Effect of treatment with GLP-1R agonists on the urinary peptidome of T2DM patients

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Abstract

Type II diabetes mellitus (T2DM) accounts for approximately 90% of all diabetes mellitus cases in the world. Glucagon-like peptide-1 receptor (GLP-1R) agonists have established an increased capability to target directly or indirectly six core defects associated with T2DM, while, the underlying molecular mechanisms of these pharmacological effects are not fully known. This exploratory study was conducted to analyze the effect of treatment with GLP-1R agonists on urinary peptidome of T2DM patients. Urine samples of thirty-two T2DM patients from the PROVALID study (A Prospective Cohort Study in Patients with T2DM for Validation of Biomarkers) collected at pre- and post-treatment with GLP-1R agonist drugs were analyzed by CE-MS. In total, 70 urinary peptides were significantly affected by GLP-1R agonist treatment; generating from 26 different proteins. The downregulation of MMP proteases, based on the concordant downregulation of urinary collagen peptides was highlighted. Treatment also resulted in downregulation of peptides from SERPINA1, APOC3, CD99, CPSF6, CRNN, SERPINA6, HBA2, MB, VGF, PIGR and TTR, many of which were previously found to be associated with increased insulin resistance and inflammation. The findings indicate potential molecular mechanisms of GLP-1R agonists in the context of management of T2DM and prevention or delaying the progression of its associated diseases.

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Abbreviations:

ADAMTS5, A disintegrin and metalloproteinase with thrombospondin motifs 5; APOC3, apolipoprotein C-III; AUC, area under the curve; BMI, body mass index; CD99, CD99 antigen; CE-MS, capillary electrophoresis coupled to mass spectrometry; COL1A1, collagen alpha-1(I) chain; COL1A2, collagen alpha-2(I) chain; COL3A1, collagen alpha-1(III) chain; CRNN, Cornulin; CPSF6, cleavage and polyadenylation specificity factor subunit 6; eGFR, estimated glomerular filtration rate; FDR, false discovery rates; GLP-1R, Glucagon-like peptide-1 receptor; HbA1C, Hemoglobin A1C; HBA, hemoglobin subunit alpha; KEGG, Kyoto Encyclopedia of Genes and Genomes; MB, myoglobin; MMP, matrix metalloproteinase proteases; PIGR, polymeric immunoglobulin receptor; PROVALID, Prospective Cohort Study in Patients with T2DM for Validation of Biomarkers study; SBP/DBP, systolic/diastolic blood pressure SERPINA1, alpha-1-antitrypsin; SERPINA6, corticosteroid-binding globulin; T2DM, Type II diabetes mellitus; TTR, transthyretin; VGF, neurosecretory protein VGF

Keywords: CE-MS, GLP-1R agonists, peptidomics, T2DM, urine biomarker

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Abstract:

Type II diabetes mellitus (T2DM) accounts for approximately 90% of all diabetes mellitus cases in the world. Glucagon-like peptide-1 receptor (GLP-1R) agonists have established an increased capability to target directly or indirectly six core defects associated with T2DM, while, the underlying molecular mechanisms of these pharmacological effects are not fully known. This exploratory study was conducted to analyze the effect of treatment with GLP-1R agonists on urinary peptidome of T2DM patients. Urine samples of thirty-two T2DM patients from the PROVALID study (A Prospective Cohort Study in Patients with T2DM for Validation of Biomarkers) collected at pre- and post-treatment with GLP-1R agonist drugs were analyzed by CE-MS. In total, 70 urinary peptides were significantly affected by GLP-1R agonist treatment; generating from 26 different proteins. The downregulation of MMP proteases, based on the concordant downregulation of urinary collagen peptides was highlighted. Treatment also resulted in downregulation of peptides from SERPINA1, APOC3, CD99, CPSF6, CRNN, SERPINA6, HBA2, MB, VGF, PIGR and TTR, many of which were previously found to be associated with increased insulin resistance and inflammation. The findings indicate potential molecular mechanisms of GLP-1R agonists in the context of management of T2DM and prevention or delaying the progression of its associated diseases.

Statement of significance:

In 2021, the International Diabetes Federation (IDF) predicted a five-fold increase in the worldwide adult population of diabetes mellitus to 783 million in 2045; of which type II diabetes mellitus (T2DM) is said to account for approximately 90% of all cases. T2DM is characterized as a chronic disease and is diagnosed with increased levels of blood glucose or hyperglycemia. Since no cure exists, efforts to control and treat T2DM have escalated. Treatment with glucagon-like peptide-1 receptor (GLP-1R) agonists have dramatically transformed patient care guidelines for T2DM. In comparison to the other anti-hyperglycemic medications, GLP-1R agonists have established an increased capability to target directly or indirectly six out of the eight core defects associated with T2DM, also portraying beneficial cardiovascular and renal outcomes. The focus of research thus needs to shift from investigating the pharmacological effects of GLP-1R agonists, to understanding the underlying molecular mechanisms. In this exploratory study, we aimed to assess the effect of treatment with GLP-1R agonists on the urinary peptidome of T2DM patients, in an untargeted peptidomics approach. The urinary peptides identified indicate potential effect of GLP-1R agonists on reduction of insulin resistance and inflammation in the context of prevention, delaying the progression and management of T2DM.

Introduction

In 2021, the International Diabetes Federation predicted a five-fold increase in the worldwide adult population of diabetes mellitus, from 150 million in 2000[1] to 783 million in 2045[2]; of which type II diabetes mellitus

(T2DM) is said to account for approximately 90% of all cases[3]. T2DM is characterized as a chronic disease[4] and is diagnosed with increased levels of blood glucose or hyperglycemia[5]. T2DM has been associated with macrovascular complications, such as atherosclerosis, myocardial infarction and diabetic foot syndrome; as well as microvascular complications, such as neuropathy, retinopathy, and nephropathy[6-8]. Approximately 44% of all T2DM cases have been linked to obesity, correlating an exponential increase in their worldwide prevalence[9, 10]. Additionally, the World Health Organization predicts 57.8% of the world population to be overweight and obese by 2030[11].

Since no cure exists, efforts to control and treat T2DM have escalated[12]. The American Diabetes Association recommends healthy lifestyle changes as the first-in-line response, followed by metformin-based pharmaceutical interventions[13]. The approach of lowering glucose plasma levels remains the most sought-after treatment and has recently witnessed novel and effective advances. These alongside metformin, commonly include treatment with glucagon-like peptide-1 receptor (GLP-1R) agonists, sodium-glucose co-transporter-2 (SGLT-2) inhibitors, dipeptidyl peptidase-4 (DPP-4) inhibitors and insulin pumps followed by continuous monitoring of glucose levels[8]. Furthermore, research focusing on treatment paradigms targeting the 'ominous octet' of T2DM, implicated in the development and progression of hyperglycemia, has gained momentum in the last couple of decades.

The GLP-1R agonists have dramatically transformed patient care guidelines for T2DM[14]. In comparison to the other antihyperglycemic medications, GLP-1R agonists have established an increased capability to target directly or indirectly six out of the eight core defects ('ominous octet') associated with T2DM[15-17]. In response to food intake, the intestinal L-cells dependent secretion of GLP-1 is significantly impaired in T2DM patients, which affects the pancreatic β cells dependent insulin secretion; eventually resulting in hyperglycemia[18-20]. The synthetically produced GLP-1R agonists stimulate GLP-1R, in a similar fashion as the native GLP-1. Treatment benefits of peptide-based GLP-1R agonists include glycemic control, weight loss, delayed onset of macroalbuminuria, prevention of clinical hyperglycemic episodes and cardiovascular events, and reduced estimated glomerular filtration rate (eGFR) decline; that in turn indirectly result in the inhibition of glucagon secretion and increased secretion of insulin with minimal hypoglycemic risks[8, 21-23].

Since their first approval in 2005, seven GLP-1R agonists drugs have been developed for glycemic control in T2DM patients. In 2021, Trujillo*et al.* , described fourteen head-to-head trials conducted on these seven GLP-1R agonists, the results of which continue to establish the benefits of this class of drugs in treatment of T2DM patients. The authors further highlighted studies that have published beneficial cardiovascular and renal outcomes of the treatment; however, the results, in terms of the magnitude for glycemic control and adverse effects are not consistent[24]. The focus of research thus needs to shift from investigating the pharmacological effects of GLP-1R agonists, to understanding the underlying molecular mechanisms.

Recent advances in the analyses of urinary peptidome, have paved way for the detection of alterations at both pathological as well as physiological levels in chronic diseases[25]. Urine-based omics studies have especially garnered relevance, due to the ease of sample collection, longer stability of the peptides, complex composition (from blood, kidney, and bladder) and the possibility of larger cohort studies because of its non-invasive approach. In addition, the water soluble and charged nature of urinary peptides enable their uncomplicated mass spectrometric (MS) based detections[26]. The urinary peptidomic analysis in this study was performed by a capillary electrophoresis coupled to mass spectrometry (CE-MS) technique, which supports the identification of naturally occurring urinary peptides and peptidomic changes in response to drug interventions[27]. Therefore, in this exploratory study, we aimed to assess the effect of treatment with GLP-1R agonists on the urinary peptidome of T2DM patients, in an untargeted peptidomics approach.

Materials and Methods

Study population and sample collection

Used were urine samples of thirty-two T2DM patients, administering anti-hypertensive medications at the primary health care level, recruited within the Prospective Cohort Study in Patients with T2DM for Validation of Biomarkers (PROVALID) study. PROVALID is an observational, prospective cohort study in

five European countries with detailed information on inclusion and exclusion criteria of the patient selection previously provided[28]. The study was approved by the Ethics Committee of the Medical University of Innsbruck (Nr. 1188/2020). Consent was obtained from all the patients. Urine samples were collected from the patients at their visit just before GLP-1R agonists prescription and labelled as pre-treatment samples. The urine samples collected at the first visit after the treatment initiation were labelled as post-treatment samples. All the sixty-four urine samples were stored at -20°C until peptidomic analysis.

Sample preparation and CE-MS analysis

The standard operating protocols describing urine sample preparation and extraction of peptides followed in this study, have been applied in numerous studies as reviewed previously [29, 30]. The CE-MS analysis was conducted as described in detail by Zurbig *et al.* [31], utilizing a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA) coupled with a Micro-TOF II MS (Bruker Daltonic, Bremen, Germany) instrument. Literary evidence on the advantages of CE-MS analysis in terms of reproducibility, sensitivity, precision and accuracy are extensively available[32]. The relative peptide intensities were normalized based on an internal standard of 29 stable collagen peptides, that can be detected naturally in urine samples of healthy and diseased. This calibration was performed for normalizing the variability in peptide intensities[33]. The resulting peptides and their normalized intensity values were stored in an internal Microsoft SQL database[34], which enabled the comparison of the pre- and post-treatment urinary peptide profiles. For identification of the peptide sequences, MS/MS based analysis by a Dionex Ultimate 3000 RSLS nanoflow system (Dionex, Camberley, UK) or a Beckman P/ACE MDQ CE that was coupled to an Orbitrap Velos MS instrument (Thermo Fisher Scientific Inc., Boston, MA, USA) was performed, as described previously[35].

Statistical analysis

All statistical analyses in this study were performed using R programming (R version 4.2.2, R Foundation for Statistical Computing, Vienna, Austria with IDE: R Studio Version 1.2.5, Boston, MA, USA). As a pre-requisite for the analysis, thresholds of 30% (i.e., [?] 10 out of 32 datasets) peptide frequency (in at least one treatment group) were applied. Alongside, area under the receiver operating curve (ROC) curve (AUC) values were calculated by the DeLong approach, to compare the urinary peptide profiles between the pre- and post-treated samples; the selected urinary peptides passed a threshold of AUC [?] 0.60. The normally distributed and continuous datasets generated from CE-MS based peptide profiles of the urine samples, obtained from pre-treatment (n =32) and post-treatment (n =32); were compared by a paired Wilcoxon rank-sum test, using the row_wilcoxon_paired() function from the matrixTests package. A p -value < 0.05 considered statistically significant, was further adjusted for false discovery rates (FDR) by the Benjamini-Hochberg method[36]. All the plots in this manuscript were created using the ggplot() function from the ggplot2 package[37].

Bioinformatic analysis

Bioinformatics analysis was employed to place the findings of urinary peptidomics within the biological context. Given that changes in peptide levels might be indicative of alterations in protease activity, the proteolytic events responsible for the secretion of the statistically significant GLP-1R agonists based urinary peptides were investigated by the open-source online tool "Proteasix" (http://www.proteasix.org, accessed on 6 March 2023)[38]. In brief, Proteasix retrieves information from literature and databases like MEROPs, UniProt Knoweldgebase (KB) and CutDB. The "Observed Prediction tool" of Proteasix with search parameters set to default, was utilized in this study. In addition, the relevant parental proteins corresponding to the statistically significant urinary peptides were subjected to network and pathway enrichment analysis utilizing the default settings of the online STRING database[39, 40] (http://www.string-db.org, accessed on 15 May 2023). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, which contains pathway maps representing the current understanding of molecular interaction and reaction networks, was utilized for enrichment analysis. Default settings were used, and pathways with a predicted significance of $p\text{-}value < 0.05$ (FDR) were considered statistically significant.

Results

Study design

The exploratory and untargeted urinary peptidomic approach in this study, was conducted to analyze the effect of GLP-1R agonist treatment on the urinary peptidome of T2DM patients, as illustrated in Figure 1. Briefly, urine samples from thirty-two T2DM patients collected at two time points: pre-treatment and post-treatment, with a time interval of 11.5 +- 3.72 months in between were analyzed. Intervention with GLP-1R agonist was introduced at 4.4 +- 4.11 months from pre-treatment visit and the treatment duration until the post-treatment visit was 6.9 +- 3.59 months.

Clinical information

The clinical information of all the thirty-two T2DM patients participating in this study, from pre- and post-treatment visits with GLP-1R agonists are provided in the Table 1. The mean age for the patients at first sample collection was 63.7 +- 7.25 years and 56.3% of them were females. As depicted in the Table 1, no statistically significant difference between the clinical levels of Hemoglobin A1C (HbA1C), body weight, body mass index (BMI), systolic and diastolic blood pressure (SBP/DBP), eGFR, albuminuria to creatinine ratio and urinary creatinine was observed after the treatment with GLP-1R agonists.

Peptidomic analysis

This exploratory urinary peptidomic analysis resulted in a list of 329 sequenced peptides to be further investigated. Statistical assessment including Benjamini-Hochberg FDR adjustment yielded a list of 70 statistically significant peptides (adjusted p -value < 0.05) affected by the GLP-1R agonist treatment, detailed information of the peptide identifications is provided in Table S1. The distribution of peptide intensity of these 70 statistically significant peptides (red spots) amongst the 329 peptides was also examined (Figure 2A). Volcano plot assessing the regulation of all the 329 peptides in response to the treatment (Figure 2B), highlighted the downregulation of urinary peptides in majority of the statistically significant peptides (66 out of 70 peptides), the vast majority in downregulation could also be visualized on comparison of the urinary peptide profiles from pre-treatment (Figure 2C) and post-treatment (Figure 2D), as obtained from the CE-MS analysis.

The 70 statistically significant peptides were identified as fragments of 26 proteins, as shown in Table 2. Most of the peptides (59 out of 70) originated from the collagen family of proteins and 41 out of the 59 collagen peptides belonged in majority to three collagen proteins, namely, collagen alpha-1(III) chain (P02461; COL3A1; n =16), collagen alpha-1(I) chain (P02452; COL1A1; n =15) and collagen alpha-2(I) chain (P08123; COL1A2; n =10). All the remaining identified 11 (out of 70) urinary peptides came from different proteins, alpha-1-antitrypsin (P01009; SERPINA1), apolipoprotein C-III (P02656; APOC3), CD99 antigen (P14209; CD99), cleavage and polyadenylation specificity factor subunit 6 (Q16630; CPSF6), Cornulin (Q9UBG3; CRNN), corticosteroid-binding globulin (P08185; SERPINA6), hemoglobin subunit alpha (P69905; HBA1; HBA2), myoglobin (P02144; MB), neurosecretory protein VGF (O15240; VGF), polymeric immunoglobulin receptor (P01833; PIGR) and transthyretin (P02766; TTR). Four out of the 70 urinary peptides showing a statistically significant upregulation on treatment with GLP-1R agonists in T2DM patients, as observed in Figure 2B, were characterized as COL3A1 (n =3) and COL1A2 (n =1) peptides (Table 2).

Since several peptides from COL1A1 and COL3A1 were found significantly associated with treatment (Figure 2E and 2F, respectively), we investigated the alignment of the peptides in their protein structures. The identified peptide sequences were aligned with the primary structure of the proteins, as shown in Figure 2G and Figure 2H, respectively for COL1A1 and COL3A1. For both proteins, peptides appeared equally distributed and no specific hot spot became apparent.

Bioinformatic analysis

To uncover plausible molecular mechanisms responsible for the observed impact of GLP-1R agonists treatment on urinary peptides in T2DM patients, the proteases potentially responsible for cleavage of the 70

statistically significant peptides were investigated using Proteasix. In total, 10 endopeptidases were retrieved as a result of the default search with the "Observed Prediction tool" of Proteasix, putatively responsible for cleaving 38 urinary peptides (36 downregulated and 2 upregulated) out of the 70 urinary peptides. The results are provided in Table S2. Most of the predicted endopeptidases belonged to the matrix metalloproteinase (MMP) family of proteases (7 out of 10 proteases), responsible for cleaving peptides at both the N' and C' terminals. Further proteases predicted as potentially responsible for cleaving the N' terminal belonged to the cathepsin family (CTSL and CTSD) while that cleaving at the C' terminus was A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5). Notably, the proteases MMP2, MMP9 and MMP13 were mapped to at least 6 cleavage sites each.

The protein-protein interactome was constructed using 26 parental proteins identified from 70 urinary GLP-1R agonist-associated peptides, using the STRING database. The network consisted of 27 nodes and 113 edges, as depicted in Figure 2I. The protein-protein interaction enrichment yielded a significant *p-value* $< 1.0e^{-16}$. While, most of the collagen proteins can be observed to interact with all the other collagen proteins, interestingly none of these interacted with the non-collagen proteins. Furthermore, a protein-protein interaction network within the non-collagen proteins could also be observed, indicating their involvement in the pathophysiology of T2DM. Within this network, a total of 9 KEGG pathways were predicted to be significantly enriched, including pathways related to protein digestion and absorption, ECM-receptor interaction, AGE-RAGE signaling pathway in diabetic complications, amoebiasis, relaxin signaling, focal adhesion, human papillomavirus infection, PI3K-Akt signaling pathway, small cell lung cancer and platelet activation, detailed results are provided in Table S3.

Discussion

In the last decade, GLP-1R agonists have been the recommended and preferred second line treatment for T2DM patients. Despite the various advantages of GLP-1R agonists over other anti-hyperglycemic drugs, the underlying molecular mechanisms of treatment with GLP-1R agonists have not been studied in-depth. Aiming to understand the effect of GLP-1R agonist treatment on T2DM patients, the urinary peptidome of thirty-two T2DM patients were analyzed for the first time in this study with CE-MS. The untargeted peptidomic analysis coupled with statistical tools identified 70 statistically significant (adjusted for multiple testing) urinary peptide fragments in abundance between the pre- and post-treatment samples. These urinary peptides generated from 26 parental proteins. Uniform distribution of intensity of the 70 peptides (red spots in Figure 2A), emphasized that their observed significant change with GLP-1R agonist treatment was not a function of abundance in the urine samples. For most of these 70 peptides, a comparative analysis further revealed a combined downregulation with GLP-1R agonist treatment (66/70 peptides).

In total, 59 out of the 70 statistically significant urinary peptides, majorly generated from three prominent collagen proteins COL3A1 (n =16), COL1A1 (n =15) and COL1A2 (n =10). Recently, He *et al.* ,[41] reported about the high abundance of collagen peptides observed in urine samples, as a result of proline hydroxylation which plausibly inhibits its reabsorption in the kidney. 55 out of the 59 collagen peptides were observed to be significantly downregulated with GLP-1R agonists treatment in the T2DM urinary proteome, while 4 peptides (COL3A1;n =3 and COL1A2; n =1) showed a significant upregulation on treatment which could plausibly be attributed to the variation in the post-translational modification by hydroxylation of proline residues in the peptides and varied proteolytic cleavage. In-line with the extensive recent report by Mavrogeorgis *et al.* ,[42] all the urinary collagen peptides identified in this study were devoid of the signal peptide, N-terminal pro-peptide and C-terminal pro-peptide; corresponding only to the mature protein region (Figure 2G and 2H). The observed downregulation of the collagen peptides in our study could potentially represent rather an attenuated degradation of the mature collagen protein, instead of resulting from protein synthesis or protein assembling processes. Rossing *et al.[43]* and Genovese *et al.* ,[44] have earlier speculated that the decrease of urinary collagen proteins could attribute to decreased proteolysis of collagen molecules, resulting from an increased resistance to proteolytic cleavage or an increased expression of protease inhibition.

To further corroborate the above speculation, endopeptidases responsible for putatively cleaving 38 statistically significant collagen peptides were majorly accounted to the MMP family of proteases (89% of the

cleavage sites, i.e., 34 out of the 38 urinary peptides) by MMP2 (21.1%), MMP9 (21.1%) and MMP13 (15.5%). The suggested downregulation in the activity of MMP peptidases as observed by decrease in intensity of collagen peptides in this study, is in-line with literature. Down-regulation in expression of MMP9 was observed on treatment with Liraglutide in a study that included induced-DM rabbit models[45]. In an another study, 45% and 60% reduction in activity of MMP2 and MMP9, respectively, in addition to 60% reduced COL1A1 levels, was observed in a male C57BL/6 mice on treatment with Semaglutide (GLP-1R agonist type)[46]. Research groups exploring the effect of Exenatide (GLP-1R agonist type) treatment on tumor necrosis factor-α human coronary artery smooth muscle cells[47] and human retinal pigment epithelium cells[48], reported the downregulated expression of MMP2 and MMP9, respectively on treatment. Another study analyzing atherosclerosis associated biomarkers in T2DM female subjects, reported decrease in MMP2 and MMP9 levels, with an increase in GLP-1 and GLP-1R levels[49]. Interestingly, treatment of Human SW1353 with Dulaglutide (GLP-1R agonist type)[50] and Fibroblast-like synoviocytes cell lines with Exenatide[51], also resulted in the downregulation of MMP13 and ADAMTS5 proteases.

On the other hand, collectively, all the non-collagen peptides (11 out of 70) showed lower abundance after GLP-1R agonist treatment and each generated from a different protein namely, SERPINA1, APOC3, CD99, CPSF6, CRNN, SERPINA6, HBA2, MB, VGF, PIGR and TTR. In studies analyzing the effect of Liraglutide (GLP-1R agonist type) on T2DM patients, Rafiullah*et al.* ,[52] reported the downregulation of the urinary protein SERPINA1 and Adiels *et al.* ,[53] reported decreased secretion of APOC3, respectively with treatment. No literature was found reporting regulation of the other non-collagen peptides by GLP-1R agonist treatment. However, impact of diabetes and/or obesity on these molecules has been reported in the literature. CD99 transcripts have been stated to up-regulate in T2DM profiles[54] and Pasello *et al.* ,[55] reported the proteins involvement in biological processes such as cell death and inflammation. CPSF6 indirectly modulates glucose homeostasis and insulin secretion[56]; while, HBA2, a commonly known marker for anemia and β-thalassaemia, interferes with glycemic markers of T2DM patients[57].

Increased levels of TTR have been associated with glucose intolerance, obesity and decreased pancreatic β-cells percentage in T2DM[58, 59]. Along the same lines, increased levels of SERPINA6 have been identified in obese patients and is reported to play a crucial role in glucose homeostasis, along with reducing insulin resistance and inflammation[60, 61]. Benchoula *et al.* ,[62] in their extensive review reported that VGF is expected to induce obesity, while also playing a role in lipolysis and insulin secretion, hence, acting as a potential target in T2DM therapy. CRNN is reportedly associated with the immune system and acts as an inflammation marker in chronic diseases[63, 64]. In addition, elevated levels of MB, a known cardiac marker, were reported in T2DM patients[65] and has been associated with insulin resistance, dyslipidemia and abnormal glucose metabolism with elevated levels acting as a biomarker for diabetic kidney disease[66]. Similarly, inflammatory mediators have been reported to increase PIGR protein levels in the renal tubular cells, linking its role in renal injury[67]. As a result of this exploratory study and literature search, we report the plausible molecular mechanisms affected by the treatment of GLP-1R agonists on the pathophysiology of T2DM, as hypothesized from the functions of the down-regulated non-collagen proteins in Figure 3. The results in this study may therefore indicate towards the beneficial effect of GLP-1R agonists in the context of management of T2DM and prevention or delaying the progression of its associated diseases.

Regardless of the novel findings, this study comes with its own limitations. Firstly, the large difference in time points between the pre-treatment and administration of GLP-1R agonists of 4.4 ± 4.11 may have resulted in unidentified variations of clinical parameters as well as the composition of urinary peptides, which were not accounted in this study. Secondly, the administering of multiple anti-hypertensives and GLP-1R agonist drugs at varied dosages and different combinations to the T2DM patients may have produced different effects of the treatment, which were also not analyzed in this study. Thirdly and surprisingly, within the follow-up we did not observe significant changes in Hb1Ac and BMI. The study was not powered to detect such changes, which would require about 10 times the number of subjects to be included, however, this does help in eliminating the argument that the reported changes in this study could be a result of weight loss, and instead support the proteins' role in T2DM pathophysiological mechanisms. Fourthly, since only one urinary peptide was identified per non-collagen protein, the reported effects of GLP-1R agonist treatment on these

proteins cannot be definitive and require further experimental studies with increased power. However, to overcome this short-coming, we additionally, performed a paired Wilcoxon test on a cohort of thirty-two T2DM patients administering only the anti-hypertensive drugs and no GLP-1R agonists drugs from the same PROVALID study, that were matched to the GLP-1R agonist treated cohort by age, sex, BMI, SBP, DBP and eGFR. Interestingly, we did not identify any statistically significant urinary peptides between the paired urine samples collected at a similar time difference as in this study. Finally, we could identify 15 urinary peptides downregulated by GLP-1R agonist treatment in our study, whose elevated levels have been previously reported as markers of heart failure[68]. This observation may further indicate the positive effect of GLP-1R agonist treatment.

To conclude, this untargeted peptidomic analysis to identify the effect of GLP-1R agonists treatment on the urinary peptidome of T2DM patients, indicated as a prominent finding the downregulation of MMP proteases, as identified by the downregulation of urinary collagen peptides on GLP-1R agonists treatment. Treatment with GLP-1R agonists also resulted in the decrease of SERPINA1, APOC3, CD99, CPSF6, CRNN, SERPINA6, HBA2, MB, VGF, PIGR and TTR peptides; indicating a potential benefit as many of these proteins express increased levels in T2DM patients. The results also merit the possibility of larger cohort studies to further understand the impact on underlying molecular mechanisms such as insulin resistance and inflammation, behind the findings.

Associated data

Data will be made available upon request directed to the corresponding author. Proposals will be reviewed and approved by the investigators and collaborators based on scientific merit. After approval of a proposal, data will be shared through a secure online platform.

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Conflict of Interest Statement:

The authors declare no conflict of interest. Author H.M. is the founder and co-owner of Mosaiques Diagnostics (Hannover, Germany). Authors E.M. and J.S. are employed by Mosaiques Diagnostics.

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Legends to Figures

Figure 1: Study design. Urine samples from thirty-two T2DM patients were collected at two time points: pre-treatment and post-treatment with the intervention of GLP-1R agonists at 4.4 +- 4.11 months from first sample collection. Naturally occurring urinary peptides were quantified in the urine samples by CE-MS analysis, followed by statistical and bioinformatic analysis of the CE-MS generated urinary peptide profiles.

Figure 2 : **Results of the urinary peptidomic analysis.**(A) Distribution of peptide intensity for all the 329 sequenced urinary peptides identified in this study, red dots indicate the statistically significant peptides. (B) Volcano plot depicting the regulation of the 329 peptides in response to GLP-1R agonist treatment. (C) Urinary peptide profiles of the 70 significant peptides in pre-treatment, as obtained from CE-MS analysis. (D) Urinary peptide profiles of the 70 significant peptides in post-treatment, as obtained from CE-MS analysis. (E) Box and Whisker plots depicting the down-regulation of all the COL1A1 peptides, in response to GLP-1R agonists treatment. (F) Box and Whisker plots depicting the up- and down-regulation of all the COL3A1 peptides, in response to GLP-1R agonists treatment. (G) Alignment of identified peptide sequences in the primary structure of protein COL1A1. (H) Alignment of identified peptide sequences in this study in the primary structure of protein COL3A1. (I) Protein-protein interaction network including the 26 parental proteins giving the origin to 70 GLP-1R agonists-associated peptides. *In, (G) and (H), the amino acids in green and red depict the down-regulated and up-regulated peptide sequences, respectively.*

Figure 3: Hypothesis. The beneficial effects of GLP-1R agonists treatment on the different pathophysiological pathways associated with T2DM as suggested by the role of the down-regulated non-collagen peptides, respectively in each pathway.

Tables

Table 1: Clinical information of the T2DM patients in mean +- SD; from pre- and post-treatment with GLP-1R agonists

Clinical information[a]	T2DM patients (n=32)	T2DM patients (n=32)	*p-value*[b]
	Pre-treatment	Post-treatment	

Clinical information[a]	T2DM patients (n=32)	T2DM patients (n=32)	*p-value*[b]
Age (years)	63.7 ± 7.25	63.7 ± 7.25	
Sex (% Females)	56.3	56.3	
HbA1C (%)	8.0 ± 1.38	8.0 ± 1.45	0.731
BW (kg)	98.3 ± 16.13	98.8 ± 20.28	0.771
BMI (kg/m^2)	33.2 ± 6.28	33.2 ± 6.93	0.915
SBP (mmHg)	139.2 ± 14.35	137.1 ± 12.61	0.385
DBP (mmHg)	81.1 ± 7.74	81.3 ± 8.21	0.906
eGFR (ml/min/1.73m^2)	67.7 ± 14.46	67.6 ± 15.55	0.965
UACR (mg/g)	33.6 ± 58.55	25.5 ± 44.80	0.248
UCREA (mg/dl)	103.9 ± 41.58	96.6 ± 50.72	0.429

[a]*HbA1C: Hemoglobin A1C; BW: Body weight; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; eGFR: estimated glomerular filtration rate; UACR: Albuminuria to Creatinine ratio; UCREA: Urinary Creatinine*

[b]*p-values were obtained from paired Wilcoxon rank sum (continuous variables) and Chi-squared test (categorical variable)*

Table 2: List of 26 proteins yielding the 70 statistically significant urinary peptides, in response to GLP-1R agonists treatment. (— refers to down regulation and — refers to upregulation)

UniProt ID	**Gene Symbol**	**Protein Name**	**Number of peptides**	**Nu**
			Total	*Regu*
P02461	COL3A1	Collagen alpha-1(III) chain	16	— (
P02452	COL1A1	Collagen alpha-1(I) chain	15	—
P08123	COL1A2	Collagen alpha-2(I) chain	10	— (
P02458	COL2A1	Collagen alpha-1(II) chain	3	—
P02462	COL4A1	Collagen alpha-1(IV) chain	2	—
P05997	COL5A2	Collagen alpha-2(V) chain	2	—
P12107	COL11A1	Collagen alpha-1(XI) chain	2	—
P27658	COL8A1	Collagen alpha-1(VIII) chain	1	—
Q5TAT6	COL13A1	Collagen alpha-1(XIII) chain	1	—
Q05707	COL14A1	Collagen alpha-1(XIV) chain	1	—
Q07092	COL16A1	Collagen alpha-1(XVI) chain	1	—
P39060	COL18A1	Collagen alpha-1(XVIII) chain	1	—
P25067	COL8A2	Collagen alpha-2(VIII) chain	1	—
P29400	COL4A5	Collagen alpha-5(IV) chain	1	—
Q14031	COL4A6	Collagen alpha-6(IV) chain	1	—
P01009	SERPINA1	Alpha-1-antitrypsin	1	—
P02656	APOC3	Apolipoprotein C-III	1	—
P14209	CD99	CD99 antigen	1	—
Q16630	CPSF6	Cleavage and polyadenylation specificity factor subunit 6	1	—
Q9UBG3	CRNN	Cornulin	1	—
P08185	SERPINA6	Corticosteroid-binding globulin	1	—
P69905	HBA1; HBA2	Hemoglobin subunit alpha	1	—
P02144	MB	Myoglobin	1	—
O15240	VGF	Neurosecretory protein VGF	1	—
P01833	PIGR	Polymeric immunoglobulin receptor	1	—
P02766	TTR	Transthyretin	1	—