are not oxalate-binding proteins (see their identities in *Supplementary* Table S1), were also submitted to the PRATT tool to search for such functional domain. The data revealed that none of these non-oxalate-binding kidney proteins had the domain "*L-x(3,5)-R-x(2)-[AGILPV]*" in their sequences (*Supplementary* Table S1). These data implicate the specificity of this functional domain, which is responsible for oxalate-binding.

The present study identified histone H2A (spot #14) as one of the nuclear oxalate-binding proteins in porcine kidney, consistent to the data reported in a previous study demonstrating that histone serves as a known oxalate-binding protein in kidney homogenate and stone matrix [13]. These concordant results strengthened that our oxalate-affinity chromatographic column is reliable and effective for isolation and enrichment of oxalate-binding proteins. In previous studies, one third of oxalate-binding proteins have been found in mitochondria of renal cells [6]. There were a number of mitochondrial oxalate-binding proteins identified in this study, including mitochondrial precursor of ATP synthase subunit beta (spot #13), mitochondrial Aacyl-CoA dehydrogenase (spot #19), mitochondrial NADH:ubiquinone oxidoreductase 51 kDa subunit (spot #22), and mitochondrial malate dehydrogenase 2 NAD (spot #24).

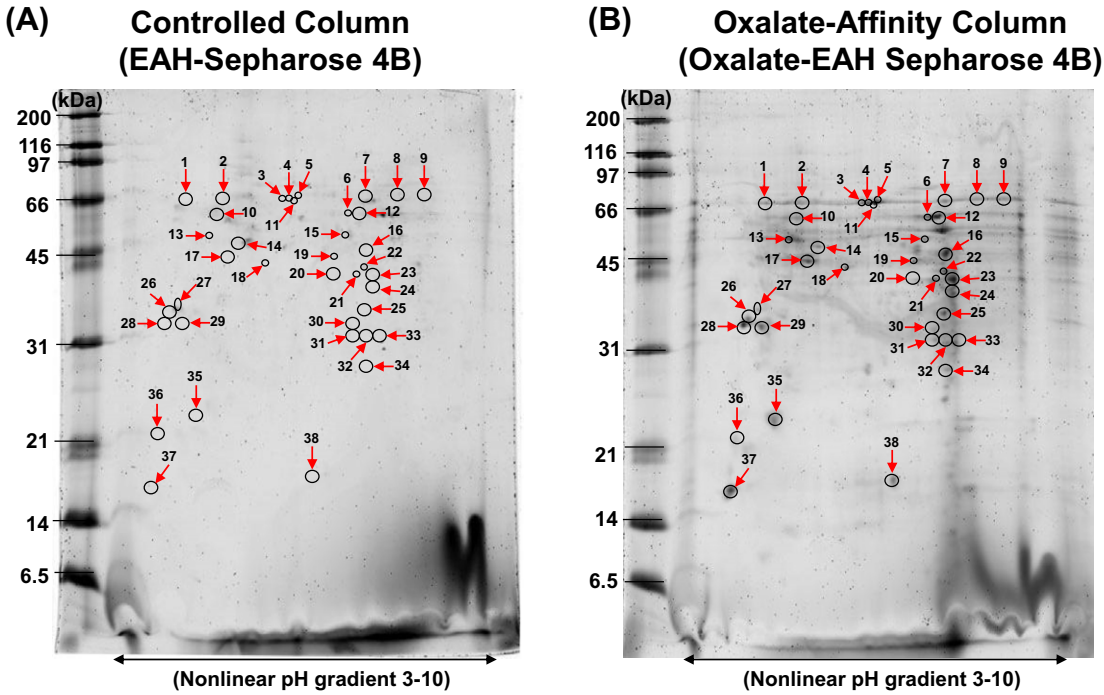

Fig. 1. 2-DE map of oxalate-binding proteins in porcine kidney. Porcine kidney proteins were passed through either controlled column containing EAH-Sepharose 4B beads (A) or oxalate-affinity column containing oxalate-conjugated EAH-Sepharose 4B beads (B). The eluate fractions from each column were pooled and the recovered proteins were resolved by 2-DE, and visualized by SYPRO Ruby staining. Protein spots that were present only in the sample derived from oxalate-affinity chromatographic column or had intensity levels > 3-fold as compared to those obtained from the controlled column were subjected to identification by Q-TOF MS and MS/MS analyses (see details in Table 1).

Table 1
Summary of oxalate-binding proteins in porcine kidney identified by Q-TOF MS and/or MS/MS analyses.

Spot No.	Protein name	NCBI ID	Identified by	Identification scores(MS, MS/MS)	%Cov (MS, MS/MS)	No. of matched peptides (MS, MS/MS)	p*I*	MW (kDa)
1	Albumin	gi|833798	MS, MS/MS	96, 24	20, 2	12, 1	5.92	71.36
2	Albumin	gi|833798	MS, MS/MS	92, 63	26, 6	13, 3	5.92	71.36
3	Albumin	gi|833798	MS, MS/MS	81, 77	22, 4	11, 2	5.92	71.36
4	Albumin	gi|833798	MS, MS/MS	123, 69	33, 4	15, 2	5.92	71.36
5	Albumin	gi|124257959	MS	105, NA	28, NA	14, NA	5.92	71.55
6	Catalase	gi|356460899	MS	114, NA	30, NA	12, NA	6.60	60.18
7	Heterogeneous nuclear ribonucleoprotein M	gi|158455026	MS	65, NA	32, NA	12, NA	9.18	58.37
8	Albumin	gi|833798	MS, MS/MS	103, 159	28, 4	14, 2	5.92	71.36
9	Albumin	gi|833798	MS	90, NA	24, NA	13, NA	5.92	71.36
10	Catalase	gi|356460899	MS	132, NA	36, NA	15, NA	6.60	60.18
11	Albumin	gi|833798	MS, MS/MS	141, 101	34, 6	16, 3	5.92	71.36
12	Catalase	gi|356460899	MS, MS/MS	125, 154	41, 13	14, 15	6.60	60.18
13	ATP synthase subunit beta, mitochondrial precursor	gi|54792127	MS	148, NA	43, NA	16, NA	5.19	56.32
14	Histone H2A.2	gi|31979	MS/MS	NA, 15	NA, 12	NA, 1	9.52	13.64
15	Adenylate kinase 7-like, partial	gi|149592814	MS	74, NA	15, NA	14, NA	7.09	136.97
16	60S ribosomal protein L32-like	gi|296188853	MS/MS	NA, 19	NA, 9	NA, 1	10.58	16.14
17	TNFAIP3-interacting protein 2 isoform 2	gi|239787094	MS	73, NA	34, NA	9, NA	6.12	37.17
18	Sp110 nuclear body protein isoform 6	gi|332815728	MS	78, NA	22, NA	12, NA	9.02	79.66
19	Aacyl-CoA dehydrogenase, mitochondrial	gi|113018	MS/MS	NA, 25	NA, 3	NA, 1	8.63	46.93
20	Fructose-1,6-bisphosphatase	gi|3288991	MS	103, NA	35, NA	11, NA	6.85	36.97
21	Coiled-coil domain-containing protein 40	gi|332263895	MS	74, NA	16, NA	14, NA	5.35	130.47
22	Mitochondrial NADH:ubiquinone oxidoreductase 51 kDa subunit	gi|263364	MS/MS	NA, 7	NA, 7	NA, 1	9.94	14.61
23	p21(CDKN1A)-activated kinase 7	gi|193787901	MS	81, NA	32, NA	11, NA	6.91	65.17
24	Mitochondrial malate dehydrogenase 2, NAD	gi|89574151	MS, MS/MS	109, 30	51, 4	10, 1	8.39	29.92
25	Fibrinogen betaB 1-118	gi|223130	MS/MS	NA, 70	NA, 11	NA, 1	6.17	12.89
26	Adenylosuccinate lyase, partial	gi|334347573	MS	74, NA	25, NA	9, NA	6.36	50.93
27	Transformation/transcription domain-associated protein	gi|344244337	MS	89, NA	11, NA	19, NA	8.30	228.20
28	Uncharacterized protein C3orf77-like	gi|332215705	MS	78, NA	13, NA	18, NA	8.17	192.59
29	Tissue-type plasminogen activator-like	gi|334312618	MS	95, NA	23, NA	11, NA	8.65	65.03
30	Tubulin polymerization-promoting protein family member 3-like isoform 1	gi|332227511	MS	81, NA	46, NA	8, NA	9.18	19.10
31	60S ribosomal protein L32-like	gi|296188853	MS/MS	NA, 19	NA, 9	NA, 1	10.58	16.14
32	CD164 antigen, sialomucin, isoform CRA_c	gi|119568736	MS/MS	NA, 21	NA, 22	NA, 1	12.00	7.27
33	60S ribosomal protein L32-like	gi|296188853	MS/MS	NA, 19	NA, 9	NA, 1	10.58	16.14
34	CD164 antigen, sialomucin, isoform CRA_c	gi|119568736	MS/MS	NA, 20	NA, 22	NA, 1	12.00	7.27
35	CD164 antigen, sialomucin, isoform CRA_c	gi|119568736	MS/MS	NA, 20	NA, 22	NA, 1	12.00	7.27
36	Retinitis pigmentosa GTPase regulator	gi|262050614	MS/MS	NA, 13	NA, 0	NA, 1	8.40	169.82
37	78 kDa glucose-regulated protein, partial	gi|829365	MS/MS	NA, 25	NA, 60	NA, 1	4.66	30.76
38	Transformation/transcription domain-associated protein isoform 2, partial	gi|380799023	MS	76, NA	9, NA	25, NA	8.60	380.25

NCBI = National Center for Biotechnology Information.
%Cov = %Sequence coverage [(number of the matched residues/total number of residues in the entire sequence) × 100%].
NA = Not applicable.

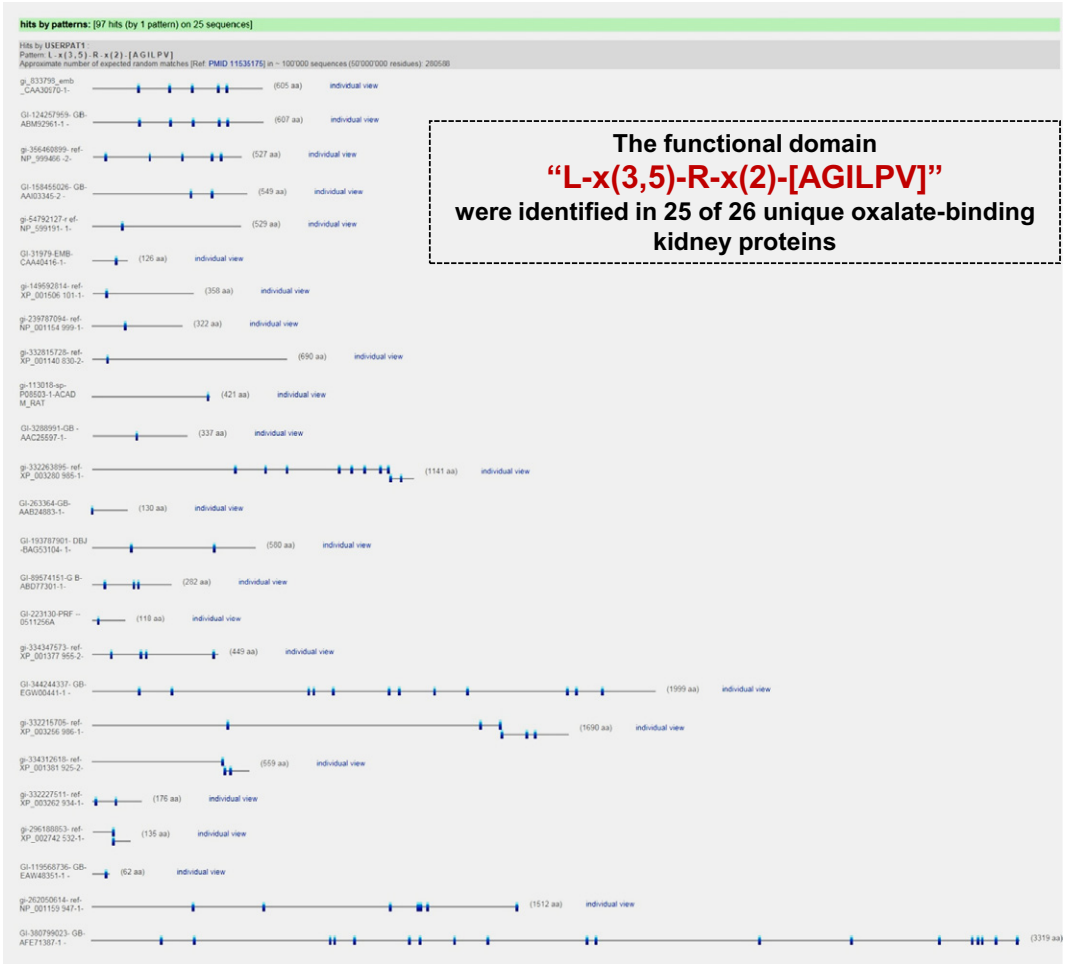

Fig. 2. Identification of oxalate-binding domain. The FASTA format of amino acid sequences of all the identified proteins listed in Table 1 were submitted to the PRATT search tool (http://www.ebi.ac.uk/Tools/pratt/) [15,16]. The data showed the domain "*L-x(3,5)-R-x(2)-[AGILPV]*" in the sequences of 25 of 26 unique proteins identified in Table 1. Among these, 15 had more than one oxalate-binding domain and the greatest number was found in transformation/transcription domain-associated protein isoform 2 (19 sites).

A number of oxidative stress regulatory proteins were identified as oxalate-binding proteins in our present study, including three isoforms of catalase (spots #6, 10, 12), mitochondrial NADH:ubiquinone oxidoreductase 51 kDa subunit (spot #22), and 78 kDa glucose-regulated protein (GRP78) (spot #37). Interestingly, catalase is responsible for catalyzing hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) [18]. There is evidence demonstrating that oxalate can induce renal cell injury by lipid peroxidation through reactive oxygen species such as superoxide anion, hydroxyl radicals and hydrogen peroxide. The accumulation of these free radicals is correlated with the decreased level of antioxidant enzymes including catalase, and catalase activity is decreased in rat kidney treated with oxalate [19]. Therefore, the decrease in level and activity of catalase might result to the reduced ability of this enzyme to bind to oxalate. *In vivo* binding of this enzyme to oxalate may be mediated by anti-oxidative processes to protect cell injury from oxidative stress.

Moreover, some metabolic enzymes were identified as oxalate-binding proteins in this study, e.g. malate dehydrogenase (spot #24). This enzyme has been proposed to bind with oxalate since its catalytic subunit binds with malate, which has a molecular structure similar to oxalate. Interestingly, malate dehydrogenase has been also identified in a previous study by Park, et al. [20] using an oxalate-affinity chromatography to purify malate synthase. These findings suggest that the ability of oxalate to bind with this enzyme may be due to the molecular mimicry of oxalate structure to the specific enzyme substrates.

Interestingly, we also identified many forms of albumin (spots #1–5, 8, 9, and 11) and three isoforms of CD164 (sialomucin) (spots #32, 34 and 35) as the oxalate-binding proteins in porcine kidney. Albumin is widely known as a sticky protein that can bind non-specifically to other proteins and various surfaces, whereas sialomucin has been reported as the secreted or membrane-associated mucin that can act as an adhesion receptor [21,22]. By their properties, it might be postulated that these two proteins could bind non-specifically to the oxalate-affinity column. However, the negative identification of the oxalate-binding domain in the non-oxalate-binding kidney proteins strengthened that albumin and sialomucin were really the oxalate-binding proteins based on the oxalate-binding domain found in their sequences.

In summary, several oxalate-binding proteins were identified from porcine kidney in our present study by a combination of oxalate-affinity purification and proteomics approach. Sequence analysis revealed that almost all of these identified proteins had the oxalate-binding domain "*L-x(3,5)-R-x(2)-[AGILPV]*". These data offer many opportunities for further investigations to address functional significance of these oxalate-binding proteins in renal physiology and pathogenic mechanisms of kidney stone disease.

Acknowledgments

This study was supported by Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative, and the Thailand Research Fund (RTA5380005 to VT). VT is also supported by "Chalermphrakiat" Grant, Faculty of Medicine Siriraj Hospital.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.015.

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Clin Chem Lab Med 2012;50(9):1697–1698 © 2012 by Walter de Gruyter • Berlin • Boston. DOI 10.1515/cclm-2012-0101

Letter to the Editor

Phosphate inhibits calcium oxalate crystal growth and crystallization through reducing free calcium ions: a morphological analysis and calcium consumption assay

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Keywords: calcium oxalate; crystal growth; inhibitor; nephrolithiasis; phosphate.

There are several lines of evidence demonstrating that phosphate serves as a calcium oxalate (CaOx) stone inhibitor, yet also promotes epitaxial deposition (or heterogeneous nucleation) of CaOx through precipitated calcium phosphate (CaP) crystals (1, 2). However, these dual modulatory effects of phosphate on CaOx crystals had been previously examined by indirect methods (e.g., spectrophotometric turbidimetry, dual constant composition). In the present study, we re-evaluated the modulatory effects of phosphate on CaOx crystals using a direct method (i.e., morphological study) to directly measure size, number and total mass of CaOx crystals upon exposure to various dosages of phosphate. Moreover, mechanism of phosphate action on CaOx crystals was addressed using a calcium consumption assay.

CaOx monohydrate (COM) crystals were prepared in the absence (control) or presence of phosphate (NaH_2PO_4) at various concentrations (0.5, 2.5, 4.0, 5.5 and 7.0 mM) (see also Supplemental data, Methods, which accompanies the article at http://www.degruyter.com/view/j/cclm.2012.50.issue-9/issue-files/cclm.2012.50.issue-9.xml). Note that physiologic concentrations of phosphate in the normal urine were 2.5–5.5 mM. After 1-h incubation, COM crystal morphology was examined under phase-contrast microscopy (CKX41, Olympus Co. Ltd., Tokyo, Japan). Quantitative analyses of COM crystal size (reflecting crystal growth), number (reflecting crystallization rate) and total mass (reflecting the final product of crystallization and growth) were performed using ImageMaster™ 2-D Platinum Software (GE Healthcare, Uppsala, Sweden), as described previously (3).

The data showed that the size, number and total mass of COM crystals were significantly reduced by phosphate treatment in a dose-dependent manner (Figure 1; see also Supplemental data, Figure S1). The degrees of inhibition were up to 62.5±1.3, 55.4±3.9, and 83.3±1.2% for crystal size, number and total mass, respectively. Note that phosphate did not alter pH of the solution (data not shown); hence, its inhibitory effect did not involve pH-induced changes in CaOx solubility. Precipitation of amorphous CaP crystals was observed in the samples treated with 2.5–7.0 mM phosphate; however, epitaxial deposition of CaOx on these precipitates was not detected (Figure 1).

To address the mechanism of action of phosphate on CaOx crystals, we performed a recently established calcium consumption assay (4). Briefly, 3 µL of the solution from COM crystallization reaction was mixed with 200 µL of Arsenazo III reagent (BioSystems S.A., Barcelona, Spain) to measure free calcium ions remained in the solution (see details of this assay and calculation in the Supplemental data, Methods). The data showed that all dosages of phosphate caused significant reduction (consumption) of free calcium ions in the crystallization solution (Supplemental data, Figure S2). This result was consistent to many clinical datasets demonstrating that phosphate therapy could lower urinary calcium levels in hypercalciuric patients; thus, reducing CaOx saturation state and the stone risk.

Taken together, our data revealed that phosphate significantly inhibited crystallization and growth of COM crystals, while epitaxial deposition of CaOx crystals was not detected in our present study. This direct evidence underscores the beneficial role of phosphate as a CaOx stone inhibitor and supports its use in clinical practice.

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Received February 16, 2012; accepted February 29, 2012; previously published online March 24, 2012

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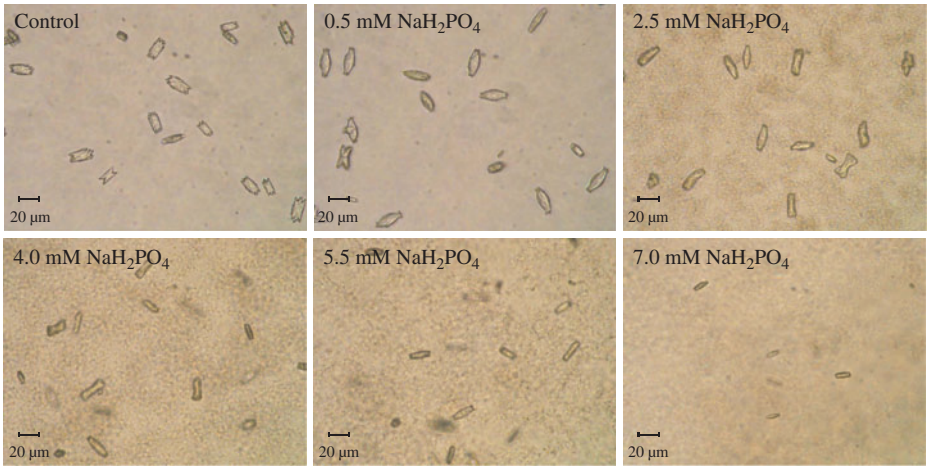

Figure 1 Morphological study of effects of phosphate on COM crystals.
COM crystallization was carried out using 5.0 mM $CaCl_2$ and 0.5 mM NaC_2O_4 in 10 mM Tris-HCl buffer containing 90 mM NaCl (pH 7.4), in the absence (control) or presence of phosphate (NaH_2PO_4) at various concentrations. Quantitative data and statistical analysis are presented in Supplementary Figure S1.

Acknowledgments

This study was supported by Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative, and The Thailand Research Fund (RTA5380005) to VT.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research funding played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.

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DOI: 10.1111/j.1365-2362.2012.02674.x

PERSPECTIVE

Implementation of proteomic biomarkers: making it work

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ABSTRACT

While large numbers of proteomic biomarkers have been described, they are generally not implemented in medical practice. We have investigated the reasons for this shortcoming, focusing on hurdles downstream of biomarker verification, and describe major obstacles and possible solutions to ease valid biomarker implementation. Some of the problems lie in suboptimal biomarker discovery and validation, especially lack of validated platforms with well-described performance characteristics to support biomarker qualification. These issues have been acknowledged and are being addressed, raising the hope that valid biomarkers may start accumulating in the foreseeable future. However, successful biomarker discovery and qualification alone does not suffice for successful implementation. Additional challenges include, among others, limited access to appropriate specimens and insufficient funding, the need to validate new biomarker utility in interventional trials, and large communication gaps between the parties involved in implementation. To address this problem, we propose an implementation roadmap. The implementation effort needs to involve a wide variety of stakeholders (clinicians, statisticians, health economists, and representatives of patient groups, health insurance, pharmaceutical companies, biobanks, and regulatory agencies). Knowledgeable panels with adequate representation of all these stakeholders may facilitate biomarker evaluation and guide implementation for the specific context of use. This approach may avoid unwarranted delays or failure to implement potentially useful biomarkers, and may expedite meaningful contributions of the biomarker community to healthcare.

Keywords Biomarker, biomarker implementation, clinical proteomics, clinical studies, expert panel, proteomics.

Eur J Clin Invest 2012; 42 (9): 1027–1036

Introduction

Clinical proteomics is defined as proteome analysis intended to improve the medical practice, for example, in relation to diagnosis, prevention, prognosis or therapy. Its success should be judged from the conferred clinical impact after implementation of its findings in everyday practice. The last decade has been marked by significant technological advancements in proteomics, especially with regard to mass spectrometry and bioinformatic solutions for data analysis. Over 4000 manuscripts including the words 'clinical' and 'proteomics' were indexed in MEDLINE in the last decade. Multiple proteomic biomarkers have been described for a variety of diseases, and several biomarkers have shown added value over current disease-management approaches, based on validation studies (e.g. in chronic kidney disease [1–3], reviewed in [4,5]). Nevertheless, implementation of the results in medical practice appears to be scarce [6]. Despite the promising findings, the impact of clinical proteomics (and biomarkers in general) on clinical decision-making, patient management and welfare appears insufficient.

Much of the problem may still lie in suboptimal discovery and validation processes for proteomic and other highly touted biomarkers. Analytical validation must be done prior to even starting a study, and the performance characteristics of the platform must be known [7–11]. Empirical evidence has shown that even in the best-studied and most studied biomarkers from diverse fields beyond proteomics, initial expectations may be inflated, and true effects may be much smaller than originally believed [12,13]. As others have pointed out, a plethora of factors can before, during and after sample analysis complicate biomarker discovery and validation and lead to false discoveries [14]. Nevertheless, it is to be expected that, as these factors are more clearly recognised, discovery and validation processes might be improved to a point where they are no longer the main bottleneck to progress. Enhanced attention is already given to clinical proteomics workflows, with special emphasis on experimental design of biomarker discovery, standardisation of procedures, data analysis and interpretation of results [11,15–17]. Mandatory requirements for contributors have, to some degree, been adopted by scientific journals (e.g. http://www.mcponline.org/site/cpmeeting/cguidelines.pdf). Technological bottlenecks associated with the transformation of discoveries into potential clinical assay are being identified and addressed [18–21]. Hopefully, if these efforts and insights become systematically exploited and implemented, valid biomarkers may start accumulating in the foreseeable future, perhaps even at a rapid pace. However, even then, successful biomarker discovery and qualification alone does not suffice for successful clinical implementation. The objective of this article is to highlight the critical hurdles downstream of biomarker discovery and verification, and to suggest potential ways to overcome them.

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Challenges in transforming biomarker discoveries into clinical application

Frequently, the discovery of biomarkers is considered a successful endpoint of clinical proteomics. Discovery and publication is a prerequisite for biomarker development, but it must be transformed into the ultimate goal of this particular translational proteomics research: clinical application. While highly ranked publications can have a prompt and major personal impact on the involved scientists (e.g. increased funding and advancement of academic career), the actual implementation requires substantially more time and is associated with diverse, unforeseeable challenges, which can bring the process to a standstill. Many scientists appear unwilling to venture down this tortuous and uncertain path.

In addition, different categories of biomarkers exist, depending on their intended use, as defined, for example, by Khleif *et al.* [22]: A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention. At least, four different categories of biomarkers should be differentiated: (i) diagnostic biomarkers (early detection biomarkers, disease classification); (ii) predictive biomarkers (predict patients likely to respond to a specific agent, predict patients likely to have an adverse event to a specific agent); (iii) metabolism biomarkers (dose defining); and (iv) outcome biomarkers (forecast response, progression or recurrence).

Implementation requires demonstration of clinical validity and utility, and benefit for the patient [23,24]. This process is demanding in time, as well as clinical, scientific and financial resources, and generally requires large studies. These include the assessment of performance on introduction of the novel biomarker over and above routinely available information; randomised trials to test improvement in clinical outcomes by using the biomarker; and late implementation and dissemination studies to show that the biomarker was successfully applied in everyday practice, with improved outcomes in large populations and a concomitant decrease in the cost of care – or, at least, without a substantial increase [25]. Such implementation testing is likely to take years, often exceeding a decade, and continues even after the biomarker has been applied and used widely in the community.

Biomarkers are actively sought for the majority of diseases associated with major societal and economic burden in developed countries (e.g. dementia, renal and cardiovascular disease, and most malignancies). However, it may generally take many years to clearly demonstrate the value of incorporating these biomarkers in management decisions in randomised trials that are evaluated based on hard endpoints. Possible solutions with shorter time horizons include the use of surrogate endpoints and/or the analysis of biomarkers in already available collections of samples with known outcomes. Surrogate endpoints may sometimes be misleading [26]. Therefore, there is debate about whether clinical implementation should be based only on results from studies that assess hard endpoints, or whether lower-level evidence that can be assessed faster may suffice. An expedited approval and implementation process based on surrogate and/or retrospective evidence may carry the risk of introducing expensive, useless or even harmful tests [27]. On the other hand, withholding an apparently beneficial test may deny a benefit for patients. As a consequence, a decision needs to be made at an early point in time whether evidence based on surrogates will be acceptable: for example, whether there is the potential for major health gains by introducing a biomarker with only modest evidence to support its use, or whether a hard endpoint must be assessed before implementation.

Evaluation of biomarkers based on analysis of previously collected samples with known outcome can be useful for the preliminary assessment of predictive or diagnostic efficacy, and the efficient reclassification of participants into informative risk categories with different implications for preventive or therapeutic intervention. However, such studies may involve a selection bias and do not guarantee that the use of biomarkers would improve the clinical outcome.

Another major impediment to implementation is that scientists are generally not well informed about the required steps from initial discovery to translation into a clinically useful assay. In fact, a clear road map towards implementation does not exist and guidance is scarce. Furthermore, regulatory requirements, if existing and applicable, are generally unknown to most researchers. This uncertainty and lack of adequate knowledge, in combination with the aforementioned need for substantial efforts and funding to demonstrate clinical validity, utility and added value of biomarkers over current clinical standards through, in principle, large trials, generally bring the further development of discovery findings to a standstill.

Performance of biomarkers superior to that for current standards does not automatically result in actual clinical implementation for several additional reasons: (i) biomarkers, even if facilitating substantial improvements in patient assessment, may initially fall short in influencing patient management, owing to the lack of appropriate interventions; (ii) patients may not wish to know about their risk of disease, especially if there is no proven intervention available; (iii) beyond the value at the clinical level, a biomarker must prove its cost-effectiveness [17], and this may differ by country, healthcare system and study approach applied by health economists; and (iv) physicians may resist changing the *status quo* in daily clinical practice, especially if this change is associated with personal financial consequences (procedures profitable for the physicians are not likely to be replaced).

Substantial uncertainty exists about the road towards implementation of biomarkers. There are multiple, interrelated steps where a plethora of different parties are involved, including researchers, clinicians, healthcare providers, funding and regulatory agencies, legislative, educative, and health insurance bodies, industry and patient groups. Each of these groups views the implementation from a different and unique angle, and cross-communication is often challenging. This results in fragmentation and severe gaps in the flow of information that may impede the clinical translation of research advancements.

A real-life example

The following typical scenario exemplifies how various challenges may create interactive hurdles for implementation of properly qualified biomarkers. Early detection of diabetic nephropathy, followed by appropriate therapy, is expected to prevent progression to advanced renal disease [28,29], improve quality of life and life expectancy of patients, and reduce the societal economic burden. Considering that biomarkers have been discovered and that associations of specific proteins or protein patterns have been validated in blinded studies (e.g. [2]), the next step would be to further validate these markers in prospective clinical trials. Of paramount importance are the definition of the endpoint(s) (in this case, detection of progression of chronic kidney disease – for example, end-stage renal disease with need for renal replacement therapy) and proof of an at least incremental superiority to existing diagnostic standards (e.g. assessment of albuminuria). The ideal study for such a chronic, slowly progressive disease with a substantial proportion of diabetic patients not affected requires the prospective collection of samples over many years in a large population for the accumulation of sufficient data to evaluate a hard endpoint. This long time-frame is a major setback for everybody. To exemplify the challenge, several of the major trials in the area are listed in Table 1. In most trials, only surrogate endpoints based on albuminuria were assessed, and, for several of the trials, hard endpoints for chronic kidney disease (doubling of serum creatinine concentration or end-stage renal disease) were not reported. Unfortunately, collection of follow-up data to assess hard endpoints at a later point in time was generally not foreseen. Apparent benefit of intervention based on a surrogate parameter (reduction of albuminuria) has been demonstrated in currently manifested stages of disease [28,29], but one needs to extrapolate whether a benefit would apply also to earlier stages. Patients with diabetes may be reluctant to be informed

Table 1 Duration, demographic parameters and outcome of major trials testing early intervention in diabetic nephropathy

	DIRECT I	DIRECT II	HOPE	BENEDICT	ADVANCE	ROADMAP
Treated (*N*)	1662	951	1808	601	5569	2232
Placebo (*N*)	1664	954	1769	603	5571	2215
Age (years)	31	57	65	62	66	57·7
Diabetes type	Type I	Type II	Type II	Type II	Type II	Type II
Diabetes duration (years)	9·1	8·8	11	8	8	6
BP (Systol/diastol)	117/73	133/78	142/80	151/88	145/81	136/81
Active treatment	Candesartan	Candesartan	Ramipril	Trandolapril	Perindopril/Indapamide	Olmesartan
Duration of treatment (years)	4·7	4·7	4·5	3·6	4·3	3·2
Incidence of new microalbuminuria (%)	5·0/5·0	12·0/13·0	33/38	5·8/11	19·6/23·6	8·2/9·8
Doubling of Screa (*N*)	NA	NA	NA	NA	55/45	23/23
ESRD (*N*)	NA	NA	10/8	NA	25/21	0/0
Death (*N*)	14/13	37/35	196/248	12	408/471	26/15
Rate of death (%/year)	0·17	0·80	2·76	0·28	1·83	0·29
Rate of onset ESRD (%/year)	NA	NA	0·11	NA	0·10	0·00
Rate of doubling of Screa (%/year)	NA	NA	NA	NA	0·21	0·43

BP, blood pressure; Screa, serum creatinine; ESRD, end stage renal disease; NA, not accessible.
Data reported were extracted from DIRECT I [41], DIRECT II [42,43], HOPE [44], BENEDICT [28], ADVANCE [45] and ROADMAP [29]. While most trials demonstrated a positive effect of intervention when assessing a surrogate parameter, albuminuria, a benefit based on hard endpoints was generally not demonstrated and was frequently not even assessed. The events are shown as number of events or percentage, as appropriate, in the active treatment/control arm.

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about early signs of kidney disease (e.g. years before recurrent microalbuminuria), given that many current therapeutic options are not proven to be effective at that stage, while potentially more effective intervention measures are still under development. We are hence faced with a fundamental question: Shall we implement such biomarkers now and employ current intervention strategies based on the assumption they will bring a significant benefit at early stages of disease, or shall we, prior to implementation, investigate whether the intervention strategies bring a significant benefit, either based on surrogate endpoints (e.g. albuminuria) or on hard endpoints? Thus, while prevention of diabetic nephropathy (or any other disease) is a worthy goal, the implementation path is far less clear.

Given these considerations, one may reflect that perhaps research in these specific areas should not be even initiated, because it will not result in any tangible impact on the current situation; or that the current situation should be altered in a way that positive results from research have a realistic chance to be implemented to improve the current clinical status. The latter option is certainly preferable, but the goals need to be rigorously defined, and critical issues must be clearly identified to advance.

An agenda to facilitate implementation of valid biomarkers

The implementation problem is gaining increasing recognition, and actions towards improving the situation have been initiated: the substantial financial need to support biomarker validation and qualification studies has been acknowledged by funding agencies – prominent examples are the recent EU FP7 calls for proposals for collaborative projects (http://ec.europa.eu/research/participants/portal/page/fp7_calls [30]). Additional examples include the Joint Programming Initiative in Neurodegenerative Diseases, which is funding the first pilot call for research projects in 'Optimisation of biomarkers and harmonisation of their use between clinical centres'; and the new ERA-Net TRANSCAN, which has proposed the topic 'Validation of biomarkers for personalised cancer medicine'. In the aforementioned example of diabetic nephropathy, a clinical trial (PRIORITY; FP7 2012–2016) is being launched, exploring the potential benefit of intervention on early diabetic kidney injury, based on a panel of urinary protein biomarkers [31]; and the 'Early Prevention of Diabetes Complications in Europe' (e-PREDICE) was also funded to investigate changes in biomarkers for microvascular damage, endothelial function, oxidation and inflammation, conferred as a result of different drug treatments designed for the early prevention of diabetic complications. Similar efforts are also underway in many other fields and in other countries, for example, in the USA, the Early Detection Research Network of the National Cancer Institute (http://edrn.nci.nih.gov) [32]. These are certainly major advances, shifting the emphasis from biomarker discovery to clinical application. Nevertheless, the implementation process, as a whole, still appears to be substantially under-funded.

Aiming to facilitate successful implementation of research findings in medical practice, the European Medical Research Council (EMRC) recently published the ESF/EMRC 'forward look' (http://www.esf.org/emrc; May 2011), describing the different hurdles in the process. To spear-head an implementation road map and identify the special turns it must take in the case of proteomics findings, the European Kidney and Urine proteomics COST Action, during its regular meeting (Madrid 2011), organised a session on clinical implementation of research findings pertinent to kidney diseases. In this meeting, researchers, clinicians and representatives from biobanks, industry, funding and regulatory agencies were invited to present their views of the implementation process. Jointly, these initiatives showed that biomarker research should adhere to a much more organised format, taking into consideration the needs and perspectives of the whole spectrum of involved parties: from scientists in the discovery laboratory to end-users (patients, physicians), including regulatory bodies. We suggest the following steps to facilitate implementation of clinical proteomics findings (Fig. 1):

- Perform initial discovery and validation for the specific context of use. If positive;
- Approach a suitable multidisciplinary panel (described in detail below) to evaluate evidence, and if positive, to provide guidance for further study design;
- Apply for funding and, in parallel, request samples from biobanks, when available, or initiate new sample collection (considering the panel's recommendations);
- Perform biomarker evaluation;
- Approach the panel for evaluation of the additional data and, if positive, for guidance for clinical study design;
- Apply for funding and perform intervention study to evaluate expected benefit. Preferably, hard endpoints should be assessed, if they can be reached in a reasonable period of time. If this is not possible, and the biomarkers are considered to have potentially life-saving clinical potential, validated surrogate endpoints may be employed, mandating additional follow-up to assess the hard endpoints;
- Approach the panel for evaluation of the evidence from the intervention study. If positive;
- Implement in clinical practice – perhaps on a limited, conditional basis until information on hard endpoints is considered robust enough;