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Diabetes mellitus (DM) is a complex disease characterized by insufficient insulin production and resultant hyperglycemia with alterations in fat and protein metabolism. With time these alterations cause secondary cellular dysfunctions and vascular damage including diabetic nephropathy, retinopathy, neuropathy, and macrovascular disease or vascular alterations. The most common types of DM are type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D is associated with destruction of insulin-producing β-cells in the islets of Langerhans in the pancreas, typically by an autoimmune mechanism, leading to insufficient insulin production. In contrast, T2D is caused by insulin resistance combined with insufficient insulin synthesis and is often associated with obesity.

Although all forms of DM are characterized by hyperglycemia and β-cell dysfunction, the pathogenesis of DM is variable, comprising different degrees of β-cell dysfunction, apoptosis, inflammation and immune responses. Proteome analysis holds the promise of delivering substantial insight into the pathophysiological changes associated with different types of DM. Urine represents an excellent specimen for proteome analysis, as it can be obtained in high quantities without the need for special collection procedures [1], shows higher stability than blood [2,3], and enables the identification of valid biomarkers for renal, as well

patients samples. As shown in **Figure 3C**, the corresponding ROC analysis resulted in an AUC value of 88% (95%-CI of 81–94%). Urine samples of both cohorts (discovery and validation set) were derived from patients without any measurable renal function loss. To verify if DN could interfere in the discrimination between T1D and T2D patients, the DTspP was applied to a further cohort of DM subjects with impaired renal function (n = 369). This classification resulted in an AUC in ROC analysis (**Figure 3D**) of 85% (95% CI of 81–88%; classification factors are listed in **Table S2**).

To identify those peptides significantly differentiating T1D and T2D patients in the validation cohort without (n = 108) and with (n = 369) renal impairment, we applied Mann-Whitney U-testing in these cohorts. This held true in the validation set for 70 markers in the normoalbuminuric patients group and 86 peptides in the kidney disease cohort. 57 candidates were significant in both groups ($P<0.05$) (**Table S3/set II**).

## Sequencing of DM specific and DM type specific biomarkers

We applied tandem mass spectrometry to obtain peptide

evidence of chronic kidney disease, these changes were significantly more pronounced in T2D than in T1D, despite the lower ACR and higher estimated GFR in T2D patients. This corresponds to the morphological observation that along with increased β-cell apoptosis, pancreatic islets from T2D patients contain amyloid deposits and resulting fibrosis [12,13]. In this context it is worth mentioning that extracellular matrix (ECM) homeostasis is maintained by the balance between tissue inhibitor of metalloproteinases (TIMP) and matrix metalloproteases (MMP). Decreased activity of certain MMPs (e.g. MMP-2, -3, and -9), as described in diabetes [14–16], would account for our finding of decreased urinary excretion of collagen fragments, since less collagen filaments would in this case be cleaved from the ECM. Our data support the hypothesis that physiological degradation of ECM components, especially collagen fibers, may be disturbed as a result of DM and this phenomenon would subsequently result in morphologically observed increased ECM deposits [15–17]. These data indicate a demand for further research to investigate the detailed relationship between MMPs/TIMPs/ECM in DM-associated complications in a systems approach, as recently suggested [18,19].

In addition, the data on the differences in urinary collagen fragments may indicate that the mechanism of attenuation of collagen degradation is different in T1D and T2D. In addition to MMPs, advanced glycemic end products (AGEs) are prominent candidates possibly responsible for a disturbance in collagen breakdown and chemical modification of collagen [20,21]. While we could not find reports indicating significant differences in protease activity or AGE status between T1D and T2D, both

glucose transport in late rodent pregnancy [30]. This effect could contribute to pregnancy associated changes in glucose homeostasis in gestational diabetes.

While uromodulin has previously been reported to be decreased in patients with DM [31–33], we observe the up-regulation of a uromodulin fragment without a C-terminal arginine residue (**Table 3**). This may be a result of increased proteolytic activity in DM, resulting in decrease of the parental protein, but increase in degradation products. However, this hypothesis requires further investigation.

Several approaches aiming at the analysis of differently regulated proteins in body fluids from patients with T1D and

In conclusion, this work gives clear and valid evidence, based on a multicenter cohort, of differences in the urinary proteome of T1D versus T2D patients with normal renal function, validated also in those with chronic kidney disease. Future studies should enable identification of not yet sequenced differentially expressed peptides and determine how these differences can be exploited for disease monitoring and therapeutic issues. However, the vast amount of data reported here and available today clearly suggest that alterations in the remodeling of extracellular matrix, and likely in endogenous proteolytic activity, are among the hallmarks of DM. These pathophysiological changes likely represent promising targets for pharmacological intervention, aiming specifically at prevention of diabetes-associated vascular complications. Further, the alterations in urinary ECM degradation products show significant differences between T1D and T2D.

## Patient characteristics and study design

Urine samples were collected as described previously [43], in agreement with the protocol established by HUPO (www.hupo.org/research/urine) and EuroKUP (www.eurokup.org). In short,

## Sample preparation

A 0.7 mL aliquot of urine was thawed immediately before use and diluted with 0.7 mL 2 M urea, 10 mM $NH_4OH$ containing 0.02% SDS. In order to remove high molecular weight compounds of urine, samples were filtered using Centrisart ultracentrifugation filter devices (20 kDa molecular weight cut-off; Sartorius, Goettingen, Germany) at 3,000 g until 1.1 mL of filtrate was obtained. Subsequently, filtrate was desalted using a PD-10 column (GE Healthcare, Sweden) equilibrated in 0.01% $NH_4OH$ in HPLC-grade water. Finally, samples were lyophilized and stored at 4°C. Shortly before CE-MS analysis, lyophilisates were resuspended in HPLC-grade water to a final protein concentration of 0.8 μg/μL as verified by BCA assay (Interchim, Montlucon, France).

## Urinary proteome analysis

CE-MS analysis was performed as described previously [2,40]. By this procedure the average recovery rate in the preparation procedure was ~85% and the limit of detection was ~1 fmol [40]. Mass resolution was controlled to be above 8,000 enabling resolution of monoisotopic mass signals for $z \leq 6$. After charge deconvolution, mass deviation was <25 ppm for monoisotopic resolution and <100 ppm for unresolved peaks ($z > 6$). The analytical precision of the set-up was assessed by reproducibility achieved for repeated measurements of the same replicate and by the reproducibility achieved for repeated preparations and measurements of the same urine sample [40]. To ensure high data consistency, a minimum of 950 peptides/proteins had to be detected with a minimal MS resolution of 8,000 in a minimal migration time interval of 10 minutes. By following this set-up, CE-MS enabled reproducible and robust high-resolution urinary proteome analysis.

## Data processing

Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaiquesVisu software [48]. For noise filtering, signals with $z > 1$ observed in a minimum of 3 consecutive spectra with a signal-to-noise ratio of at least 4 were considered. MosaiquesVisu employs a probabilistic clustering algorithm and uses both isotopic distribution (for $z \leq 6$) as well as conjugated masses for charge-state determination of peptides/proteins. The resulting peak list

characterizes each polypeptide by its mass and its migration time. TOF-MS data were calibrated utilizing 80 reference masses exactly determined by Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). For calibration, linear regression is performed. Both capillary electrophoresis (CE)-migration time and ion signal intensity (amplitude) show variability, mostly due to different amounts of salt and peptides in the sample and were consequently normalized. Reference signals of more then 1700 urinary polypeptides were used for CE-time calibration by local regression [49]. For normalization of analytical and urine dilution variances, MS signal intensities were normalized relative to 29 ''housekeeping'' peptides with small relative standard deviation. For calibration, local regression is performed [50]. The obtained peak lists characterized each polypeptide by its molecular mass [Da], normalized CE migration time [min] and normalized signal intensity. To avoid artifacts (specific individual peptides) only detected peptides with frequency >20% were deposited, matched, and annotated in a Microsoft SQL database allowing further statistical analysis. For clustering, peptides in different samples were considered identical, if mass deviation was ≤50 ppm for small or ≤75 ppm for larger peptides. Due to analyte diffusion effects, CE peak widths increase with CE migration time. In the data clustering process this effect was considered by linearly increasing cluster widths over the entire electropherogram (19 min to 45 min) from 2–5%. After calibration, mean deviation of migration time was controlled to be below 0.35 minutes. All annotated data are available in **Table S5C**.

## Statistical analysis

**Patients' data analysis.**