Proteomic changes in response to crystal formation in *Drosophila* Malpighian tubules

Vera Y. Chung [1*]

Rebecca Konietzny [2*]

Philip Charles[2]

Benedikt Kessler[2]

Roman Fischer[2]

Benjamin W. Turney [1]

[1] Oxford Stone Group, Department of Urology, Nuffield Department of Surgical Sciences, University of Oxford, OX3 9DU, UK

[2] Target Discovery Institute, Nuffield Department of Medicine, University of Oxford, OX3 7FZ, UK

*shared first author

Keywords:
Drosophila, Malpighian tubules, stone, proteome, mass spectrometry, gel assisted sample preparation

Abstract:

Kidney stone disease is a major health burden with a complex and poorly understood pathophysiology. *Drosophila* Malpighian tubules have been shown to resemble human renal tubules in their physiological function. Herein, we have used *Drosophila* as a model to study the proteomic response to crystal formation induced by dietary manipulation in Malpighian tubules. Wild-type male flies were reared in parallel groups on standard medium supplemented with lithogenic agents: control, Sodium Oxalate (NaOx) and Ethylene Glycol (EG). Malpighian tubules were dissected after two weeks to visualize crystals with polarized light microscopy. The parallel group was dissected for protein extraction. A new method of Gel Assisted Sample Preparation (GASP) was used for protein extraction. Differentially abundant proteins ($p<0.05$) were identified by label-free quantitative proteomic analysis in flies fed with NaOx and EG diet compared with control. Their molecular functions were

further screened for transmembrane ion transporter, calcium or zinc ion binder. Among these, 11 candidate proteins were shortlisted in NaOx diet and 16 proteins in EG diet. We concluded that GASP is a proteomic sample preparation method that can be applied to individual *Drosophila* Malpighian tubules. Our results may further increase the understanding of the pathophysiology of human kidney stone disease.

Introduction:

Over the last century, with the increase in prosperity and availability of nutritious food, the incidence of renal stones has progressively increased worldwide.[1] The lifetime prevalence of nephrolithiasis is between 5% and 12% in developed countries such as United States and most European states.[2] In developing countries, the incidence of stone disease is also increasing in particular as a consequence of hot climate in some geographical regions.[3] The incidence of stone disease leads to increased financial burden in the diagnosis and treatment. Despite the advances in minimally invasive surgery in recent decades, little is known as to the fundamental molecular pathophysiology of kidney stone disease. The quest for effective medical treatment and prevention is ongoing.

Previous pilot studies have suggested that *Drosophila* could be used as a cost-effective model for kidney stone disease.[4] The advantages of *Drosophila* over other animal models include similar anatomical structure and philological function of Malpighian tubule to human renal tubules, brief life cycle and the ease and low cost of rearing.[5-8] *Drosophila* has also been reported to reliably develop calcium oxalate (CaOx) crystals with dietary supplements of stone-forming agents.[4] The simplicity and transparent nature of Malpighian tubules allows for direct observation and quantification of crystals, which is not possible in other animal models.

To better understand the underlying biological process of human kidney stone disease, it would be helpful to know how drosophila proteomic profiles are regulated secondary to crystal formation. The objective of this study is to assess the proteomic changes in response to crystal formation in *Drosophila* Malpighian tubules, using gel-aided sample preparation (GASP)[9] combined with nano-liquid chromatography tandem mass spectrometry (nLC-MS/MS) analysis.

Results:

Inducing crystallization in Malpighian tubules

Crystal formation in the Malpighian tubule of male Canton S flies was observed as early as one week of ingestion of lithogenic diets, including either sodium oxalate (NaOx) or ethylene glycol (EG). The control group had a basal incidence of crystal formation around 20%. The overall rate of crystal formation was significantly higher in flies fed with NaOx and EG diets, 73 % and 84 % respectively (Table 1).

The birefringent crystals were most often observed in the distal segment of the Malpighian tubules. No crystals were observed in the hindgut. Fig 1 shows the different morphology of crystals in three experimental conditions. The crystal composition has been shown to be predominantly CaOx monohydrate or CaOx dihydrate by energy-disperse X-ray spectroscopy and scanning electron microscopy in previous studies [4,10].

Qualitative analysis of detected proteins in three biological replicates in Mascot analysis

Initially, the MASCOT (v2.5.1, Matrix Science) search engine was used to interrogate the Malpighian tubule proteome in order to generate a comprehensive list of all detected proteins in three experimental conditions. Only proteins common in the three biological replicates were further analyzed in sub-categories. We then used LC-Progenesis IQ software as an individual analysis to identify differentially abundant ($p<0.05$) proteins in control as compared to treated flies and to quantify their fold changes. These proteins identified from MASCOT and Progenesis were screened against Flybase for their molecular functions. We further narrowed down the functionally significant proteins by choosing those functioning as transmembrane ion transporters, calcium or zinc ion binders, which might be relevant in crystal formation.

In the initial experiment using gel-aided sample preparation (GASP), we identified a total of 748 proteins from the Malpighian tubules and hindgut of one fly compared to 859 proteins from five flies (Supplementary Fig 1) from a nLC-MS/MS one hour gradient. In addition, we used a pool of five flies and nLC-MS/MS analysis with an optimized two hour gradient, in which more proteins (1217 proteins present in all biological replicates of the control group, 1142 in sodium oxalate group and 983 in ethylene glycol group) were detected in all samples (MASCOT) (Fig 2a-c). The biological triplicates showed close clustering as shown in the Principal Component Analysis (Supplementary Fig 2). We then compared the common proteins found in the biological triplicates of the three dietary conditions (Fig 2d).

All proteins identified by Mascot analysis were searched in Flybase database for their molecular functions. Candidate proteins which function as transmembrane ion transporters, calcium, zinc or magnesium ion binders that may play a role in crystal formation were shortlisted. Among the 57 proteins which were common to the NaOx and EG groups, only Vha100-1 fulfills this criteria whereas the other 56 proteins do not. Vha100-1(protein accession number: Q6NLA3; Flybase ID: FBgn0028671) functions as proton transporting ATPase which might be of interest in CaOx crystal formation (Fig 2d).

Label-free quantitation of proteins identified in Malpighian tubules of Drosophila

In total 1982 proteins were identified by nLC-MS/MS and Mascot/Progenesis analysis. Using a cutoff of $p<0.05$, 211 proteins were differentially synthesized between NaOx and control groups (Fig 3a, Supplementary Table 1), and 314 proteins between EG and control groups (Fig 3b, Supplementary Table 2). Molecular functions were analyzed and categorized by PANTHER Classification System (Protein ANalysis THrough Evolutionary Relationships, version 10.0) (Fig 4a and 4b). Among those differentially abundant proteins, their molecular functions were screened for transmembrane ion transporter, calcium, zinc or magnesium binders that could be relevant in crystal pathogenesis. 11 candidate proteins were selected in NaOx group and 16 in EG group (Table 2a and 2b). Positive fold changes indicated up regulation compared with control and negative fold changes indicated down regulation.

Discussion:

Kidney stone disease is a major health problem worldwide but the underlying pathophysiology remain largely unknown and effective medical treatment is scarce. Using the *Drosophila* as a model for kidney stone disease, we have revealed a comprehensive profile of altered proteome in response to induced crystal formation in Malpighian tubules. To our knowledge, this is the first study to identify the possible candidate proteins that may play an important role in tubular crystal formation.

The recently described Gel-aided sample preparation (GASP) method[9] has been applied for the first time on *Drosophila* Malpighian tubules. The GASP method is very reliable especially with low protein amounts compared to the known in-gel, in-solution digestion methods in mass spectrometry. In our hands the GASP protocol, shows more consistency with biological replicates due to lesser samples loss during preparation. Using the GASP method, we were able to identify similar number of proteins from five *drosophila* Malpighian tubules and ten

tubules (859 compared to 867, respectively, on a one hour gradient). The sensitivity of the GASP method for 1, 5 and 10 tubules are shown in Supplementary Fig 1.

GASP shows high reproducibility of biological replicates and gave more protein identifications in our hands than the in-solution method (data not shown). As GASP has been previously optimized to be scalable, ease of use and sensitivity, it appears to be well suited for low abundant samples that require a flexible application to other body parts and/or fluids of flies and will have a significant impact of future applications in fly research.

The chosen label-free method for Mass Spectrometry relies on a good alignment of the chromatograms for quantitation but is more time and cost effective compared to a SILAC approach.[11]

Among the 57 proteins present in both NaOx and EG group but not in control group in MASCOT analysis, Vha100-1 should be pointed out as an interesting candidate protein. Vha100-1 which is known to be involved in the pathogenesis of renal tubular acidosis and calcium homeostasis, both being recognized pathways of calcium crystal formation. None of the other 56 candidate proteins identified have previously been shown to have a role in recognized pathways of calcium crystal formation.

Vha100-1 stands for vacuolar H+ ATPase 100kD subunit 1, which is one of the many isoforms of V-type proton transporting ATPase, mainly involved in reduction of intracellular pH and ATP hydrolysis coupled proton transport.[12] The human ortholog of Vha100-1 is Hsap\ATP6V0A4. Located on chromosome 7q33-34, Hsap\ATP6V0A4 is known to play a key role in the pathogenesis of renal tubular acidosis. The homozygous mutation of ATP6V0A4 results in distal renal tubular acidosis, which is a hereditary disorder of renal stone disease.[13] The kidney fails to produce an appropriately acid urine in the presence of systemic metabolic acidosis, due to failure of hydrogen ion secretion or bicarbonate reabsorption in the distal nephron. This results in hyperchloremic metabolic acidosis and is usually accompanied by nephrocalcinosis or nephrolithiasis.[14] Heterozygous mutation of V-ATPase B1 subunit in human has been shown to cause incomplete distal renal tubular acidosis.[15] Vha100-1 is also involved in the process of calmodulin binding, a calcium-binding proteins with many roles, indicating Vha100-1 may have a modulating effect in calcium metabolism and calcium related metabolic pathways. It has been reported that insect calcitonin-like diuretic peptides stimulated V-ATPase activity in *Drosophila* Malpighian tubules resulting urine acidification hence crystallization.[16]

V-ATPase plays a central role in many aspects of cellular function in the kidney as well in other organs.[17,18] These range from proton secretion in order to acidify the extracellular milieu, to intracellular acidification of vesicles and regulation of a variety of processes that range from lysosomal degradation, ligand receptor dissociation and intracellular

trafficking.[19] The holoenzyme is comprised of many different subunits, each having different functions that are involved not only in the activity of the enzyme but also to its intracellular regulation and targeting. It might be an important agent in the common pathways in kidney crystal pathogenesis as several other V-ATPase subunits has been identified in the Progenesis analysis as well. We have observed both Vha68-2 (Vacuolar H^+ ATPase 68kDa subunit 2) and VhaSFD (Vacuolar H^+ ATPase SFD subunit) were up-regulated in NaOx group.

Among the differentially abundant proteins, we have identified 11 proteins in NaOx groups and 16 proteins in EG group that function as transmembrane ion transporters, calcium, zinc or magnesium ion binders. We made the hypothesis these groups of proteins are more important in the process of crystal formation based on previous published work.[20-23] We have previously looked into a different cohort of flies for the corresponding transcriptive changes (data not shown), but the candidate proteins cannot be correlated at the mRNA level.

A recent study using the *Drosophila* model suggested that zinc and possibly magnesium had an essential role in driving heterogeneous nucleation that eventually results in crystal formation.[24] Chi et al proposed that zinc facilitates calcification given the presence of non-trace levels of zinc in fly crystals, human xanthine stones and CaOx plaques. By inhibiting zinc transporter genes a suppression of *Drosophila* crystals formation were observed. In this study, numerous zinc binding proteins were differentially abundant in both NaOx and EG diets. Although the particle structure and mechanism of crystallization reported by Chi et al are different from the CaOx crystals induced in the current experiment, our results also support the hypothesis that zinc pathways may be important in crystal formation.

JYalpha, as well as nervana 1, are proteins that function as sodium: potassium exchanging ATPase. We found this group of protein of interest because sodium and potassium transport has been implicated in calcium ion transport across renal tubules and hemostasis.[25] It was shown in rat and rabbit model that the basal-lateral plasma membrane contain sodium/calcium exchange system which mediates the counter-transport of calcium and sodium across the basal cell border.[26] Decreased Na+/K+ ATPase activity were described in idiopathic hypercalciuria.[27] This transporter might be differentially synthesized as a result of CaOx crystal formation.

The altered proteome probably represents changes at a cellular level rather than in the extracellular environment. These differentially abundant proteins could be directly involved in CaOx formation or could be a regulated response to the obstructive uropathy, metabolic acidosis and failure to maintain electrolyte or acid-base balance in Malpighian tubules. A significant proportion of detected proteomic changes such as those with binding, catalytic and enzyme regulator activity could as well be the result of inflammatory responses or cellular adaptation to the lithogenic agents or crystal formation.

During the screening of candidate proteins, we made the hypothesis that those functioning as transmembrane ion transporter, calcium or zinc binding proteins might be more critical in crystal formation but other proteins might also be relevant. More studies will be desirable to elucidate the biological process of how these differentially abundant proteins function as a whole in the process of CaOx crystal formation. Though previous researchers [4,10] have shown the crystals inside the Malpighian tubules to be CaOx, there was the possibility given that the relative elemental composition was altered during prolonged feeding. One of the other limitation of the current study is the lack of functional validation of the candidate proteins. Further work will be needed to evaluate if changing the expression of candidate proteins will have any impact on the crystal formation process. Assisted by powerful transgenic resources, these proteins could be targeted to allow manipulation of stone formation and develop therapeutic agents in human kidney stone disease in the future.

To conclude, our study has applied a new proteomic sample preparation method GASP on *Drosophila* model to narrow down the potential protein targets involved in human stone pathogenesis. These results may further increase the understanding of the pathophysiology of human kidney stone disease.

Material and methods:

***Drosophila* stocks:**

Wild-type (Canton S) *D. melanogaster* were used. Male flies were selected and maintained in plastic vials containing standard medium (maize 18.6%, yeast 3.8%, soya 2.2%, agar 3.1% and water 72.3%) in a 25^{o}C, 50-60% humidity incubator.

Lithogenic agents:

Different concentrations of crystal forming agents at a physiological dose were added into standard fly medium. Three conditions were prepared: control (standard fly medium), 0.05% sodium oxalate and 0.5% Ethylene Glycol. Biological triplicates were raised in each condition.

Fly maintenance:

Figure 5: Workflow for fly maintenance

New emergent flies were collected from eclosion under light anesthesia. Flies were housed at a density of 25 flies per vial. At day three male flies were randomly divided into three diet groups (control, NaOx, EG) and kept on these diets for two weeks. Every third day the male flies were transferred into fresh medium. The proteomic study contains five male flies for each diet group in biological triplicates of Malpighian tubules and hindgut (Figure 5).

Malpighian tubule preparation:

After CO_2 anesthesia the excretory organs (Malpighian tubules and hindgut) were dissected in Schneider's *Drosophila* medium. Freshly dissected tubules and hindgut from 15 flies were prepared for polarized light microscopy examination. Groups were dissected in parallel and processed for protein extraction with 5 flies pooled from each biological triplicate and analyzed separately.

Birefringence microscopy:

Malpighian tubules and hindgut were prepared fresh on SuperFrost slides and observed immediately under normal and polarized white light with an Olympus BX60 microscope. The tubules with crystal formation were photographed and scaled.

Proteomic sample preparation and quality control:

For protein extraction, Malpighian tubules and hindgut samples were homogenized by tissue grinder (4 minutes) and vortexing (2 minutes) respectively in 180µl of cocktail buffer consisting of 7M Urea, 2M Thiourea, 4% CHAPS and 0.2% Triton X-100 with freshly added 10µl of 1M tris (2-carboxyethyl) phosphine (TCEP) and 10µl of 1M iodoacetamide (IAA) and incubated for 30min at room temperature. During method development we compared the Gel-aided sample preparation (GASP) (6) to an in-solution digestion protocol on one Malpighian tubules and hindgut. Briefly, the steps for the GASP protocol includes (i) protein extraction, (ii) co-polymerization of proteins with monomeric acrylamide, (iii) shredding of gel, (iv) depletion of small molecules, (v) proteolysis, and (vi) peptide recovery. The other steps for Gel-aided sample preparation (GASP), an in house developed trypsin digestion and peptide extraction procedure, were performed according to the protocol described elsewhere (6). Alternatively, for the in-solution protocol, samples were subjected to a Methanol/Chloroform extraction.[28] The precipitated protein pellet was resuspended in 6M Urea and diluted to 2M Urea prior to the addition of trypsin as described (6). Digested samples were analyzed on a Dionex Ultimate 3000 UPLC coupled to a hybrid quadrupole-

orbitrap instrument (Q Exactive, Thermo Scientific).[29] Samples were desalted online (PepMAP C18, 300µm x5mm, 5 µm particle, Thermo) for 1 minute at a flow rate of 20 µl/min and separated on a nEASY column (PepMAP C18, 75 µm x 500mm, 2 µm particle, Thermo) over 120 Minutes using a gradient of 2%-35% Acetonitrile in 5% DMSO with 0.1% Formic acid at 250nl/min. Scans were acquired at a resolution of 70,000 @ 200m/z and the 15 most abundant precursors were selected for HCD fragmentation.

Mass spectrometry data analysis:

Peak list files were generated with MSConvert (Proteowizard v3.0.5211) using the 200 most abundant peaks/spectrum. The SwissProt *D. melanogaster* reference proteome (retrieved 30/03/2015) (21,361 sequences; 14,358,849 residues) database was used for searches in Mascot (v2.5.1, Matrix Science), PEAKS (v7, Bioinformatics Solutions). Searches were performed with fixed modification for Propionamide (C) and variable modifications for Oxidation (M) and Deamidated (N). A mass deviation of 10 ppm for MS1 and 0.05 Da for MS2 spectra were applied for all analysis. A decoy database search was implemented in order to get a probability score threshold regarding search engines and a general false discovery rate of 1% was set. Raw data were individually searched in Mascot or Peaks. All proteins were searched against Flybase[30] to investigate reported molecular function. Ion transporters, water channels and calcium dependent proteins were short listed and compared to the results from Progenesis (v2.0.5556.29015). For label-free quantification raw data were submitted to Progenesis.

Acknowledgement:

We appreciate the help of Dr. Markus Toegel, Dr. Tudor Fulga from Weatherall Institute of Molecular Medicine, University of Oxford for the generous support during the experiments. We also appreciate the help of Mr. Mark Sullivan for reviewing the manuscript.

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Figure 1. *Crystal formation in Malpighian tubules 10-40x magnification.* The control diet showed the normal structure of a pair of Malpighian tubules joining into a single ureter, which further secretes into hindgut. The crystal morphology was small and extensive in the NaOx treatment group. In contrast (ethylene glycol) EG enriched diet induced crystals which were bigger, poly-angular with a jewel-like gloss.

Figure 2A-D. *Number of Malpighian tubule proteins identified by mass spectrometry using a two hour LC gradient and MASCOT analysis*. We identified 1217 proteins present in all biological replicates in the control group (A), 1142 in sodium oxalate (NaOx) group (B) and 983 in ethylene glycol (EG) group (C), respectively. Venn diagram comparing the proteins identified by MASCOT analysis in three experimental conditions (D). Comparing 1142 proteins in NaOx group, 984 in EG group and 1217 in control groups, the majority of proteins identified (792) were common to the three dietary conditions. Among the 57 proteins common to both experimental conditions (NaOx and EG), Vha100-1 was one interesting candidate proteins probably related to CaOx crystal formation.

Figure 3A-B. *Volcano plot of differentially expressed proteins in Malpighian tubules after treatment.* Volcano plots of protein fold changes for control versus sodium oxalate (A) and versus ethylene glycol (B). The magnitude of the change is plotted on the x-axis (log2 of diet versus control) against the significance of the change (-log10 of Progenesis p-values, which are equivalent to a two-tailed Student's t-test of arcsinh-transformed normalised protein abundances) on the y-axis. Proteins were $p<=0.05$ are highlighted in dark circles.

Figure 4A-B. *211 proteins were differentially expressed between NaOx and control groups (A); 314 proteins between EG and control groups (B).* Molecular function were analyzed and categorized by PANTHER Classification System (Protein ANalysis THrough Evolutionary Relationships, version 10.0). The functional categories were similar between NaOx and EG group, with proteins with catalytic activity being predominant.

Supplementary Figure 1. *Number of proteins identified in one fly versus five and ten flies by nLC-MS/MS using an one hour gradient.* Number of proteins identified by mass spectrometry using a one hour LC gradient and MASCOT analysis. We identified 748 proteins from one fly, 859 proteins from five and 867 proteins from 10 flies, respectively.

Supplementary Figure 2. *Principal components analysis (PCA) graph of three biological replicates*. Flies treated with NaOx (purple) or EG (orange) were compared to control (blue),

demonstrating a clear overall effect on the corresponding Drosophila Malpighian tubule proteomes.