Title: Peptidomic analysis of urine from youths with early type 1 diabetes reveals novel bioactivity of uromodulin peptides in vitro

Authors: Julie A. D. Van1,2*,†, Sergi Clotet-Freixas2, Joyce Zhou1, Ihor Batruch3, Chunxiang Sun4, Michael Glogauer4, Luca Rampoldi5,6, Yesmino Elia7, Farid H. Mahmud7, Etienne Sochett7, Eleftherios P. Diamandis3,8, James W. Scholey1,2,9, Ana Konvalinka1,2,9

Affiliations:

1Institute of Medical Science, University of Toronto, Toronto, Canada.

2Toronto General Hospital Research Institute, University Health Network, Toronto, Canada.

3Department of Laboratory Medicine and Pathobiology, Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, University of Toronto, Toronto, Canada.

4Faculty of Dentistry, University of Toronto, Toronto, Canada.

5Molecular Genetics of Renal Disorders Unit, Division of Genetics and Cell Biology, IRCCS San Raffaele Scientific Institute, Milan, Italy.

6Vita-Salute San Raffaele University, Milan, Italy.

7Hospital for Sick Children, Toronto, Canada.

8Department of Clinical Biochemistry, University Health Network, University of Toronto, Toronto, Canada.

9Department of Medicine, Division of Nephrology, University Health Network, Toronto, Canada.

Running title: Urinary peptidomics in early type 1 diabetes

Correspondence: Julie A. D. Van, University of Toronto, Institute of Medical Science, 1 King’s College Circle, Room MS4368, Toronto ON, M5S 1A8, Canada

Email: julie.van@mail.utoronto.ca; Phone: +1 416 978 6870; Fax: +1 416 978 8765
Urinary peptidomics in early type 1 diabetes

ABSTRACT

Chronic hyperglycemia is known to disrupt the proteolytic milieu, initiating compensatory and maladaptive pathways in the diabetic kidney. Such changes in intrarenal proteolysis are captured by the urinary peptidome. To elucidate the early kidney response to chronic hyperglycemia, we conducted a peptidomic investigation into urines from otherwise healthy youths with type 1 diabetes and their non-diabetic peers using unbiased and targeted mass spectrometry-based techniques. This cross-sectional study included two separate cohorts for the discovery (N = 30) and internal validation (N = 30) of differential peptide excretion. Peptide bioactivity was predicted using PeptideRanker and subsequently verified in vitro. Proteasix and the Nephroseq database were used to identify putative proteases responsible for peptide generation and examine their expression in diabetic nephropathy. A total of 6550 urinary peptides were identified in the discovery analysis. We chose to further examine a subset of 162 peptides that were quantified across all thirty samples. Of the 15 differentially excreted peptides (P < 0.05), seven derived from a C-terminal region (589SGSVIDQSRVLNLGPI607) of uromodulin, a kidney-specific protein. Increased excretion rates of five uromodulin peptides were replicated in the validation cohort using parallel reaction monitoring (P < 0.05). One of the validated peptides (SGSVIDQSRVLNLGPI) activated NFκB and AP-1 signalling, stimulated cytokine release, and enhanced neutrophil migration in vitro. In silico analyses highlighted several potential proteases such as hepsin, meprin A, and cathepsin B to be responsible for generating these peptides. In summary, we identified a urinary signature of uromodulin peptides associated with early type 1 diabetes before clinical manifestations of kidney disease and discovered novel bioactivity of uromodulin peptides in vitro. Our present findings lay the groundwork for future studies to validate peptide excretion in larger and broader populations, to investigate the role of bioactive uromodulin peptides in high glucose conditions, and to examine proteases that cleave uromodulin.

Keywords: diabetes, peptidomics, bioinformatics, urine, mass spectrometry, parallel reaction monitoring
# ABBREVIATIONS

| Abbreviation | Description |
|--------------|-------------|
| ACR          | Albumin/creatinine ratio |
| Ang          | Angiotensin |
| BH           | Benjamini-Hochberg |
| BMI          | Body mass index |
| DCCT         | Diabetes Control and Complications Trial |
| DMEM         | Dulbecco's Modified Eagle Medium |
| EDIC         | Epidemiology of Diabetes Intervention and Complication |
| eGFR         | Estimated glomerular filtration rate |
| ELISA        | Enzyme-linked immunosorbent assay |
| FDR          | False discovery rate |
| GPI          | Glycosylphosphatidylinositol |
| HbA1c        | Glycated hemoglobin A |
| HKUPP        | Human Kidney and Urine Proteome Project |
| HUPO         | Human Proteome Organization |
| IL           | Interleukin |
| LFQ          | Label-free quantitation |
| MS/MS        | Tandem mass spectrometry |
| SCX-HPLC     | Strong cation exchange high-performance liquid chromatography |
| TLR4         | Toll-like receptor 4 |
INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia. It arises from either an insufficient production of insulin as a result of an autoimmune attack on beta cells of the pancreas (referred to as type 1 diabetes) or insulin resistance as a result of decreased sensitivity (referred to as type 2 diabetes) (1). Type 1 diabetes typically emerges in childhood or adolescence; whereas type 2 diabetes typically appears later in life and is often complicated by hypertension, obesity, or cardiovascular disease at time of diagnosis (2). Despite differences in etiology, both types predispose individuals to serious complications that threaten survival and quality of life (3).

A common microvascular complication is diabetic kidney disease. It is in fact the leading cause of end-stage renal disease, accounting for 30-50% of incident cases in Canada and worldwide (4, 5). The current standard of care is geared toward managing diabetes and delaying the onset of renal disease. Landmark clinical trials in diabetes, namely the Diabetes Control and Complications Trial (DCCT) and the Epidemiology of Diabetes Intervention and Complications (EDIC), have demonstrated that intensive glycemic control significantly reduces the risk of kidney disease (6); however, maintaining “normal” blood glucose levels may not be attainable for most individuals with diabetes. The search for better personalized and targeted therapies has been under way for decades; however, the greatest hurdle is our limited understanding of the early mechanisms responsible for initiating diabetic kidney disease.

Omics approaches have previously been employed to fill these gaps in knowledge. In a review of the existing literature on urinary peptidomics in diabetes, we noted that the primary focus has been placed on later stages of diabetes, in which individuals with diabetes already present with some degree of impaired renal function (7). Furthermore, studies rarely extended their interpretation of differentially excreted peptides beyond their potential roles as biomarkers. Applying a mechanistic approach to urinary peptidomics may provide new insights into the proteolytic milieu of the diabetic kidney. This approach combines the global study of naturally occurring peptides with the pursuit of elucidating mechanisms underlying normal and disease states (8, 9). Peptides represent an intriguing class of analytes because they are generated through the action of proteases on protein precursors. Akin to a chemical reaction, changes
in peptide (product) levels reflect changes in protein (substrate) expression, proteolytic (enzyme) activity, or both. Furthermore, proteolysis of zymogens, prohormones, and other precursors is often required for protein maturation, activation, and regulation. Such is the case for angiotensinogen, which has no known bioactivity until it is cleaved by renin and subsequently angiotensin-converting enzyme to form the peptide hormone angiotensin (Ang) II: the principal regulator of blood pressure. As a result, peptides not only reflect proteolytic activity, but they may also act as potent bioactive mediators of disease.

Dysregulation of proteolytic activity has previously been linked to early compensatory and maladaptive changes in the diabetic kidney (10–12); although it is not fully understood. In this study, we aimed to elucidate the early kidney response to chronic hyperglycemia using urinary peptidomics. To do so, we carefully selected a population of otherwise healthy youths with type 1 diabetes and their non-diabetic peers as healthy controls, which enabled us to study the effect of chronic hyperglycemia in the diabetic kidney without potential confounding effects of comorbidities and medications. We identified a urinary signature of 15 differentially excreted peptides in the discovery cohort of thirty youths. Targeted methods using parallel reaction monitoring were developed and used to validate differential peptide excretion in a second cohort. We then employed in silico analyses to predict peptide bioactivity and to identify the potential proteases responsible for generating the peptides. Lastly, we demonstrated, for the first time to our knowledge, that a uromodulin peptide, whose excretion was elevated in those with diabetes, exhibited pro-inflammatory bioactivity.

EXPERIMENTAL PROCEDURES

Experimental Design and Statistical Rationale

This cross-sectional study included a discovery cohort and an internal validation cohort. Each cohort included 30 otherwise healthy youths with and without type 1 diabetes; each individual served as a biological replicate.
For the discovery phase, we performed power calculations to determine the appropriate total cohort size \((N = 30)\) required to achieve a study power of 80% using the independent two-sample \(t\)-test and Benjamini-Hochberg (BH) multiple testing correction with a false detection rate (FDR) of 0.0001 and an effect size \(d\) of 2. We thus obtained urine samples from 15 youths with type 1 diabetes and 15 healthy age- and sex-matched controls for the discovery phase.

For the validation phase, we performed power calculations based on the observed effect sizes from the discovery phase. To achieve a study power of 80% and an FDR of 0.05, we determined that the minimum number of samples to validate the top peptide candidates as determined by the BH adjustment was 9 per group (Supplemental Table S1). To match the discovery analysis, we collected thirty urine samples for the validation cohort.

A total of 60 second-morning midstream urine samples were collected from 60 youths. All power calculations were performed with G*Power software.

**Study Population**

All sixty youths were under 19 years of age; had no history of hypertension, persistent microalbuminuria, renal disease, macrovascular disease, and chronic inflammatory disease; and had never used corticosteroid, anti-inflammatory, or anti-hypertensive drugs. In this study, youths with type 1 diabetes mellitus were considered to be in the earliest and uncomplicated stage of the natural history of diabetic kidney disease. Youths with diabetes were recruited and initially screen from multiple diabetes clinics in the Greater Toronto Area (e.g., Hospital for Sick Children, Markham-Stouffville Hospital, Credit Valley Hospital, and Charles H. Best Centre); while youths without diabetes were either family members of youths with type 1 diabetes or were healthy volunteers recruited at the Hospital for Sick Children and Toronto General Hospital.

Relevant clinical characteristics such as age, sex, glycated hemoglobin (HbA1c), blood pressure, body mass index (BMI), urinary albumin/creatinine ratios (ACR), and estimated glomerular filtration rate (eGFR) were compared between groups. We converted blood pressure and BMI into z-scores to account
Urinary peptidomics in early type 1 diabetes

for age and sex differences in youths (13). eGFR was determined using the Larsson’s formula (eGFR = 77.24 × CysC⁻¹.2623). For the discovery cohort, clinical characteristics were measured at the time of study enrolment for both groups (Table 1) and at time of urine collection for youths with diabetes only (Supplemental Table S2). The mean time between the measurement of clinical characteristics and the urine collection was 2.7 ± 0.5 years and 2.5 ± 0.7 years for youths with diabetes and without diabetes, respectively. Groups were matched by age at time of urine collection and sex. For the validation cohort, we obtained clinical characteristics at or near the time of urine collection (Table 2). Groups were matched based on age. The research ethics boards at the Hospital for Sick Children and Mount Sinai Hospital approved this study.

Collection, Handling, and Storage of Urines

We followed the Standard Protocol for Urine Collection and Storage, created by the Human Kidney and Urine Proteome Project (HKUPP) and the Human Proteome Organization (HUPO) (14). Following collection, all fresh urine samples were immediately kept at 4°C until further processing. Urine samples were centrifuged at 1000 g for 10 minutes to remove intact cells and debris. The supernatants were then aliquoted in either 15- or 50-mL Falcon tubes as well as 1.5-mL Eppendorf tubes and stored at -80°C. This initial processing step was completed within 3 hours of urine collection to obviate the need for urine preservatives and protease inhibitors. Samples were de-identified and randomized so that investigators were blinded to experimental groups during sample processing.

Discovery Peptidomics

We employed previously published methods for the identification and quantification of the naturally occurring urine peptidome (15). Our workflow is summarized in Figure 1. All thirty urine samples were processed and analysed together in a single batch to avoid variability due to day-to-day sample preparation. Frozen samples were thawed, vortexed, and centrifuged at 1000 g for 10 minutes to remove any remaining cells and debris. Sample volumes were normalized to 90 μmol of creatinine to normalize for degree of hydration. After adjusting the pH to 8 using ammonium bicarbonate, we used Vivaspin Centrifugal
Urinary peptidomics in early type 1 diabetes

Concentrators (VivaProducts) with 10-kDa cut-off membranes to isolate urinary peptides. We added dithiothreitol to a final concentration of 2 mM and iodoacetamide to a final concentration of 4 mM to reduce and alkylate disulfide bonds. The peptides were then passed through solid-phase extraction using Oasis HLB cartridges (Waters Corporation), after adjusting pH to 4 with formic acid. To remove urinary pigments, we added ethyl acetate, vortexed and centrifuged the sample, and then discarded the supernatant. The extracted peptides were speed-vacuumed until the final sample volume was less than 200 µL and subsequently stored at -20°C until further processing.

Frozen peptides were thawed, topped off to 500 µL in strong cation exchange high-performance liquid chromatography (SCX-HPLC) running buffer (0.26 M formic acid in 5% acetonitrile), and immediately loaded onto a PolySULFOETHYL ATM column (The Nest Group, Inc.) containing a hydrophilic, anionic polymer (poly-2-sulfoethyl aspartamide). We ran a 1-hour SCX-HPLC fractionation method on a linear gradient using the running buffer with incremental increases in 1M ammonium formate as the elution buffer. We collected twelve fractions per sample at a rate of one fraction per two min after the start of the elution gradient. All fractions were stored at -20°C until the entire fractionation step for all 30 samples was completed. Overall, this fractionation step was carried out continuously for 2-3 weeks.

In order to reduce time per sample on the mass spectrometer, we selected, thawed, and pooled seven consecutive fractions, which covered the largest area of peptide abundance on the chromatogram. Pooled fractions were further desalted using Bond Elut OMIX C18 tips (Agilent Technologies), eluted in 5 µL of 65% acetonitrile, and diluted with 60 µL of 0.1% formic acid in pure mass spectrometry-grade water. After desalting, we immediately loaded 40 µL of each of the three pooled fractions per sample onto a 96-well plate in individual wells, from which 18 µL from each well were injected onto a 0.3 cm C18 pre-analytical column (IntegraFrit capillary, New Objective; inner diameter of 150 µm; 5 µm bead size; Agilent Pursuit C18, Agilent Technologies) and then loaded onto a C18 resolving analytical column with dimensions 15 cm x 75 µm ID (PicoTip emitter, 8 µm tip, New Objective Agilent Pursuit C18, 3 µm bead size). These columns were operated on an EASY-nLC1000 system (Thermo Fisher Scientific), coupled to a Q Exactive
Urinary peptidomics in early type 1 diabetes

Plus hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific) using a nano-electrospray ionization source. Each fraction ran on a 60-minute gradient in data-dependent acquisition mode with full MS1 scan from 400 – 1500 m/z with a resolution of 70,000 and subsequent MS2 scan of the top 12 parent ions with a resolution of 17,500. Xcalibur software (v. 3.0.63; Thermo Fisher Scientific) was utilized to generate RAW files of each run. Mass spectrometry data have been deposited onto the ProteomeXchange Consortium via the PRIDE (16) partner repository with the dataset identifier PXD012210 (http://www.ebi.ac.uk/pride/archive/login; username: reviewer85516@ebi.ac.uk; password: ht3PGbCg).

All mass spectrometry raw data were analyzed by MaxQuant software (version 1.5.3.28) and were searched against the human Uniprot FASTA database (version July 2016 with 42,158 protein entries) using the built-in Andromeda search engine (17). To study endogenous peptides, we selected the “unspecific” digestion mode. Cysteine carbamidomethylation was the only fixed modification. Variable modifications included methionine oxidation, proline oxidation, and N-terminal acetylation with a maximum number of 5 modifications per peptide. The false discovery rate was set to 1% for both proteins and peptides with a minimum length of 6 amino acids and was determined by searching a randomized database. Potential contaminants were allowed in the search. The initial peptide tolerance was set to 20 ppm against a small ‘human-first-search’ database. The main search peptide mass tolerance was 4.5 ppm, and the fragment mass MS/MS tolerance was set to 0.5 Da. Matching between runs was selected. Peptide intensities derived from extracted ion currents and were used as a measure of peptide quantification.

Peptidomic data analysis was done on Perseus software (version 1.5.5.3) (18). Non-human contaminants and reverse hits were manually checked and removed. We then filtered out peptides that were not quantified in all samples so that we could examine the most robust changes in peptide intensities between groups. Peptide intensities were log-transformed to approximate a normal distribution. Differential peptide intensities between groups were then determined using the independent t-test ($P < 0.05$), followed by the BH adjustment ($Q < 0.05$).

Targeted Peptidomics
Urinary peptidomics in early type 1 diabetes

We initially developed targeted methods for selected reaction monitoring to validate the observed differences in urinary peptide excretion (using Tier 2 analyses). The top seven transitions were chosen for each precursor on the basis of magnitude, order of intensity, co-elution, and peak shape. Linearity was determined for each of the detected crude heavy-labeled peptides (Supplemental Figure S1). We ultimately developed and performed a parallel reaction monitoring assay. Complete details about method development and optimization can be found in the Supplemental Methods. The master mix and list of transitions are summarized in Supplemental Table S3.

Overall, sample preparation of urine was similar to what was described for discovery peptidomics with some notable exceptions. After thawing, urine volumes were normalized to 20 μmol of creatinine. Crude heavy-labeled peptide standards for each of the differentially excreted peptides were purchased from JPT Peptide Technologies and were spiked into the samples just prior to Vivaspin centrifugation. Steps involving SCX-HPLC fractionation and OMIX tip desalting were not performed. Instead, peptides were analyzed at the SickKids Proteomics, Analytics, Robotics, and Chemical Biology Centre (SPARC Biocentre in Toronto, Canada) facility, where they were loaded onto Evotips and injected in duplicate onto an Evosep One nLC system coupled to a Thermo Scientific Q Exactive HF-X hybrid quadrupole-orbitrap mass spectrometer on a 21-minute unscheduled gradient. Raw files were imported into Skyline software (version 4.1). We then manually reviewed each precursor ion to ensure that all transitions were captured in the integration boundaries for relative quantification. Differential peptide excretion rates (deriving from peak area ratios of endogenous-to-heavy-labeled peptides) were determined using the Mann-Whitney test ($P < 0.05$) between groups in the validation phase.

Skyline files and raw data have been deposited in Panorama Public with a ProteomeXchange identifier PXD012389 (https://panoramaweb.org/lotso19.url; username: panorama+diamandis3@proteinms.net; password: 6Zr3q+ex).

Uromodulin Protein Excretion

Urine aliquots from the internal validation cohort were thawed to 4°C, centrifuged at 2000 g for 5 minutes, and analysed in duplicate using enzyme-linked immunosorbent assay (ELISA). Human
Urinary peptidomics in early type 1 diabetes

Uromodulin ELISA (DY5144) and ancillary kits (DY008) were purchased from R&D Systems. All plates were read at 450 and 540 nm using a Perkin Elmer 2103 EnVision Multilabel Plate Reader. Protein concentrations were extrapolated from optical densities using four-parameter logistic (4-PL) regression. Urinary measurements were normalized to urinary creatinine concentrations.

Cell Culture

Immortalized proximal tubular HK-2 cells were grown in 1:1 mix of Dulbecco's Modified Eagle Medium (DMEM) and Ham’s F12 media supplemented with 10% fetal bovine serum, 10 ng/mL epidermal growth factor, 5 μg/mL transferrin, 5 μg/mL insulin, 0.05 μM hydrocortisone, 50 units/mL penicillin, and 50 μg/mL streptomycin at 37°C with 5% CO₂. Once the cells reached 50-60% confluency, cells are transfected for 16 hours, serum-starved for 24 hours, and then treated with peptide in fresh serum- and supplement-free DMEM/F12 for 24 hours.

Human primary proximal tubular epithelial cells from a male donor were purchased from Lonza. The cells (at passage 6) were grown in DMEM media supplemented with 10% fetal bovine serum, 10 ng/mL epidermal growth factor, 5 μg/mL transferrin, 5 μg/mL insulin, 5 μg/mL selenium, 0.05 μM hydrocortisone, 50 units/mL penicillin, and 50 μg/mL streptomycin at 37°C with 5% CO₂. Once the cells reached 60-70% confluency, cells are serum-starved for 24 hours and subsequently treated with peptide in fresh serum- and supplement-free DMEM for 24 hours.

Luciferase Activity Assay

HK-2 and human primary proximal tubular epithelial cells were transfected with Renilla luciferase control reporter vector pRL-TK and a luciferase reporter for either NF-κB or AP-1. Purified peptides (70%) were purchased from JPT Peptide Technologies and were used in all in vitro experiments. Uromodulin protein (>95% purity) was purchased from Millipore Sigma (catalogue number AG733). Cells were serum-starved for 24 hours and treated for 24 hours with the uromodulin peptides (0.1 to 10 μM), uromodulin protein (0.2 to 20 μg/mL), angiotensin II (Ang II, 0.1 μM) or lipopolysaccharide (LPS, 0.1 μg/mL). In certain experiments, cells were pre-treated with TAK-242 (0.3 μM) or gallein (10 μM) for...
1 hour. Reporter activities were measured using the Promega dual-luciferase assay kit. The luciferase activity was normalized to the Renilla luciferase activity.

**Neutrophil Chemotaxis Assay**

Human primary neutrophils were isolated from peripheral blood from a healthy volunteer. Neutrophils were incubated with purified uromodulin peptide (1 µM) for 15 minutes at 37°C and then placed on a 5% bovine serum albumin-coated microscope cover glass (22 × 40 mm) for 10 minutes at 37°C. The cover glass was inverted onto Zigmund chambers: 100 µL of Hank's Balanced Salt Solution media in the right chamber, and 100 µL of media containing fMLP (1 µM) in the left chamber. We recorded neutrophil movement for 15 minutes at a rate of 0.05 frame per second using time-lapse video microscopy. Captured images were analyzed using cell-tracking Retrac software (version 2.1.01).

**Cytokine Release from Kidney Tubular Cells**

Human primary proximal tubular epithelial cells were serum-starved for 24 hours and subsequently treated with purified uromodulin peptides (0.1 to 10 µM) or LPS (1 µg/mL) for 24 hours. Conditioned media were collected, centrifuged at 2000 g for 10 minutes, and kept at either 4°C for immediate use or stored at -20°C. Human Interleukin (IL)-6 (ab178013) and IL-8 (ab214030) SimpleStep ELISA kits were purchased from Abcam. Conditioned media were analysed in duplicate according to kit instructions. Plates were read as previously described.

**Cytokine Measurements from Urine**

Frozen urine aliquots were analyzed in duplicate by Eve Technologies (Calgary, Canada) using the Human Cytokine Array/Chemokine Array 42-Plex with IL-18 (HD42) (Millipore MILLIPLEX). The full list of analytes can be found in the Supplemental Methods. The Luminex bead-based immunoassay was run on the Biorad BioPlex 200 Systems. Analyte concentrations were normalized to urinary creatinine concentration as a measure of urinary excretion. We selected eight analytes a priori to characterize an inflammatory signal in urine based on the existing literature (19–22): IL-1β, IL-6, IL-8, IL-18, interferon
gamma-induced protein 10 (IP-10/CXCL10), monocyte chemoattractant protein 1 (MCP-1/CCL2), macrophage inflammatory protein 1-beta (MIP-1β/CCL4), and tumour necrosis factor alpha (TNFα).

**Bioinformatics**

For the heatmap analysis, we converted log-transformed peptide intensities into z-scores and performed Euclidean hierarchical clustering using the R package pheatmap. PeptideRanker was used to predict bioactive peptides (23). Peptide sequences were aligned with precursor proteins using Peptide Extractor to visualize cleavage sites (9). We inferred protease activity using Proteasix (24). Structural information was extracted from the UniProtKB database. We used iceLogo to determine percent differences in amino acid proportion at each position and to visualize the consensus cleavage sites at the N- and C-termini of urinary peptides (25). The MEROPS database (version 12.1, https://www.ebi.ac.uk/merops/index.shtml) was manually searched to identify proteases with specific cleavage sites and to collate cleavage site sequence logo motifs (26, 27).

**Statistics**

Normal distribution of each variable was examined using the Shapiro-Wilks normality test. We compared clinical characteristics between groups using the t-test for continuous variables and the chi-squared test for categorical variables. Pearson correlations were performed on log-transformed urinary peptide excretion rates and uromodulin protein excretion. Differences in luciferase activities and protein concentrations were determined using the independent t-test. Differential excretion of cytokines and chemokines was determined using the Mann-Whitney test on positive, non-zero data only. Imputation of zero-value intensities was performed with Perseus software from a normal distribution with a downshift of 1.8 and width of 0.3 standard deviations to determine fold changes for the cleavage site analysis. Plots were created with R software.

**RESULTS**

*Urinary peptidomic analyses of type 1 diabetes*
Overall, 6550 peptides deriving from 751 protein precursor groups were identified using our urinary peptidomics workflow (Figure 2A, Supplemental Tables S4 and S5). After the removal of false hits, unquantified peptides, and contaminants, our MaxQuant search provided label-free quantitation (LFQ) for 6323 peptides from 731 unique protein precursors (Supplemental Tables S6 and S7): 5708 peptides were found in youths with diabetes, 5011 peptides originated from controls, and 4396 peptides were common to both groups (Figure 2B). Of the 615 peptides found exclusively in the non-diabetic group, only ten peptides were found in at least ten samples—all deriving from the C-peptide region of insulin (Supplemental Table S6). Notably, one C-peptide fragment (EAEDLQVGQVELGGGPGAGSLQP) was identified in all fifteen urine samples from youths without diabetes and in none from those with diabetes. On the other hand, no peptide was exclusively identified in urine from all youths with diabetes.

The most frequently observed protein precursors, associated with at least 100 urinary peptides, were serum albumin, collagen alpha-1(I) chain, hemoglobin subunit alpha and subunit beta, keratin type II cytoskeletal 6A, and apolipoprotein A1. Together, these six proteins accounted for 17% of the quantified peptidome (Supplemental Table S7).

A subset of 162 peptides were found in all thirty samples (Supplemental Table S8), representing 2.6% of the total peptidome. Although this subset mainly consisted of medium-to-high-abundance peptides, the range of intensities spanned four orders of magnitude (Figure 2C). As expected, low-abundance peptides were not consistently detected across samples. We examined the tissue origins of peptides using the Human Protein Atlas database (28). Roughly 80% of unique peptides derived from proteins with known expression in the kidneys. Kidney-specific or -enriched proteins accounted for 15% of the 162 peptides, compared to 4% of the total peptidome (Figure 2D). We thus decided to focus our further analyses on these 162 peptides as they reflected the most robust and consistent changes in the peptidome.

Fifteen peptides were differentially excreted between study groups ($P < 0.05$), deriving from alpha-2-HS-glycoprotein, albumin, apolipoprotein A1, clusterin, latent transforming growth factor beta binding protein 4, Na⁺/K⁺-transporting ATPase subunit gamma, and uromodulin (Figure 2E, Table 3). All
but three peptides were more abundant in urines from youths with diabetes (Figure 2F, Figure 3). After BH adjustment, four uromodulin peptides and one clusterin peptide remained statistically significant ($Q < 0.05$). Unsupervised hierarchical clustering based on normalized peptide intensities segregated the two groups based on diabetes status, leaving one youth with diabetes among the controls (Figure 2F).

**Validation of differentially excreted peptides**

Parallel reaction monitoring takes advantage of mass spectrometry-based techniques to quantify multiple peptides simultaneously on quadrupole-orbitrap hybrid instrument using heavy-labeled internal standards (29). We successfully developed targeted methods for the relative quantification of six peptides: UMOD-1, UMOD-2, UMOD-3, UMOD-4, UMOD-5, UMOD-7. Each of the thirty urine samples were analyzed in duplicate injections onto the mass spectrometer with median technical coefficients of variation less than 12% for each peptide (Supplemental Table S9). All peptides except UMOD-2 were differentially excreted between groups in the validation cohort (Figure 4A, Table 4). Fold changes were similar to those from the discovery phase. Notably, UMOD-2 was the lone peptide whose urinary excretion decreased in diabetes (fold change of 0.75, $P = 0.0672$). We also demonstrated that total uromodulin protein excretion was markedly reduced in diabetes (fold change of 0.28, $P = 0.0061$), similar to UMOD-2 (Figure 4B), although there was no significant correlation between peptide and protein excretion (Figure 4C, Supplemental Table S10). We observed that the uromodulin peptides strongly correlated with one another, except for UMOD-2 (Figure 4C, Supplemental Table S10). These findings suggest UMOD-2 may derive separately from the other uromodulin peptides. To better understand this, we sought to examine whether the downward trend of UMOD-2 would be reflected when applying other urinary normalization methods (Supplemental Table S11). In contrast to urinary excretion, urinary concentration of UMOD-2 was mildly increased in diabetes (fold change of 1.1, $P = 0.7718$). A similar trend was observed when UMOD-2 was normalized to total uromodulin protein (fold change of 2.8, $P = 0.0561$). The direction of change of the other uromodulin peptides remained consistent despite different normalization methods.

**Novel bioactivity of uromodulin peptides**
Urinary peptidomics in early type 1 diabetes

We next investigated whether these uromodulin peptides might exert a functional or biological role in the kidney. PeptideRanker was used to predict bioactivity of the differentially excreted peptides (Table 5). This server was trained using databases of known bioactive peptides (PeptidesDB, CAMP, and BIOPEP) (30–32), providing probabilities of bioactivity based on amino acid composition (23). Ang II, Ang (1-7), and vasopressin were also included as positive controls in our analysis. The top uromodulin peptides, UMOD-1 and UMOD-2, outscored Ang (1-7) with scores of 0.47 and 0.46, respectively.

To verify the in silico prediction, we first performed NFκB and AP-1 luciferase activity assays in HK-2 cells. Both transcription factors are activated downstream of toll-like receptor 4 (TLR4) activation as part of the pro-inflammatory response (33). Chronic activation of inflammation is thought to contribute to diabetic kidney disease progression (34, 35). As an initial screen, cells were treated for 24 hours with graded concentrations (0.1, 1, and 10 μM) of 70%-purified uromodulin peptides, UMOD-1, UMOD-2, and UMOD-3 (Figure 5A). Ang II was used as a positive control based on previous findings that it promotes inflammation and oxidative stress in the kidney (36–38). At 0.1 μM, UMOD-1 and UMOD-2 significantly stimulated NFκB activity (P < 0.05). As predicted, UMOD-3 failed to elicit a response, even at the highest tested dose of 10 μM.

Previous studies have shown that uromodulin protein activates NFκB in immune cells via TLR4 (39, 40). We thus inhibited TLR4 by pre-treating cells with TAK-242 (resatorvid) for 1 hour, which significantly reduced LPS- and uromodulin peptide-induced NFκB activity (P < 0.05) (Supplemental Figure S2, Figure 5B). LPS was used as a positive control for canonical TLR4-mediated NFκB activation (41, 42). Activation of NFκB signaling by uromodulin peptides, UMOD-1 and UMOD-2, may thus be mediated by TLR4 signalling. The same peptides also triggered an AP-1 response, which was attenuated with gallein, an inhibitor of G protein βγ subunit-dependent signalling (Supplemental Figure S3, Figure 5C). Interestingly, uromodulin protein failed to stimulate NFκB luciferase activity in HK-2 cells at varying concentrations of 0.2, 2, and 20 μg/mL (Figure 5D). These concentrations were selected to cover the reported range from 100 ng/mL to 25 μg/mL for urinary uromodulin concentration (5–8). In this study, the median urinary concentration for uromodulin protein was 2.5 μg/mL for youths with
diabetes and 4.7 μg/mL for their non-diabetic peers. Similar results were observed in primary proximal tubular epithelial cells (Supplemental Figure S4).

Next, we conducted a neutrophil chemotaxis assay. Human blood neutrophils were first treated with one of the three previously tested uromodulin peptides, then placed in a Zigmond chamber, and finally subjected to a N-formyl-methionyl-leucyl-phenylalanine (fMLP) attractant gradient. Compared to untreated neutrophils or neutrophils treated with UMOD-3, UMOD-1 and UMOD-2 significantly enhanced neutrophil migration toward fMLP \((P < 0.05)\) (Figure 5E).

To bridge NFκB signaling and neutrophil chemotaxis, we examined whether UMOD-1 might induce cytokine release from primary proximal tubular epithelial cells. Interleukin (IL)-8 was specifically chosen as a neutrophil chemokine; while IL-6 was selected for its pro-inflammatory action. Both cytokines are downstream products of TLR-4 mediated NFκB signaling. We demonstrated that UMOD-1 promotes IL-6 and IL-8 secretion into serum-starved conditioned media of primary proximal tubular epithelial cells at 0.1 to 10 μM \((P < 0.05)\) (Figures 5F-G).

**Urinary peptidomics in early type 1 diabetes**

Our in vitro findings suggested a role for uromodulin peptides in inflammation. Accordingly, we speculated whether a pro-inflammatory signal on the basis of increased urinary excretion of cytokines and chemokines could be detected in early diabetes. To do so, we used a Luminex bead-based immunoassay and adjusted urinary analyte concentrations to urinary creatinine concentrations. Eight analytes were selected a priori based on the literature on urinary markers of inflammation in diabetic kidney disease: IL-1β, IL-6, IL-8, IL-18, IP-10, MCP-1, MIP-1β, and TNFα (Figure 6). IL-1β and TNFα, however, were omitted because more than two-thirds of the samples in each group could not be reliably quantified. Of the six remaining analytes, IL-6 (median fold change of 2.1), IL-18 (median fold change of 2.5), and IP-10 (median fold change of 2.8) were excreted to a greater extent by youths with diabetes, compared to their non-diabetic peers \((P < 0.05)\).

**Cleavage site analysis of urinary peptides**
Urinary peptidomics in early type 1 diabetes

To investigate the proteolytic mechanisms altered in early diabetes, we first examined the cleavage sites associated with the N-termini and C-termini of urinary peptides. For this particular analysis, we initially included all 4396 peptides that were common to both groups and subsequently excluded peptides that were quantified in fewer than five samples per group. Missing LFQ data was imputed using the Perseus software. Of the 1941 eligible peptides, 549 peptides were increased (with a fold change of at least 1.5) and 331 peptides were decreased (with a fold change of 0.67 and below) in diabetes. We also added 94 peptides that were exclusively identified in one group if they were quantified in at least five samples: 58 from youths with diabetes and 36 from their non-diabetic peers. Thus, a total of 974 peptides was examined in our cleavage site analysis using iceLogo (25), in which the experimental set (\(N=607\) peptides that were elevated in diabetes) was compared to the reference set (\(N=367\) downregulated peptides) (Figure 7A).

We noted a preponderance of proline residues at or near the N- and C-termini of urinary peptides, especially peptides that were downregulated in diabetes (Figure 7B-C). In fact, proline residues accounted for 25% of all C-termini of downregulated peptides, compared to 14% of C-termini of peptides elevated in diabetes. Additionally, there was a higher percentage of trypsin-like cleavages (i.e., at the carboxyl side of an arginine or lysine residues) resulting in the N-terminus of urinary peptides downregulated in diabetes; the opposite trend seems to be true at the C-terminus of peptides elevated in diabetes (\(P<0.05\)). Unlike the non-diabetic profile, the sequence motifs for diabetes were more diverse and highlighted a broader range of residues such as leucine, phenylalanine, and isoleucine. Notably, the top motif for the N-terminus cleavage site (L\(^*\)LV) corresponded to renin (26), which is secreted by juxtaglomerular cells of the kidney.

We next focused on the proteolytic mechanisms responsible for the uromodulin peptides. Using the Peptide Extractor(9), we mapped all of the differentially excreted peptides to a specific region near the C-terminus of uromodulin: \(^{589}\text{GSVIDQSRVLNLGPIR}^{607}\) (Figure 7D). Adjacent regions include the hepsin consensus cleavage site at \(^{586}\text{FR}^{589}\) and a glycosylphosphatidylinositol (GPI) anchor site at S\(^{614}\), which anchors the uromodulin protein onto the cell membrane of the loop of Henle and distal tubular
Urinary peptidomics in early type 1 diabetes

cells. This peptide region contains the external hydrophobic patch (EHP) motif, 589VLNLGPITRK607, which inhibits uromodulin polymerization and function (43). Given that urinary protein excretion of uromodulin was decreased in youths with diabetes (Figure 4B), we hypothesize that changes in peptide excretion could reflect changes in proteolytic activity.

Proteasix (24) was used to predict the proteases responsible for generating the uromodulin peptides. A total of 156 combinations of predicted proteases and cleavage sites met the MEROPS threshold score. For each cleavage site, we selected the proteases according to the predicted probability as follows: 1) predicted probability fell above the 99th percentile of the population distribution of all sequences, 2) predicted probability was below, but was the closest to the 99th percentile, and 3) predicted probability was the furthest away from the MEROPS threshold score. After applying the criteria, nineteen protease/cleavage sites combinations remained (Table 6), and the predicted cleavage sites are illustrated in Figure 7E. Of these predicted proteases, eight predominantly cleave at the carboxyl side of arginine or lysine (Supplemental Figure S5). According to our earlier sequence logo motif analyses, we speculate that the N-terminal cleavage of the peptide region (589SGSVIDQSRVLNLGPITRK607) occurs in normal, physiologic conditions (Figure 7B), whereas the C-terminal cleavage events are increased in diabetes (Figure 7C). This is supported by an increased percentage of cleavage events that are at the carboxyl side of isoleucine (UMOD-1, UMOD-3, and UMOD-7), lysine (UMOD-6), threonine (UMOD-4) of uromodulin peptides elevated in diabetes.

We also searched the Nephroseq v5 database (www.nephroseq.org, November 2018, University of Michigan, Ann Arbor, MI) for expression data in diabetic nephropathy and found nine proteases whose expression was significantly altered in cases of diabetic nephropathy ($P < 0.05$): a disintegrin and metallo-proteinase domain-containing protein 10, cathepsin B granzyme A, hepsin, kallikrein-6, matrix metalloproteinase-2, matrix metalloproteinase-9, neprilysin, and plasmin. Hepsin has previously been identified as a putative protease responsible for the N-terminal cleavage of UMOD-1 and UMOD-2. Meprin A and cathepsin B were predicted to cleave at carboxyl side of G590, resulting in the inert peptide UMOD-3. Neutrophil elastase was mapped to the C-terminus of UMOD-1, UMOD-3, and UMOD-7.
DISCUSSION

It is generally accepted that chronic hyperglycemia disrupts the proteolytic milieu (10–12). The resulting peptide products could thus be used to examine the upstream interactions between proteolytic enzymes and protein substrates (9). In this study, we report that otherwise healthy youths with type 1 diabetes excrete a urinary peptide profile that is distinct from their non-diabetic peers and that these differences precede the onset of microalbuminuria or renal function decline. Seven of the fifteen differentially excreted peptides originated from a small region (\textsuperscript{589}SGVIDQSRVLNLGPITRK\textsuperscript{607}) near the C terminus of uromodulin; and five were validated in a second cohort by parallel reaction monitoring. Furthermore, we discovered that UMOD-1, one of the validated peptides, displays novel bioactivity \textit{in vitro}, by stimulating TLR4-dependent NFκB luciferase activity in HK-2 and primary proximal tubular epithelial cells, by inducing cytokine release from primary proximal tubular epithelial cells, and by enhancing neutrophil chemotaxis. A second peptide, UMOD-2, also exhibited similar bioactivity, but was not differentially excreted in the validation cohort. Interestingly, uromodulin protein failed to elicit a pro-inflammatory response in tubular epithelial cells. \textit{In silico} analyses identified several proteases that could be responsible for generating these uromodulin peptides, such as hepsin, meprin A, cathepsin B, and neutrophil elastase. Our findings present new insights into the early kidney response to chronic hyperglycemia long before the clinical manifestation of renal dysfunction.

Uromodulin, also known as Tamm-Horsfall glycoprotein, is exclusively expressed in the loop of Henle and distal tubules of the kidney (44, 45). Naturally, its peptides have gained notoriety as potential kidney-specific biomarkers for diabetic nephropathy (46), hypertension (47, 48), chronic kidney disease (49), systemic lupus erythematosus(50), IgA nephropathy(51), and acute rejection following renal transplantation (52). Even though the exact peptide sequences differ between peptidomic studies, they typically originate from the same region as our peptides: \textsuperscript{589}SGVIDQSRVLNLGPITRK\textsuperscript{607}. These observations suggest that perturbations in specific proteolytic events associated with kidney injury may not be unique to diabetes after all. Still, our findings demonstrate that the urinary peptidome may reveal
Urinary peptidomics in early type 1 diabetes

Early signs of kidney injury in youths with diabetes, even when they present with no clinical symptoms of renal stress or disease. Additional validation studies are needed to examine how urinary excretion of uromodulin peptides changes over the course of diabetic kidney disease.

Beyond their potential utility as indicators of injury, very little is known about these uromodulin peptides. We thus sought to investigate whether they may play a direct mechanistic role in the kidney; and, for the first time to our knowledge, we demonstrated that two peptides, UMOD-1 and UMOD-2, were capable of stimulating pro-inflammatory responses in kidney tubular epithelial cells and enhanced neutrophil migration. These findings significantly enrich our current understanding of uromodulin biology because the glycoprotein is thought to be immunologically inert in the tubules (40, 53, 54). Namely, uromodulin was shown to induce the maturation of myeloid dendritic cells by activating TLR4-dependent NFκB signalling pathways (39). Uromodulin nanoparticles stimulated the NLRP3-inflammasome in antigen-presenting cells, triggering IL-1β secretion (40). In addition, uromodulin protein expression on membranes of cultured tubular epithelial cells enhanced neutrophil adherence and trans-epithelial migration (55). Neutrophils also preferentially attached to uromodulin protein immobilized on microtiter plates; however, no such interaction was observed with soluble uromodulin (56). A recent study reported that uromodulin abated renal and systemic oxidative stress by inhibiting the transient receptor potential cation channel, subfamily M, member 2 (TRPM2), as a protective mechanism against acute kidney injury (57). Altogether, uromodulin protein seems to rely on interactions with immune cells to trigger inflammation and may be protective in the kidney. In contrast, our in vitro data suggest that uromodulin peptides could mediate pro-inflammatory processes in the kidney directly.

We next examined how these peptides might be generated. While basolateral expression has been observed, uromodulin is predominantly attached to the apical surface by a GPI anchor near the C terminus (58, 59). The bulk of the protein is subsequently shed into the urinary space following hepsin cleavage (60). More specifically, the type II transmembrane serine protease separates the active portion of the protein from our peptide region, which remains attached to the membrane and contains the inhibitory external hydrophobic patch (60–62). This particular cleavage event accounts for the N-terminus of the
two potentially bioactive peptides: UMOD-1 and UMOD-2. Highly expressed in the liver and renal tubules (63–65), hepsin is involved in cell growth and proliferation, blood coagulation, extracellular matrix remodeling (66–68). Studies have demonstrated that deficient hepsin expression produces longer, polymerization-incompetent isoforms of uromodulin containing the peptide region; results in intracellular accumulation of uromodulin; and predisposes tubules to stress (60, 62). Although renal expression of hepsin is downregulated in diabetic nephropathy (Nephroseq database), it is unclear whether hepsin expression or activity is reduced in the diabetic kidney before clinical injury.

Two other notable cleavage sites emerged from our in silico analyses: the N-terminus of UMOD-3, UMOD-4, UMOD-5, and UMOD-6 by meprin A and cathepsin B; and the C-terminus of UMOD-1, UMOD-3, and UMOD-7 by neutrophil elastase. Meprin A is a metalloproteinase found in the brush border of the proximal tubules, where it is constitutively shed into the urinary space by furin (69). In its active form, meprin A stimulates pro-inflammatory cytokine release and fibrosis in the diabetic kidney (70–73). Studies have also hinted at a protective role for meprin A, in which diminished expression and activity in the kidney were associated with later stages of diabetic kidney disease (74, 75). Urinary levels of meprin A were markedly elevated in microalbuminuric and late-stage disease, but were negligible in normoalbuminuric individuals with diabetes and non-diabetic healthy control subjects(76). Cathepsin B, a lysosome-associated enzyme, is involved in autophagy, extracellular matrix remodeling, inflammasome activation, and sodium reabsorption in the kidney (77–80). Studies have shown that cathepsin B activity is decreased following in vitro stimulation of HK-2 cells with advanced glycation end-product (82) and in the kidneys of rats with streptozotocin-induced diabetes (81). Interestingly, lysosome-derived cathepsin B expression was elevated in glomeruli of a murine model of podocyte injury; and cathepsin B knockout mice were more resistant to injury and recovered more rapidly following injection of nephrotoxic serum (83). Urinary proteomic profiling in early diabetes has demonstrated increased urinary excretion of cathepsin B and other lysosome-associated enzymes (84, 85). Lastly, neutrophil elastase is secreted from neutrophil granules during acute inflammation and promotes reactive oxygen species generation (86, 87).
Neutrophil infiltration into the kidney has been well-documented in later stages of diabetic kidney disease and contributes to disease progression and fibrosis (88–90).

While it is possible that each peptide is generated \textit{de novo} from uromodulin protein, we posit that they derive from peptide intermediates, similar to angiotensinogen and its peptide products. The 452-residue precursor is first shortened into the inactive Ang I decapeptide (1DRVYIHPFHL) by renin. Subsequent angiotensin-converting enzyme activity produces the bioactive Ang II octapeptide (1DRVYIHPF), which promotes sodium reabsorption via aldosterone secretion (91). Alternate processing of the Ang I peptide results in a myriad of bioactive and inert peptide sequences (92–95). Notably, Ang (1-7) and Ang II (1-8) have opposing biological effects (96, 97), underscoring the significance of a single residue. Likewise, the bioactive UMOD-1 (SGSVIDQSRVLNLGPI) and the inert UMOD-3 (SVIDQSRVLNLGPI) peptides differ in two residues at the N-terminus, suggesting that the loss of the serine-glycine residues at the N-terminus may render a peptide inactive and could act as a means to “turn off” peptide bioactivity. Interestingly, UMOD-2 excretion did not correlate with any of the other uromodulin peptides and may thus originate from a separate proteolytic mechanism. Although UMOD-2 was not differentially excreted in the validation cohort, it displayed a downward trend in diabetes. Its presence may be related to normal renal physiology or reflect decreased total uromodulin protein expression, rather than dysregulated processing in chronic hyperglycemia. Future studies will be necessary to define the proteolytic pathways and putative enzymes responsible for uromodulin processing.

Our study has several strengths. First, we carefully selected our study population to avoid possible confounding effects of concurrent medications and comorbidities. This allowed us to examine the early kidney response to chronic hyperglycemia in a homogenous cohort of youths with and without diabetes. Second, we conducted an internal validation of uromodulin peptides in a second, independent cohort. Finally, we provided three pieces of evidence of novel bioactivity of UMOD-1, whose urinary excretion was elevated in early type 1 diabetes, using three different cell systems. Our findings may open
Urinary peptidomics in early type 1 diabetes

a new field of investigation into the mechanisms responsible for early kidney injury in type 1 diabetes and into therapies that target prevention, rather than management, of diabetic kidney disease.

Our study also has some important limitations. First, the cause-effect relationships between uromodulin peptides and chronic hyperglycemia cannot be fully discerned in a cross-sectional study. Second, the study population did not include individuals at later stages of diabetic kidney disease, and it is thus difficult to infer whether these peptides are associated with progressive disease. Finally, we were unable to experimentally verify the protease prediction due to the lack of a model system, which mimics the appropriate kidney cell type and naturally expresses uromodulin protein. Ultimately, an in-depth investigation into these proteolytic events will better define the pathophysiologic role of uromodulin peptides in the diabetic kidney.

In summary, we identified and validated a signature of uromodulin peptides associated with early, uncomplicated type 1 diabetes in two separate cohorts. Our discovery of novel bioactivity in uromodulin peptides suggests a potential role in mediating early changes in the diabetic kidney.
**Urinary peptidomics in early type 1 diabetes**

**Author contributions:** JADV was involved with the study design, performed the peptidomic and validation experiments, analyzed the data, and wrote the majority of manuscript. SCF provided guidance and assistance with the cell experiments. XZ performed the luciferase activity assays. IB provided expertise in mass spectrometry. CS performed and described the methods for the chemotaxis assay. MG provided expertise in neutrophils and chemotaxis. LR provided expertise in uromodulin. YE secured funding. ES and FHM provided expertise in pediatric type 1 diabetes. EPD provided expertise in proteomics. JWS was involved with the study design; secured funding, provided expertise in diabetic kidney disease, and edited the manuscript. AK was involved with the study design, provided expertise in peptidomics, and edited the manuscript.

**Acknowledgements:** Special thanks to Christine Kerr, Daryl Baquillos, Laura Motran, Harriet Georgas, Denis Daneman, Veronica Talunay, Antoninus Soosaipillai, and the SPARC BioCentre facility members at SickKids Hospital in Toronto.

**Funding:** JADV is supported by the Banting & Best Diabetes Centre through the Yow Kam-Yuen Graduate Scholarship in Diabetes Research and Novo Nordisk Graduate Studentship and by an Ontario Graduate Scholarship. FHM and ES have received funding through the Juvenile Diabetes Research Foundation Canadian Clinical Trials Network (JDRF-CCTN), Canadian Institutes of Health Research (CIHR), the Heart and Stroke Foundation of Canada, and Diabetes Canada. JWS is supported by operating grants from the Heart and Stroke Foundation of Canada and the CIHR CanSOLVE-CKD SPOR program. AK is supported by a Kidney Foundation of Canada operating grant, the Kidney Research Scientist Core Education and National Training (KRESCEnt) program, Kidney Foundation of Canada Predictive Biomarker Grant, CIHR Catalyst Grant, and Canada Foundation for Innovation award.

**Data Availability:** For discovery peptidomics, mass spectrometry data have been deposited onto the ProteomeXchange Consortium via the PRIDE(16) partner repository with the dataset identifier PXD012210 (http://www.ebi.ac.uk/pride/archive/login; username: reviewer85516@ebi.ac.uk; password: ht3PGbCg). For targeted peptidomics, Skyline files and raw data have been deposited in Panorama Public
Urinary peptidomics in early type 1 diabetes

with a ProteomeXchange identifier PXD012389 (https://panoramaweb.org/lotso19.url; username: panorama+diamandis3@proteinms.net; password: 6Zr3q+ex).


References and Notes:

1. American Diabetes Association (2014) Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 37, S81–S90

2. Harris, M. I., Klein, R., Welborn, T. A., and Knuiman, M. W. (1992) Onset of NIDDM occurs at least 4-7 yr before clinical diagnosis. *Diabetes Care* 15, 815–9

3. Foley, R., Parfrey, P., and Sarnak, M. (1998) Clinical epidemiology of cardiovascular disease in chronic renal disease. *Am. J. Kidney Dis.* 32, S112–S119

4. Kim, S. J., Gill, J. S., Knoll, G., Campbell, P., Cantarovich, M., Cole, E., and Kiberd, B. (2019) Referral for Kidney Transplantation in Canadian Provinces. *J. Am. Soc. Nephrol.*, ASN.2019020127

5. Webster, A. C., Nagler, E. V, Morton, R. L., and Masson, P. (2017) Chronic Kidney Disease. *Lancet* 389, 1238–1252

6. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. (1993) *N. Engl. J. Med.* 329, 977–86

7. Van, J. A. D., Scholey, J. W., and Konvalinka, A. (2017) Insights into Diabetic Kidney Disease Using Urinary Proteomics and Bioinformatics. *J. Am. Soc. Nephrol.*, ASN.2016091018

8. Schulz-Knappe, P., Schrader, M., and Zucht, H.-D. (2005) The peptidomics concept. *Comb. Chem. High Throughput Screen.* 8, 697–704

9. Guerrero, A., Dallas, D. C., Contreras, S., Chee, S., Parker, E. A., Sun, X., Dimapasoc, L., Barile, D., German, J. B., and Lebrilla, C. B. (2014) Mechanistic peptidomics: factors that dictate specificity in the formation of endogenous peptides in human milk. *Mol. Cell. Proteomics* 13, 3343–51

10. Thrailkill, K. M., Clay Bunn, R., and Fowlkes, J. L. (2009) Matrix metalloproteinases: their
Urinary peptidomics in early type 1 diabetes

potential role in the pathogenesis of diabetic nephropathy. *Endocrine* 35, 1–10

11. Madhusudhan, T., Kerlin, B. A., and Isermann, B. (2016) The emerging role of coagulation proteases in kidney disease. *Nat. Rev. Nephrol.* 12, 94–109

12. Cocchiaro, P., De Pasquale, V., Della Morte, R., Tafuri, S., Avallone, L., Pizard, A., Moles, A., and Pavone, L. M. (2017) The Multifaceted Role of the Lysosomal Protease Cathepsins in Kidney Disease. *Front. cell Dev. Biol.* 5, 114

13. Must, A., and Anderson, S. E. (2006) Body mass index in children and adolescents: considerations for population-based applications. *Int. J. Obes.* 30, 590–594

14. Yamamoto, T. (2010) The 4th Human Kidney and Urine Proteome Project (HKUPP) workshop. 26 September 2009, Toronto, Canada. *Proteomics* 10, 2069–70

15. Smith, C. R., Batruch, I., Bauça, J. M., Kosanam, H., Ridley, J., Bernardini, M. Q., Leung, F., Diamandis, E. P., and Kulasingam, V. (2014) Deciphering the peptidome of urine from ovarian cancer patients and healthy controls. *Clin. Proteomics* 11, 23

16. Vizcaíno, J. A., Deutsch, E. W., Wang, R., Csordas, A., Reisinger, F., Rios, D., Dianes, J. A., Sun, Z., Farrah, T., Bandeira, N., Binz, P.-A., Xenarios, I., Eisenacher, M., Mayer, G., Gatto, L., Campos, A., Chalkley, R. J., Kraus, H.-J., Albar, J. P., Martinez-Bartolomé, S., Apweiler, R., Omenn, G. S., Martens, L., Jones, A. R., and Hermjakob, H. (2014) ProteomeXchange provides globally coordinated proteomics data submission and dissemination. *Nat. Biotechnol.* 32, 223–226

17. Cox, J., Neuhauser, N., Michalski, A., Scheltema, R. A., Olsen, J. V., and Mann, M. (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.* 10, 1794–805

18. Tyanova, S., Temu, T., Sinitecyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., and Cox, J. (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat. Methods* 13, 731–740
Urinary peptidomics in early type 1 diabetes

19. Navarro-González, J. F., and Mora-Fernández, C. (2008) The role of inflammatory cytokines in diabetic nephropathy. *J. Am. Soc. Nephrol.* 19, 433–42

20. Ruster, C., and Wolf, G. (2008) The Role of chemokines and chemokine receptors in diabetic nephropathy. *Front. Biosci.* 13, 944

21. Navarro-González, J. F., Mora-Fernández, C., Muros de Fuentes, M., and García-Pérez, J. (2011) Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy. *Nat. Rev. Nephrol.* 7, 327–40

22. Wolkow, P. P., Niewczas, M. A., Perkins, B., Ficociello, L. H., Lipinski, B., Warram, J. H., and Krolewski, A. S. (2008) Association of urinary inflammatory markers and renal decline in microalbuminuric type 1 diabetics. *J. Am. Soc. Nephrol.* 19, 789–97

23. Mooney, C., Haslam, N. J., Pollastri, G., and Shields, D. C. (2012) Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity. *PLoS One* 7, e45012

24. Klein, J., Eales, J., Zürbig, P., Vlahou, A., Mischak, H., and Stevens, R. (2013) Proteasix: a tool for automated and large-scale prediction of proteases involved in naturally occurring peptide generation. *Proteomics* 13, 1077–82

25. Colaert, N., Helsens, K., Martens, L., Vandekerckhove, J., and Gevaert, K. (2009) Improved visualization of protein consensus sequences by iceLogo. *Nat. Methods* 6, 786–787

26. Rawlings, N. D., Barrett, A. J., Thomas, P. D., Huang, X., Bateman, A., and Finn, R. D. (2018) The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. *Nucleic Acids Res.* 46, D624–D632

27. Rawlings, N. D., and Barrett, A. J. (1999) MEROPS: the peptidase database. *Nucleic Acids Res.* 27, 325–331

28. Pontén, F., Jirström, K., and Uhlen, M. (2008) The Human Protein Atlas--a tool for pathology. *J.
Urinary peptidomics in early type 1 diabetes

Pathol. 216, 387–93

29. Gallien, S., Kim, S. Y., and Domon, B. (2015) Large-Scale Targeted Proteomics Using Internal Standard Triggered-Parallel Reaction Monitoring (IS-PRM). Mol. Cell. Proteomics 14, 1630–1644

30. Iwaniak, A., Minkiewicz, P., Darewicz, M., Sieniawski, K., and Starowicz, P. (2016) BIOPEP database of sensory peptides and amino acids. Food Res. Int. 85, 155–161

31. Thomas, S., Karnik, S., Barai, R. S., Jayaraman, V. K., and Idicula-Thomas, S. (2010) CAMP: a useful resource for research on antimicrobial peptides. Nucleic Acids Res. 38, D774-80

32. Liu, F., Baggerman, G., Schoofs, L., and Wets, G. (2008) The construction of a bioactive peptide database in Metazoa. J. Proteome Res. 7, 4119–31

33. Newton, K., and Dixit, V. M. (2012) Signaling in Innate Immunity and Inflammation. Cold Spring Harb. Perspect. Biol. 4, a006049–a006049

34. Gurley, S. B., Ghosh, S., Johnson, S. A., Azushima, K., Sakban, R. B., George, S. E., Maeda, M., Meyer, T. W., and Coffman, T. M. (2018) Inflammation and Immunity Pathways Regulate Genetic Susceptibility to Diabetic Nephropathy. Diabetes 67, 2096–2106

35. Lin, M., Yiu, W. H., Wu, H. J., Chan, L. Y. Y., Leung, J. C. K., Au, W. S., Chan, K. W., Lai, K. N., and Tang, S. C. W. (2012) Toll-Like Receptor 4 Promotes Tubular Inflammation in Diabetic Nephropathy. J. Am. Soc. Nephrol. 23, 86–102

36. Konvalinka, A., Zhou, J., Dimitromanolakis, A., Drabovich, A. P., Fang, F., Gurley, S., Coffman, T., John, R., Zhang, S.-L., Diamandis, E. P., and Scholey, J. W. (2013) Determination of an angiotensin II-regulated proteome in primary human kidney cells by stable isotope labeling of amino acids in cell culture (SILAC). J. Biol. Chem. 288, 24834–47

37. Lodha, S., Dani, D., Mehta, R., Bhaskaran, M., Reddy, K., Ding, G., and Singhal, P. C. (2002) Angiotensin II-induced mesangial cell apoptosis: role of oxidative stress. Mol. Med. 8, 830–40

38. Wolf, G., Bohlender, J., Bondeva, T., Roger, T., Thaiss, F., and Wenzel, U. O. (2006) Angiotensin
Urinary peptidomics in early type 1 diabetes

II Upregulates Toll-Like Receptor 4 on Mesangial Cells. *J. Am. Soc. Nephrol.* 17, 1585–1593

39. Säemann, M. D., Weichhart, T., Zeyda, M., Staffler, G., Schunn, M., Stuhlmeier, K. M., Sobanov, Y., Stulnig, T. M., Akira, S., von Gabain, A., von Ahsen, U., Hörl, W. H., and Zlabinger, G. J. (2005) Tamm-Horsfall glycoprotein links innate immune cell activation with adaptive immunity via a Toll-like receptor-4-dependent mechanism. *J. Clin. Invest.* 115, 468–75

40. Darisipudi, M. N., Thomasova, D., Mulay, S. R., Brech, D., Noessner, E., Liapis, H., and Anders, H.-J. (2012) Uromodulin Triggers IL-1 -Dependent Innate Immunity via the NLRP3 Inflammasome. *J. Am. Soc. Nephrol.* 23, 1783–1789

41. Pugin, J., Schürer-Maly, C. C., Leturcq, D., Moriarty, A., Ulevitch, R. J., and Tobias, P. S. (1993) Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proc. Natl. Acad. Sci. U. S. A.* 90, 2744–8

42. Akira, S., and Takeda, K. (2004) Toll-like receptor signalling. *Nat. Rev. Immunol.* 4, 499–511

43. Bokhove, M., Nishimura, K., Brunati, M., Han, L., de Sanctis, D., Rampoldi, L., and Jovine, L. (2016) A structured interdomain linker directs self-polymerization of human uromodulin. *Proc. Natl. Acad. Sci.* 113, 1552–1557

44. Tokonami, N., Takata, T., Beyeler, J., Ehrbar, I., Yoshifuji, A., Christensen, E. I., Loffing, J., Devuyst, O., and Olinger, E. G. (2018) Uromodulin is expressed in the distal convoluted tubule, where it is critical for regulation of the sodium chloride cotransporter NCC. *Kidney Int.* 94, 701–715

45. Zimmerhackl, L. B., Pfeiderer, S., Kinne, R., Manz, F., Schuler, G., and Brandis, M. (1991) Tamm-Horsfall-Protein excretion as a marker of ascending limb transport indicates early renal tubular damage in diabetes mellitus type I. *J. Diabet. Complications* 5, 112–4

46. Krochmal, M., Kontostathis, G., Magalhães, P., Makridakis, M., Klein, J., Husi, H., Leirer, J., Mayer, G., Bascands, J.-L., Denis, C., Zoidakis, J., Züribig, P., Delles, C., Schanstra, J. P.,
Mischak, H., and Vlahou, A. (2017) Urinary peptidomics analysis reveals proteases involved in diabetic nephropathy. *Sci. Rep.* 7, 15160

47. Mary, S., Small, H. Y., Siwy, J., Mullen, W., Giri, A., and Delles, C. (2017) Polymerization-Incompetent Uromodulin in the Pregnant Stroke-Prone Spontaneously Hypertensive Rat Novelty and Significance. *Hypertension* 69, 910–918

48. Carty, D. M., Siwy, J., Brennand, J. E., Zürbig, P., Mullen, W., Franke, J., McCulloch, J. W., North, R. A., Chappell, L. C., Mischak, H., Poston, L., Dominiczak, A. F., Delles, C., and Delles, C. (2011) Urinary Proteomics for Prediction of Preeclampsia. *Hypertension* 57, 561–569

49. Good, D. M., Zurbig, P., Argiles, A., Bauer, H. W., Behrens, G., Coon, J. J., Dakna, M., Decramer, S., Delles, C., Dominiczak, A. F., Ehrich, J. H. H., Eitner, F., Fliser, D., Frommberger, M., Ganser, A., Girolami, M. A., Golovko, I., Gwinner, W., Haubitz, M., Herget-Rosenthal, S., Jankowski, J., Jahn, H., Jerums, G., Julian, B. A., Kellmann, M., Kliem, V., Kolch, W., Krolewski, A. S., Luppi, M., Massy, Z., Melter, M., Neususs, C., Novak, J., Peter, K., Rossing, K., Rupprecht, H., Schanstra, J. P., Schiffer, E., Stolzenburg, J. U., Tarnow, L., Theodorescu, D., Thongboonkerd, V., Vanholder, R., Weissinger, E. M., Mischak, H., and Schmitt-Kopplin, P. (2010) Naturally occurring human urinary peptides for use in diagnosis of chronic kidney disease. *Mol. Cell. proteomics MCP* 9, 2424–2437

50. Pejchinovski, M., Siwy, J., Mullen, W., Mischak, H., Petri, M. A., Burkly, L. C., and Wei, R. (2018) Urine peptidomic biomarkers for diagnosis of patients with systemic lupus erythematosus. *Lupus* 27, 6–16

51. Wu, J., Wang, N., Wang, J., Xie, Y., Li, Y., Liang, T., Wang, J., Yin, Z., He, K., and Chen, X. (2010) Identification of a uromodulin fragment for diagnosis of IgA nephropathy. *Rapid Commun. Mass Spectrom.* 24, 1971–1978

52. Ling, X. B., Sigdel, T. K., Lau, K., Ying, L., Lau, I., Schilling, J., and Sarwal, M. M. (2010) Integrative urinary peptidomics in renal transplantation identifies biomarkers for acute rejection. *J.*
Urinary peptidomics in early type 1 diabetes

*Am. Soc. Nephrol.* 21, 646–53

53. El-Achkar, T. M., and Wu, X.-R. (2012) Uromodulin in kidney injury: an instigator, bystander, or protector? *Am. J. Kidney Dis.* 59, 452–61

54. Chambers, R., Groufsky, A., Hunt, J. S., Lynn, K. L., and McGiven, A. R. (1986) Relationship of abnormal Tamm-Horsfall glycoprotein localization to renal morphology and function. *Clin. Nephrol.* 26, 21–6

55. Schmid, M., Prajczer, S., Gruber, L. N., Bertocchi, C., Gandini, R., Pfaller, W., Jennings, P., and Joannidis, M. (2010) Uromodulin Facilitates Neutrophil Migration Across Renal Epithelial Monolayers. *Cell. Physiol. Biochem.* 26, 311–318

56. Toma, G., Bates, J. M., and Kumar, S. (1994) Uromodulin (Tamm-Horsfall Protein) Is a Leukocyte Adhesion Molecule. *Biochem. Biophys. Res. Commun.* 200, 275–282

57. LaFavers, K. A., Macedo, E., Garimella, P. S., Lima, C., Khan, S., Myslinski, J., McClintick, J., Witzmann, F. A., Winfree, S., Phillips, C. L., Hato, T., Dagher, P. C., Wu, X.-R., El-Achkar, T. M., and Micanovic, R. (2019) Circulating uromodulin inhibits systemic oxidative stress by inactivating the TRPM2 channel. *Sci. Transl. Med.* 11, eaaw3639

58. Jennings, P., Aydin, S., Kotanko, P., Lechner, J., Lhotta, K., Williams, S., Thakker, R. V, and Pfaller, W. (2007) Membrane targeting and secretion of mutant uromodulin in familial juvenile hyperuricemic nephropathy. *J. Am. Soc. Nephrol.* 18, 264–73

59. Rindler, M. J., Naik, S. S., Li, N., Hoops, T. C., and Peraldi, M. N. (1990) Uromodulin (Tamm-Horsfall glycoprotein/uromucoid) is a phosphatidylinositol-linked membrane protein. *J. Biol. Chem.* 265, 20784–9

60. Brunati, M., Perucca, S., Han, L., Cattaneo, A., Consolato, F., Andolfo, A., Schaeffer, C., Olinger, E., Peng, J., Santambrogio, S., Perrier, R., Li, S., Bokhove, M., Bachi, A., Hummler, E., Devuyst, O., Wu, Q., Jovine, L., and Rampoldi, L. (2015) The serine protease hepsin mediates urinary
Urinary peptidomics in early type 1 diabetes

secretion and polymerisation of Zona Pellucida domain protein uromodulin. *Elife* 4, e08887

61. Tokonami, N., Takata, T., Beyeler, J., Ehrbar, I., Yoshifuji, A., Christensen, E. I., Loffing, J., Devuyst, O., and Olinger, E. G. (2018) Uromodulin is expressed in the distal convoluted tubule, where it is critical for regulation of the sodium chloride cotransporter NCC. *Kidney Int.* 94, 701–715

62. Olinger, E., Lake, J., Sheehan, S., Schiano, G., Takata, T., Tokonami, N., Debaix, H., Consolato, F., Rampoldi, L., Korstanje, R., and Devuyst, O. (2019) Hepsin-mediated Processing of Uromodulin is Crucial for Salt-sensitivity and Thick Ascending Limb Homeostasis. *Sci. Rep.* 9, 12287

63. Tsuji, A., Torres-Rosado, A., Arai, T., Le Beau, M. M., Lemons, R. S., Chou, S. H., and Kurachi, K. (1991) Hepsin, a cell membrane-associated protease. Characterization, tissue distribution, and gene localization. *J. Biol. Chem.* 266, 16948–53

64. Wilson, P. C., Wu, H., Kirita, Y., Uchimura, K., Ledru, N., Rennke, H. G., Welling, P. A., Waikar, S. S., and Humphreys, B. D. (2019) The single-cell transcriptomic landscape of early human diabetic nephropathy. *Proc. Natl. Acad. Sci.* 116, 19619–19625

65. Wu, H., Malone, A. F., Donnelly, E. L., Kirita, Y., Uchimura, K., Ramakrishnan, S. M., Gaut, J. P., and Humphreys, B. D. (2018) Single-Cell Transcriptomics of a Human Kidney Allograft Biopsy Specimen Defines a Diverse Inflammatory Response. *J. Am. Soc. Nephrol.* 29, 2069–2080

66. Torres-Rosado, A., O’Shea, K. S., Tsuji, A., Chou, S. H., and Kurachi, K. (1993) Hepsin, a putative cell-surface serine protease, is required for mammalian cell growth. *Proc. Natl. Acad. Sci. U. S. A.* 90, 7181–5

67. Kazama, Y., Hamamoto, T., Foster, D. C., and Kisiel, W. (1995) Hepsin, a Putative Membrane-associated Serine Protease, Activates Human Factor VII and Initiates a Pathway of Blood Coagulation on the Cell Surface Leading to Thrombin Formation. *J. Biol. Chem.* 270, 66–72
Urinary peptidomics in early type 1 diabetes

68. Wilkinson, D. J., Desilets, A., Lin, H., Charlton, S., del Carmen Arques, M., Falconer, A., Bullock, C., Hsu, Y.-C., Birchall, K., Hawkins, A., Thompson, P., Ferrell, W. R., Lockhart, J., Plevin, R., Zhang, Y., Blain, E., Lin, S.-W., Leduc, R., Milner, J. M., and Rowan, A. D. (2017) The serine proteinase hepsin is an activator of pro-matrix metalloproteinases: molecular mechanisms and implications for extracellular matrix turnover. *Sci. Rep.* 7, 16693

69. Prox, J., Arnold, P., and Becker-Pauly, C. (2015) Meprin α and meprin β: Procollagen proteinases in health and disease. *Matrix Biol.* 44–46, 7–13

70. Yura, R. E., Bradley, S. G., Ramesh, G., Reeves, W. B., and Bond, J. S. (2009) Meprin A metalloproteinases enhance renal damage and bladder inflammation after LPS challenge. *Am. J. Physiol. Renal Physiol.* 296, F135-44

71. Herzog, C., Haun, R. S., Kaushal, V., Mayeux, P. R., Shah, S. V., and Kaushal, G. P. (2009) Meprin A and meprin α generate biologically functional IL-1β from pro-IL-1β. *Biochem. Biophys. Res. Commun.* 379, 904–908

72. Herzog, C., Haun, R. S., Ludwig, A., Shah, S. V, and Kaushal, G. P. (2014) ADAM10 is the major sheddase responsible for the release of membrane-associated meprin A. *J. Biol. Chem.* 289, 13308–22

73. Mathew, M. M., Han, N. V., Murugesan, A., Raj, E. A., and Prasanth, K. G. (2015) Evaluation of the protective effect of Pterocarpus marsupium on acetic acid-induced ulcerative colitis in rats. *Inflammopharmacology* 23, 195–201

74. Mathew, R., Futterweit, S., Valderrama, E., Tarectecan, A. A., Bylander, J. E., Bond, J. S., and Trachtman, H. (2005) Meprin-alpha in chronic diabetic nephropathy: interaction with the renin-angiotensin axis. *Am. J. Physiol. Renal Physiol.* 289, F911-21

75. Bylander, J. E., Ahmed, F., Conley, S. M., Mwiza, J.-M., and Ongeri, E. M. (2017) Meprin Metalloprotease Deficiency Associated with Higher Mortality Rates and More Severe Diabetic Kidney Injury in Mice with STZ-Induced Type 1 Diabetes. *J. Diabetes Res.* 2017, 1–11
Urinary peptidomics in early type 1 diabetes

76. Cao, L., Sedighi, R., Boston, A., Premadasa, L., Pinder, J., Crawford, G. E., Jegede, O. E., Harrison, S. H., Newman, R. H., and Ongeri, E. M. (2018) Undiagnosed Kidney Injury in Uninsured and Underinsured Diabetic African American Men and Putative Role of Meprin Metalloproteases in Diabetic Nephropathy. *Int. J. Nephrol.* 2018, 6753489

77. Conley, S. M., Abais, J. M., Boini, K. M., and Li, P.-L. (2017) Inflammasome Activation in Chronic Glomerular Diseases. *Curr. Drug Targets* 18,

78. Anders, H.-J., and Muruve, D. A. (2011) The Inflammasomes in Kidney Disease. *J. Am. Soc. Nephrol.* 22, 1007–1018

79. Liu, D., Wen, Y., Tang, T.-T., Lv, L.-L., Tang, R.-N., Liu, H., Ma, K.-L., Crowley, S. D., and Liu, B.-C. (2015) Megalin/Cubulin-Lysosome-mediated Albumin Reabsorption Is Involved in the Tubular Cell Activation of NLRP3 Inflammasome and Tubulointerstitial Inflammation. *J. Biol. Chem.* 290, 18018–18028

80. Larionov, A., Dahlke, E., Kunke, M., Zanon Rodriguez, L., Schiessl, I. M., Magnin, J.-L., Kern, U., Alli, A. A., Mollet, G., Schilling, O., Castrop, H., and Theilig, F. (2019) Cathepsin B increases ENaC activity leading to hypertension early in nephrotic syndrome. *J. Cell. Mol. Med.*, jcmcm.14387

81. Peres, G. B., Juliano, M. A., Simões, M. J., and Michelacci, Y. M. (2013) Lysosomal enzymes are decreased in the kidney of diabetic rats. *Biochim. Biophys. Acta* 1832, 85–95

82. Liu, W. J., Shen, T. T., Chen, R. H., Wu, H.-L., Wang, Y. J., Deng, J. K., Chen, Q. H., Pan, Q., Huang Fu, C., Tao, J., Liang, D., and Liu, H. (2015) Autophagy-Lysosome Pathway in Renal Tubular Epithelial Cells Is Disrupted by Advanced Glycation End Products in Diabetic Nephropathy. *J. Biol. Chem.* 290, 20499–20510

83. Höhne, M., Frese, C. K., Grahammer, F., Dafinger, C., Ciarimboli, G., Butt, L., Binz, J., Hackl, M. J., Rahmatollahi, M., Kann, M., Schneider, S., Altintas, M. M., Schermer, B., Reinheckel, T., Göbel, H., Reiser, J., Huber, T. B., Kramann, R., Seeger-Nukpezah, T., Liebau, M. C., Beck, B.
Urinary peptidomics in early type 1 diabetes

B., Benzing, T., Beyer, A., and Rinschen, M. M. (2018) Single-nephron proteomes connect morphology and function in proteinuric kidney disease. *Kidney Int.* 93, 1308–1319

84. Suh, M.-J., Tovchigrechko, A., Thovarai, V., Rolfe, M. A., Torralba, M. G., Wang, J., Adkins, J. N., Webb-Robertson, B.-J. M., Osborne, W., Cogen, F. R., Kaplowitz, P. B., Metz, T. O., Nelson, K. E., Madupu, R., and Pieper, R. (2015) Quantitative Differences in the Urinary Proteome of Siblings Discordant for Type 1 Diabetes Include Lysosomal Enzymes. *J. Proteome Res.* 14, 3123–35

85. Magagnotti, C., Zerbini, G., Fermo, I., Carletti, R. M., Bonfanti, R., Vallone, F., and Andolfo, A. (2019) Identification of nephropathy predictors in urine from children with a recent diagnosis of type 1 diabetes. *J. Proteomics* 193, 205–216

86. Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., and Zychlinsky, A. (2004) Neutrophil Extracellular Traps Kill Bacteria. *Science (80-. ).* 303, 1532–1535

87. Wong, S. L., Demers, M., Martinod, K., Gallant, M., Wang, Y., Goldfine, A. B., Kahn, C. R., and Wagner, D. D. (2015) Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. *Nat. Med.* 21, 815–819

88. Takahashi, T., Hato, F., Yamane, T., Inaba, M., Okuno, Y., Nishizawa, Y., and Kitagawa, S. (2000) Increased spontaneous adherence of neutrophils from type 2 diabetic patients with overt proteinuria: possible role of the progression of diabetic nephropathy. *Diabetes Care* 23, 417–8

89. Galkina, E., and Ley, K. (2006) Leukocyte recruitment and vascular injury in diabetic nephropathy. *J. Am. Soc. Nephrol.* 17, 368–77

90. Fardon, N. J. M., Wilkinson, R., and Thomas, T. H. (2002) Abnormalities in primary granule exocytosis in neutrophils from Type I diabetic patients with nephropathy. *Clin. Sci. (Lond).* 102, 69–75
Urinary peptidomics in early type 1 diabetes

91. Davis, J. O., Hartoft, P. M., Titus, E. O., Carpenter, C. C. J., Ayers, C. R., and Spiegel, H. E. (1962) The role of the renin-angiotensin system in the control of aldosterone secretion. *J. Clin. Invest.* 41, 378–389

92. Nehme, A., Cerutti, C., Dhaouadi, N., Gustin, M. P., Courand, P.-Y., Zibara, K., and Bricca, G. (2015) Atlas of tissue renin-angiotensin-aldosterone system in human: A transcriptomic meta-analysis. *Sci. Rep.* 5, 10035

93. Velez, J. C. Q., Ryan, K. J., Harbeson, C. E., Bland, A. M., Budisavljevic, M. N., Arthur, J. M., Fitzgibbon, W. R., Raymond, J. R., and Janech, M. G. (2009) Angiotensin I Is Largely Converted to Angiotensin (1-7) and Angiotensin (2-10) by Isolated Rat Glomeruli. *Hypertension* 53, 790–797

94. Domenig, O., Manzel, A., Grobe, N., Königshausen, E., Kaltenecker, C. C., Kovarik, J. J., Stegbauer, J., Gurley, S. B., van Oyen, D., Antlanger, M., Bader, M., Motta-Santos, D., Santos, R. A., Elased, K. M., Säemann, M. D., Linker, R. A., and Poglitsch, M. (2016) Neprilysin is a Mediator of Alternative Renin-Angiotensin-System Activation in the Murine and Human Kidney. *Sci. Rep.* 6, 33678

95. Brar, G. S., Barrow, B. M., Watson, M., Griesbach, R., Choung, E., Welch, A., Ruzsicska, B., Raleigh, D. P., and Zraika, S. (2017) Neprilysin Is Required for Angiotensin-(1–7)’s Ability to Enhance Insulin Secretion via Its Proteolytic Activity to Generate Angiotensin-(1–2). *Diabetes* 66, 2201–2212

96. Pereira, R. M., dos Santos, R. A. S., Teixeira, M. M., Leite, V. H. R., Costa, L. P., da Costa Dias, F. L., Barcelos, L. S., Collares, G. B., and Simões e Silva, A. C. (2007) The renin–angiotensin system in a rat model of hepatic fibrosis: Evidence for a protective role of Angiotensin-(1–7). *J. Hepatol.* 46, 674–681

97. Su, Z., Zimpelmann, J., and Burns, K. D. (2006) Angiotensin-(1–7) inhibits angiotensin II-stimulated phosphorylation of MAP kinases in proximal tubular cells. *Kidney Int.* 69, 2212–2218
Tables:

Table 1. Clinical characteristics of the discovery cohort at time of study enrollment. Data are presented as mean ± standard deviation, except for sex (frequency). P values are shown between youths with and without type 1 diabetes (T1D).

| Clinical Characteristics                  | Youths without T1D (N = 15) | Youths with T1D (N = 15) | P      |
|------------------------------------------|-----------------------------|--------------------------|--------|
| Age (years)                              | 13.3 ± 1.7                  | 13.1 ± 1.5               | 0.7403 |
| Sex (females/males)                      | 6/9                         | 6/9                      | 1.0000 |
| Glycated hemoglobin, HbA1c (%)           | 5.4 ± 0.3                   | 8.5 ± 1.1                | 0.0000 |
| Diabetes duration (years)                | 0.0 ± 0.0                   | 7.1 ± 2.7                | 0.0000 |
| Body mass index, BMI (kg/m²)             | 20.4 ± 2.1                  | 21.4 ± 5.0               | 0.4804 |
| z-score BMI                              | 0.54 ± 0.89                 | 0.65 ± 1.38              | 0.8047 |
| Systolic blood pressure, SBP (mmHg)      | 109 ± 11                    | 114 ± 10                 | 0.1952 |
| z-score SBP                              | 0.03 ± 0.91                 | 0.42 ± 0.70              | 0.2048 |
| Diastolic blood pressure, DBP (mmHg)     | 63 ± 5                      | 70 ± 7                   | 0.0062 |
| z-score DBP                              | -0.01 ± 0.43                | 0.52 ± 0.66              | 0.0144 |
| Albumin/creatinine ratio, ACR (mg/mmol)  | 0.5 ± 0.3                   | 0.7 ± 0.6                | 0.1598 |
| Estimated glomerular filtration rate, eGFR (ml/min/1.73m²) | 118 ± 25                  | 141 ± 27                 | 0.0263 |
| Time between enrolment and urine collection (years) | 2.7 ± 0.5                  | 2.5 ± 0.7               | 0.5899 |
Table 2. Clinical characteristics of the validation cohort at time of urine collection. Data are presented as mean ± standard deviation, except for sex (frequency). *P* values are shown between youths with and without type 1 diabetes.

| Clinical Characteristics                  | Youths without T1D (N = 15) | Youths with T1D (N = 15) | *P*  |
|------------------------------------------|-----------------------------|--------------------------|------|
| Age (years)                              | 16.0 ± 1.2                  | 16.5 ± 1.1               | 0.2548|
| Sex (females/males)                      | 5/10                        | 6/9                      | 0.7047|
| Glycated hemoglobin, HbA1c (%)           | 5.1 ± 0.3                   | 9.1 ± 1.6                | 0.0000|
| Diabetes duration (years)                | 0.0 ± 0.0                   | 10.4 ± 2.9               | 0.0000|
| Body mass index, BMI (kg/m^2)            | 22.5 ± 4.8                  | 23.3 ± 4.2               | 0.6781|
| *z*-score BMI                            | 0.39 ± 1.67                 | 0.64 ± 1.03              | 0.6395|
| Systolic blood pressure, SBP (mmHg)      | 120 ± 11                    | 115 ± 11                 | 0.2746|
| *z*-score SBP                            | 0.56 ± 1.05                 | 0.01 ± 1.05              | 0.1989|
| Diastolic blood pressure, DBP (mmHg)     | 64 ± 9                      | 66 ± 8                   | 0.5621|
| *z*-score DBP                            | -0.20 ± 0.71                | -0.11 ± 0.67             | 0.7548|
| Albumin/creatinine ratio, ACR (mg/mmol)  | 0.6 ± 0.2                   | 1.1 ± 1.1                | 0.1554|
| Estimated glomerular filtration rate, eGFR (ml/min/1.73m^2) | 102 ± 15                   | 115 ± 24                 | 0.0721|
**Table 3. Summary of differentially excreted peptides from the discovery phase.** P values were determined using the independent t-test and adjusted by Benjamini-Hochberg procedure (Q). Fold change represents the ratio of the mean log-transformed intensities of youths with type 1 diabetes (T1D) to healthy controls (HC). AHSG, alpha-2-HS-glycoprotein; ALB, albumin; APOA1, apolipoprotein A1; CLU, clusterin; LTBP4, latent transforming growth factor beta binding protein 4; FXYD2, Na+/K+-transporting ATPase subunit gamma; UMOD, uromodulin.

| Peptide                  | Name   | Mass (Da) | Charge | Fold Change (T1D – HC) | P         | Q         |
|--------------------------|--------|-----------|--------|------------------------|-----------|-----------|
| SGSVIDQSRVLNLGPI         | UMOD-1 | 1653.9050 | 2,3    | 2.11                   | 0.0283    | 0.3298    |
| SGSVIDQSRVLNLGPIVTR     | UMOD-2 | 1911.0538 | 2,3,4  | 0.68                   | 0.0170    | 0.2768    |
| SVIDQSRVLNLGPI          | UMOD-3 | 1509.8515 | 1,2    | 6.72                   | 0.0001    | 0.0070    |
| SVIDQSRVLNLGPIT         | UMOD-4 | 1610.8992 | 1,2,3  | 3.95                   | 0.0003    | 0.0127    |
| SVIDQSRVLNLGPITR        | UMOD-5 | 1767.0003 | 2,3,4  | 3.84                   | < 0.0001  | 0.0003    |
| SVIDQSRVLNLGPITRK       | UMOD-6 | 1895.0952 | 2,3,4  | 3.75                   | 0.0035    | 0.0820    |
| VIDQSRVLNLGPI          | UMOD-7 | 1422.8195 | 1,2    | 5.48                   | 0.0005    | 0.0159    |
| DDGGPYGESEAPPPGPGRWP    | LTBP4  | 2208.9712 | 2,3    | 0.65                   | 0.0450    | 0.4948    |
| TGLSMDDGGGSPKGDVDPF     | FXYD2  | 1735.7723 | 2      | 0.38                   | 0.0093    | 0.1901    |
| SHTSDSDVPSGVTEVVKL     | CLU-1  | 1954.9848 | 2,3    | 2.55                   | 0.0195    | 0.2891    |
| HTSDSDVPSGVTEVVKL      | CLU-2  | 1867.9527 | 2,3    | 5.87                   | 0.0001    | 0.0096    |
| LSALEETYTKKLTQ          | APOA1  | 1636.8672 | 2,3    | 5.80                   | 0.0217    | 0.2948    |
| YGEMADCCAKQEPERNECFLQ   | ALB-1  | 2634.0607 | 2,3    | 2.12                   | 0.0138    | 0.2503    |
| VRYTCKVPQVSTPTL         | ALB-2  | 1715.9934 | 2,3,4  | 4.64                   | 0.0245    | 0.3067    |
| TVVQPSVGAAAGPVVPCPGIRH  | AHSG   | 2421.3063 | 3,4    | 1.92                   | 0.0019    | 0.0529    |
Table 4. Summary of differentially excreted uromodulin peptides from the validation phase.
Differential excretion was determined using the Mann-Whitney test \((P < 0.05)\). Fold change represents the ratio of the median peptide excretion of youths with type 1 diabetes (T1D) to the median peptide excretion of healthy controls (HC). AHSG, alpha-2-HS-glycoprotein; ALB, albumin; APOA1, apolipoprotein A1; CLU, clusterin; FXYD2, Na+/K+-transporting ATPase subunit gamma; LTBP4, latent transforming growth factor beta binding protein 4; UQ, unable to quantify; UMOD, uromodulin.

| Peptide          | Name   | Fold Change (T1D:HC) | P       |
|------------------|--------|----------------------|---------|
| SGSVIDQSRVLNLGPI | UMOD-1 | 2.91                 | 0.0183  |
| SGSVIDQSRVLNLGPI | UMOD-2 | 0.75                 | 0.0672  |
| SVIDQSRVLNLGPI  | UMOD-3 | 12.83                | 0.0002  |
| SVIDQSRVLNLGPI  | UMOD-4 | 4.16                 | 0.0006  |
| SVIDQSRVLNLGPI  | UMOD-5 | 4.41                 | 0.0004  |
| SVIDQSRVLNLGPI  | UMOD-6 | UQ                   | ---     |
| VIDQSRVLNLGPI   | UMOD-7 | 6.94                 | 0.0024  |
Table 5. Probability of bioactivity of differentially excreted peptides, as predicted by Peptide Ranker. The asterisk (*) denotes the peptides that were included as a positive control.

| Peptide                      | Protein/Name | Score |
|------------------------------|--------------|-------|
| CYFQNCPRG/Vasopressin*       | AVP          | 0.90  |
| DDGGPYGESEAPAPPGPGRWP        | LTBP4        | 0.81  |
| DRVYIHP/Angiotensin II*      | AGT          | 0.69  |
| TVQSVGAAGPVPPCPGRIRH         | AHSG         | 0.63  |
| YGEMADCCAKQEPERNECFLQ        | ALB-1        | 0.59  |
| SGSVIDQSRVNLNLGPI            | UMOD-1       | 0.47  |
| SGSVIDQSRVLNGLPITR           | UMOD-2       | 0.46  |
| SVIDQSRVNLGLPITR             | UMOD-6       | 0.35  |
| DRVYIHP/Angiotensin (1-7)*   | AGT          | 0.34  |
| TGLSMDGGGSPKGDVDPF           | FXYD2        | 0.33  |
| SVIDQSRVNLGLPI               | UMOD-3       | 0.29  |
| SVIDQSRVLNGLPIT              | UMOD-4       | 0.29  |
| SVIDQSRVLNGLPITR             | UMOD-5       | 0.29  |
| VIDQSRVNLGLPI                | UMOD-7       | 0.24  |
| SHTSDSDTPSVGTELVVKL          | CLU-1        | 0.19  |
| HTSDDVPSGVTEVVKL             | CLU-2        | 0.17  |
| LSALEELYTKKNLTQ              | APOA1        | 0.12  |
| VRYTKKVQVSTPTL               | ALB-2        | 0.11  |
**Table 6. Predicted proteases responsible for the generation of the differentially excreted uromodulin peptides.** The asterisk (*) denotes a known cleavage site. The arrows (↑ and ↓) indicate the direction of the average fold change in Nephroseq gene expression datasets on kidney biopsies from adults with diabetic nephropathy, compared to living or cadaveric donors. The studies are referenced as follow: (1) Woroniecka Diabetes TubInt, (2) Schmid Diabetes TubInt, (3) Ju CKD Glom, (4) Ju CKD TubInt, (5) Woroniecka Diabetes Glom, and (6) ERCB Nephrotic Syndrome TubInt.

| Protease (Gene Symbol) | MEROPS ID | Predicted Cleavage Sites | Fold change (ref.) |
|------------------------|-----------|--------------------------|--------------------|
| A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) | M12.210 | GPIT|RKGV | ↑ 1.7 (1,2) |
| Cathepsin B (CTSB) | C01.060 | FRSG|SVID | ↑ 1.9 (1) |
| Granzyme A (GZMA) | S01.135 | TRFR|SGSV PITR|KGVQ | ↑ 4.6 (1,3-6) |
| Hepsin (HPN) | S01.224 | TRFR|SGSV* PITR|KGVQ | ↓ 1.7 (2,3) |
| Kallikrein-2 (KLK2) | S01.161 | TRFR|SGSV | No change |
| Kallikrein-4 (KLK4) | S01.251 | ITRK|GVQA | No change |
| Kallikrein-6 (KLK6) | S01.236 | TRFR|SGSV | ↓ 4.1 (4,5) |
| Matrix metalloproteinase-2 (MMP2) | M10.003 | RSGS|VIDQ | ↑ 2.7 (3,6) |
| Matrix metalloproteinase-9 (MMP9) | M10.004 | GPIT|RKGV | ↑ 15.2 (6) |
| Matrix metalloproteinase-12 (MMP12) | M10.009 | GPIT|RKGV | No change |
| Matrix metalloproteinase-13 (MMP13) | M10.013 | GPIT|RKGV | No change |
| Meprin A (MEP1A) | M12.002 | FRSG|SVID | No change |
| Neprilysin (MME) | M13.001 | RSGS|VIDQ | ↓ 2.3 (3-5) |
| Neutrophil elastase (ELANE) | S01.131 | LGPI|TRKG | No change |
| Plasminogen (PLG) | S01.233 | TRFR|SGSV | ↓ 2.7 (3-4) |
| Matriptase-2 (TMPRSS6) | S01.308 | ITRK|GVQA | No change |
| Matriptase-3 (TMPRSS7) | S01.072 | PITR|KGVQ | Not found in database |
Urinary peptidomics in early type 1 diabetes

Figures:

Figure 1. Schematic outline of the urinary peptidomic protocol for the discovery phase.
Urinary peptidomics in early type 1 diabetes

Figure 2. General characterization of the urinary peptidomes of 15 youths with type 1 diabetes and 15 healthy controls. (A) Flow diagram describing the urinary peptidome. (B) Venn diagram of urinary peptides. The dotted line represents the 100% cut-off, in which 162 peptides were quantified in every sample. (C) Range of intensities of peptides found in one to thirty urines. Low-abundance peptides were found in relatively few samples, compared to high-abundance peptides. The range of intensities of the 162 peptides found in all 30 samples is highlighted in red. (D) Tissue origins of the total peptidome and the subset of 162 peptides using the Human Protein Atlas. (E) Volcano plot of the 162 recurring peptides. Fifteen peptides were differentially excreted (independent two-sample Student’s t-test, \( P < 0.05 \), purple); however, only five survived the Benjamini-Hochberg adjustment (\( Q < 0.05 \), red). (F) Heatmap representation of the 15 differentially excreted peptides with unsupervised clustering of samples. Log-transformed peptide intensities were converted into \( z \)-scores using means, wherein higher scores are indicated in red and lower scores in blue.
Figure 3. Boxplots for the fifteen differentially excreted peptides found in the discovery cohort (\(P < 0.05\)). Differential excretion was determined using the independent two-sample t-test after log-transforming peptide intensities (\(P < 0.05\)). HC, youths without T1D; T1D, youths with type 1 diabetes.
Figure 4. Evaluation of uromodulin peptides in validation cohort of thirty youths with and without type 1 diabetes. Differential excretion was determined using the Mann-Whitney test ($P < 0.05$). (A) Urinary excretion of uromodulin peptides measured by parallel reaction monitoring. (B) Urinary excretion of uromodulin protein measured using a commercial enzyme-linked immunosorbent assay kit. (C) Representative correlation plots between uromodulin peptides and protein. HC, youths without T1D; T1D, youths with type 1 diabetes.
Figure 5. Uromodulin peptides are bioactive in vitro. Technical replicates are shown as open circles (n = 3). (A) NFκB luciferase activity following uromodulin peptide treatment for 24 hours in HK-2 cells at graded concentrations (0.1 to 10 μM). Angiotensin (Ang II, 0.1 μM) served as a positive control. (B) NFκB luciferase activity following uromodulin peptide treatment (0.1 μM) for 24 hours with or without TAK-242 pre-treatment (0.3 μM) for 1 hour in HK-2 cells. Lipopolysaccharide (LPS, 0.1 μg/mL) served as a positive control. (C) AP-1 luciferase activity following uromodulin peptide treatment (0.1 μM) for 24 hours with or without gallein pre-treatment (10 μM) for 1 hour in HK-2 cells. Ang II served as a positive control. (D) NFκB luciferase activity following uromodulin protein treatment for 24 hours in HK-2 cells at graded concentrations (0.2 to 20 μg/L). Lipopolysaccharide (LPS, 0.1 μg/mL) served as a positive control. (E) Neutrophil chemotaxis following uromodulin peptide incubation toward the N-formyl-methionyl-leucyl-phenylalanine (fMLP) attractant. Migration was recorded for 15 min at a rate of 0.05 frames per second (fps) using time-lapse video microscopy. (F-G) IL-6 and IL-8 protein levels in conditioned media of primary proximal tubular epithelial cells following UMOD-1 treatment for 24 hours. LPS served as a positive control (1 μg/mL).

*P < 0.05, compared to untreated cells (N).

#P < 0.05, compared to cells treated with LPS alone.

&P < 0.05, compared to cells treated with UMOD-1 alone.

^P < 0.05, compared to cells treated with UMOD-2 alone.
Figure 6. Urinary cytokine and chemokine excretion of youths with and without diabetes. Differential excretion was determined using the Mann-Whitney test ($P < 0.05$). HC, youths without T1D; T1D, youths with type 1 diabetes.
**Urinary peptidomics in early type 1 diabetes**

**Figure 7. Understanding the proteolytic mechanisms responsible for generating the urinary peptidome.** (A) Flow diagram describing the selection of urinary peptides altered in diabetes for cleavage site analysis. From each section of the Venn diagram in Figure 2B, peptides were eligible for inclusion if they were identified in at least five of the fifteen samples per group, where appropriate. Missing LFQ data was imputed using the Perseus software. Of the 1941 eligible peptides common to both groups, 549 peptides were increased (with a fold change of at least 1.5) in diabetes, and 331 peptides were decreased (with a fold change of 0.67 and below). A total of 974 peptides was examined in subsequent cleavage site analyses. (B-C) Sequence logo of N- and C-termini of urinary peptides using iceLogo. Significant differences in percentage of amino acids at each position in the cleavage are shown ($P < 0.05$). Positive values indicate increased percentages in type 1 diabetes. (D) Proteolytic map of uromodulin and the predicted proteases. Differentially excreted peptides originate from a region near the C-terminus of the precursor uromodulin protein, which is shown in bold at the bottom with predicted cleavage sites and proteases. Full-length uromodulin consists of 640 residues.