Protease profiling of different biofluids in type 1 diabetes mellitus

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Abstract

Objectives: We aimed to disclose the proteolytic events underlying type 1 diabetes and related complications through protease profiling in the bodily fluids serum, urine and saliva.

Design and methods: Zymography followed by LC-MS/MS was performed for protease identification and quantitative comparison of proteolytic activity between healthy, type 1 diabetic patients with no complications and with retinopathy and/or nephropathy. Western blotting was also accomplished for MMP-9 and MMP-2 identification and expression analysis.

Results: Only MMP-2 and MMP-9 were observed in serum with significantly increased levels and activity observed in diabetic patients. In urine and saliva other proteases besides MMPs were identified by MS and presented disease-dependent activity variations. Among these are complex MMP-9/Neutrophil gelatinase-associated lipocalin, aminopeptidase N, azurocidin and kallikrein 1 with more activity noticed in type 1 diabetes patients with nephropathy and/or retinopathy.

Conclusion: Our data highlight the usefulness of urine and saliva for the monitoring of type-1 diabetes-related proteolytic events, where aminopeptidase N, azurocidin and kallikrein 1 appear as promising screening targets for type 1 diabetes-related complications.

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Introduction

Diabetes mellitus (DM) is a chronic debilitating disease affecting over 366 million people worldwide [1,2]. Chronic hyperglycaemia is the critical factor for the development and progression of microvascular complications like nephropathy, retinopathy and peripheral neuropathy in diabetes [3-6]. Nevertheless, the molecular mechanisms influencing the severity of diabetic microvascular disease are not fully understood. Proteomic profiling of clinical species has been heralded as a powerful tool for the identification of altered biochemical pathways and biomarkers of disease states. More than 50 distinct proteins were already suggested as potential biomarkers in bodily fluids from DM patients. Alterations in the levels of distinct apolipoproteins (e.g. A-1, J, C-1) were reported in the plasma and urine of type 1 and type 2 DM, whereas Zn-α-2-glycoprotein 1 and transthyretin were found up- and downregulated, respectively, in the urine of type 2 DM [7-10]. However, little emphasis has been given to protease profiling in biofluids [11] and even less to its alterations in diabetes mellitus. Besides metalloproteases (MMPs) and kallikreins, no other proteases have been implicated in the pathophysiology of DM and related complications [12,13]. The increased serum levels of these zinc endopeptidases capable of degrading all the components of the extracellular matrix, particularly of MMP-9 and MMP-2, were already suggested as a marker of chronic kidney disease’s risk [13] and of active retinopathy [14]. Recently, McKittrick et al. [15] suggested urinary MMP activities as clinically relevant biomarkers for predicting vascular remodeling in diabetic renal and vascular complications in type 1 DM patients. In type 2 DM, kallikrein 3 was found down-regulated in urine [10]. No other proteases have been related to DM pathogenesis though more than 500 human proteases are included in the degradeome database and some have been suggested to have context-dependent disease roles [16].

In order to evaluate type 1 DM-related alterations on biofluids’ protease profile we performed a straightforward screening of the proteases present in saliva, serum and urine from diabetic patients with no complications diagnosed, with nephropathy and with retinopathy using zymography-LC-MS/MS.

Material and methods

Patients

Subjects enrolled in the present study included 15 type 1 diabetic patients: 5 with retinopathy and nephropathy (group A), 5 with retinopathy (group B) and 5 without chronic complications (group C).
followed-up by the internal medicine service of Figueira da Foz Hospital, Portugal. Five healthy volunteers (group D) were also included in the study. As inclusion criteria for diabetic patients was defined a minimum duration of diabetes of 10 years. The patients were all examined by the same internal medicine physician. The patients with nephropathy presented a urinary albumin excretion of more than 300 mg in a 24-h collection and retinopathy was diagnosed by an ophthalmologist (Supplementary data, Table S1). The protocol was approved by the Hospital Ethical Committee and followed the Helsinki Declaration. All subjects included in the study gave their written informed consent after being informed of research project’s nature. Blood, saliva and urine samples were collected from each individual.

Sample collection

Venous blood samples collected from each subject and glycated hemoglobin (HbA1c) were measured by ion-exchange chromatography using a commercial kit (BioSystems). Blood samples were centrifuged at 3500g for 10 min and serum was stored at −70 °C until analysis. The total protein content was determined using the LQ total protein kit (Cormay) in automated clinical analyzer Prestige 24 (Tokyo Boeki Medical System).

Unstimulated whole-saliva was collected from all subjects who had refrained from eating and drinking for at least 2 h (performed between 9:00 and 12:00 a.m.), by direct draining into an ice-cold saliva collection tube. Saliva samples were centrifuged at 12,000g for 30 min (4 °C) and the supernatant stored at −70 °C until analysis. The total protein content was determined with the DC protein assay kit (Bio-Rad).

A midstream urine collection was performed and the abnormal presence of leukocytes was screening with urinalysis test strips (Uritest Makromed M10). Urine samples were centrifuged at 1000g for 10 min (4 °C) and the supernatant was passed through a 10-kDa filter to concentrate samples before further analysis. Total protein content was estimated in the fraction corresponding to the retentate using the DC protein assay kit (Bio-Rad).

Zymography and protease identification by LC-MS

Zymography assays were performed as previously described by Vitorino et al. [17]. Briefly, 10 μg of protein was separated by 10% SDS-PAGE. Gels were copolymerized with porcine gelatin. After electrophoresis, gels were washed with 2.5% Triton X-100 solution and incubated at 37 °C in developing buffer (50 mM Tris–HCl pH 7.6, 10 mM CaCl₂, 10 mM ZnCl₂) for 16 h. Then, gels were stained with 0.5% w/v Coomassie Brilliant Blue (CBB) G250 for 4 h under agitation and destained with 40% methanol/10% acetic acid. In parallel, EDTA (10 mM) and phenylmethylsulfonyl fluoride (PMSF) (5 mM) were included separately in the zymogram developing buffer. Images were acquired using GelDoc XR system (Bio-Rad) and processed using Quantity One® Software (Bio-Rad).

To identify the proteases present in each zymo gel’s band, nano-LC-MS/MS was performed. So, bands were excised from the gel and digested with trypsin. The trypsin digest was resuspended in acetonitrile/formic acid solution and separated in a nano-LC-system. LC separation of trypsin digest was performed according to Vitorino et al. [17]. Protein digests were separated using an Ultimate 3000 (Dionex, LCPackings) onto a 150 mm × 75 μm Pepmap100 capillary analytical C18 column with 3 μm particle size (Dionex, LC Packings) at a flow rate of 300 nL/min. A linear gradient of 5–50% Buffer B (85% acetonitrile, 0.04% trifluoroacetic acid) was run over a period of 45 min. Peptides eluting from the capillary column were mixed with CHCA matrix solution and directly deposited onto the LC-MALDI plate for MALDI-TOF/TOF MS using 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems). An S/N threshold of 50 was used to select peaks for MS/MS analyses. The spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal Mascot software (v.2.1.0.4, Matrix Science Ltd) for peptide/protein identification based on peptide mass fingerprints and MS/MS data. Searches were performed against the SwissProt protein database (March 2009) for Homo sapiens. A MS tolerance of 30 ppm was found for precursor ions and 0.3 Da for fragment ions, as well as two missed cleavages. The confidence levels accepted for positive protein identification were above 95%.

Immunoblotting analysis

Slot blot analysis was performed according to Caseiro et al. [18]. Briefly, saliva and urine samples were diluted in Tris buffered saline (TBS) to a final protein concentration of 0.01 μg/μL and slot blotted into a nitrocellulose membrane (Whatman®, Protran®). For western blot analysis, samples were subjected to electrophoresis (12.5% SDS-PAGE), followed by blotting onto a nitrocellulose membrane (Whatman®, Protran®). The membranes were then incubated with primary antibodies (anti-MMP-2 (clone 101721) and anti-MMP-9 (clone 36020) from R&D Systems) and secondary antibody (horseradish-conjugated antimouse, GE Healthcare). Detection was carried out with enhanced chemiluminescence according to manufacturer’s instructions (GE Healthcare). Film images were acquired using GelDoc XR system (Bio-Rad) and quantitative analysis of optical density (OD) was performed with Quantity One® 1-D Analysis Software (Bio-Rad).

Statistics

Statistical calculations were performed with the GraphPad Prism version 5.0 for Windows (GraphPad Software). Mean and standard deviation were calculated and one-way analysis of variance or a Kruskal–Wallis test was performed to analyze the statistical significance of differences between groups in relation to OD measures, followed by the Bonferroni or Dunn’s multiple comparison post-hoc tests. Differences were considered statistically significant at p < 0.05.

Results

Analysis of human biofluids’ proteolytic profile

In order to screen the proteases present in the most abundant human biofluids and to compare their proteolytic profile, the gelatinolytic protease activity of serum, saliva and urine from healthy individuals was evaluated. As shown in Fig. 1, these fluids contain several proteases capable of hydrolyze gelatin but presented distinct proteolytic patterns. An overlap of the average optical density (OD) for each zymo gel lane corresponding to serum (S), saliva (Sa) and urine (U) is presented in Fig. 1 with the gel bands sequentially annotated with numbers from the lowest to the highest molecular weight. The analysis of serum gelatinolytic proteases’ profile revealed one zymo gel band with approximately 72 kDa (band 7) with prominent activity and three others of approximately 86, 92 and 225 kDa (bands 8, 9 and 12) with lower proteolytic activity. Considering the molecular weight, one might suggest the presence of pro-MMP-2, active MMP-9, pro-MMP-9 and pro-MMP-9 homodimer on these zymo bands. This assumption was supported by the complete proteolytic inhibition observed after incubation of zymo gels with 10 nM EDTA (Fig. 3D). Regarding urine, zymo bands with proteolytic activity presented lower intensity in comparison with serum. The analysis of zymogram profile suggests the presence of proteases with approximately 72, 82 and 225 kDa. The molecular weight of the band with higher proteolytic activity in urine (72 kDa; band 7) seems to correspond to pro-MMP-2. Saliva zymography revealed a completely distinct proteolytic pattern with several bands with high proteolytic activity, corresponding to proteases with approximately 43 (band 3), 53 (band 4), 82 (band 8), 92 (band 9), 130 and 225 kDa. Minor activity was detected for proteases’ bands with molecular weights
lower than 30 kDa and with 40 (band 2), 60 (band 5), 66 (band 6), 72 (band 7), and 160–200 kDa (band 11). Considering the complete proteolytic inhibition observed after incubation of zymo gels with EDTA (Fig. 1B) and western blot analysis for MMP-2 and MMP-9 (Fig. 4D), band 6 was assigned as MMP-2, band 7 as pro-MMP-2, band 8 as MMP-9 and band 9 as pro-MMP-9. According to literature [19] and based on western blot analysis, the bands with 130 kDa (band 10) and 225 kDa (band 12) correspond to the complex MMP-9/Neutrophil gelatinase-associated lipocalin and to MMP-9 homodimer, respectively.

In an effort to identify proteases in these biofluids, the zymogram bands were digested with trypsin and analyzed by LC-MS/MS. The proteases identified in each fluid, either by mass spectrometry or western blot analysis, are presented in Table 1. As expected based on zymo incubated with EDTA, western blot and on literature [20–22], several metalloproteinases were identified in all fluids analyzed, namely MMP-2, MMP-9 and pro-MMP-9. These proteases were only identified by western blotting. Besides these, other MMPs like MMP-8 and aminopeptidase N were observed on saliva and on urine, respectively. Other classes of proteases were identified by MS/MS, mainly serine proteases, which is in agreement with the moderated inhibitory effect of PMSF on zymo activity (Fig. 1C).

Most of these serine proteases, like vitamin K-dependent protein Z, involved in immune processes, complement C1r subcomponent-like protein, cathepsin A were recognized on urine. Kallikrein-1 and azurocidin were both detected in urine and saliva and myeloblastin only in saliva. Some aspartic proteases as pepsin A and cathepsin D heavy chain were also identified in urine.

Type 1 diabetes-related proteolytic profile of biofluids

The proteolytic profile of biofluids from type 1 diabetic patients with (A and B) and without (C) chronic complications was analyzed and compared with the one from healthy individuals (D). The 15 non-controlled type 1 diabetic patients included in the study were followed for more than 10 years of disease by the same team of physicians, being totally characterized regarding the associated microvascular complications. Individuals from group A presented retinopathy for approximately 12.6 ± 10.8 years and nephropathy for 4.1 ± 3.3 years, whereas individuals with only retinopathy diagnosed for approximately 7.8 ± 6.1 years were included in group B.

In respect to serum, type 1 diabetic patients’ proteolytic profile was similar to the observed for healthy controls. Nevertheless, higher activity was noticed considering zymo bands’ OD (Fig. 2). An extra band with low proteolytic activity (band 54) was observed in group C patients’ serum, at a molecular weight of approximately 130 kDa. Unfortunately, we were not succeeded in its identification. Band 51’s OD was evaluated and in all groups of patients presented higher intensity compared to healthy individuals (p < 0.05; Fig. 2C). Relatively to band 53, all patients presented higher proteolytic intensity, but only with statistical significance in patients from groups B and C. Band 51, 52 and 53 bands were assigned as MMP-2, MMP-9 and pro-MMP-9, respectively, based on their molecular weight and on previous studies [13,19]. Two zymo bands of approximately 130 (S4) and 225 kDa (S5) corresponding to complex MMP-9/Neutrophil gelatinase-associated lipocalin and to MMP-9 homodimer [19] were also observed without variation among groups (Fig. 2). Besides MMPs, no other proteases were identified in serum.

The gelatinolytic activity of saliva from diabetic patients showed an increment in several zymo bands’ activity (Fig. 3A), being possible to distinguish 9 different bands (Sa 1–Sa 9) (Fig. 4B). From OD analysis, significant differences were observed (p < 0.05) for bands Sa 6, Sa 7 and Sa 9 (Fig. 3C), identified by western blot as pro-MMP-9, MMP-9 and MMP-9 homodimer, respectively (Table 1). From the analysis of zymo band’s activity outstanding the significantly higher activity of MMP-9 in type 1 diabetics with complications like retinopathy and nephropathy (groups A and B). Besides MMPs, other proteases identified by MS presented an increased activity in the saliva collected from DM patients with complications, namely kallikrein 1 (band Sa 2) and azurocidin (band Sa 1). Compared to serum, a higher intragroup variability was noticed in saliva, particularly evidenced for kallikrein-1 (band Sa 2) and MMP-8 (band Sa 3).

Regarding urine analysis by zymography (Fig. 4A), seven distinct bands (U1–U7) of approximately 60, 72, 82, 92, 130, 190–200 and 225 kDa, respectively, were identified and presented disease-related activity alterations (Fig. 4C). The bands that correspond to active and pro-form of MMP-9 (U3 and U4) presented a gradual activity increase being higher in the patients with chronic complications, namely retinopathy and nephropathy (p < 0.001) (Fig. 4C). Indeed, more than 3-fold increase was noticed for U4 in patients from group A (Fig. 4C). The activity of MMP-9 homodimer (band U7) was high in all patient groups (p < 0.001). Most of the proteases identified by MS presented no activity differences between groups with the exception of complement C1r subcomponent-like protein (band U2), higher in DM patients with complications.

To validate data obtained from the analysis of gelatinolytic activity, slot blot assays were performed for saliva and urine, the samples that presented more bands and higher differences between groups (Fig. 5). Slot blot analysis of MMP-2 in saliva showed a gradual increase in patients but without statistical significance, corroborating
zymography results. In urine samples, slot blot analysis of MMP-2 showed variability between patients, being the observed differences no statistically significant like those observed in zymography.

Regarding MMP-9, a significant rise was observed in urine of all patients in comparison with controls ($p<0.05$), while in saliva only a slight expression increase was noticed in diabetic individuals.

Table 1

<table>
<thead>
<tr>
<th>Method</th>
<th>Band</th>
<th>Pro tease</th>
<th>Acession number</th>
<th>MW [kDa]</th>
<th>Fluid</th>
<th>Comparison between groups A vs. D B vs. D C vs. D</th>
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<tr>
<td>WB</td>
<td>S5</td>
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<td>225</td>
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<td>↑</td>
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<td>U7</td>
<td></td>
<td></td>
<td>Sa</td>
<td>↑</td>
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<tr>
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<td>S7</td>
<td>Matrix metalloproteinase-9/Neutrophil gelatinase-associated lipocalin</td>
<td>P14780/P80188</td>
<td>130</td>
<td>Sa</td>
<td>↑</td>
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<tr>
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<td>U5</td>
<td>Aminopeptidase N</td>
<td>P15144</td>
<td>109</td>
<td>U</td>
<td>↑</td>
</tr>
<tr>
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<td>S3</td>
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<td>P14780</td>
<td>92</td>
<td>S</td>
<td>↑</td>
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<tr>
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<td>↑</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>Sa</td>
<td>↑</td>
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<td>Mannan-binding lectin serine protease 2 B chain</td>
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<td>76</td>
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<tr>
<td>WB</td>
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<td></td>
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<td>Sa</td>
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<tr>
<td>Sa5</td>
<td>U2</td>
<td></td>
<td></td>
<td>Sa</td>
<td>↑</td>
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<tr>
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<td>P08253</td>
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<td>Sa</td>
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<td>54</td>
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<td>–</td>
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<td>Complement C1r subcomponent-like protein</td>
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<td>U</td>
<td>↑</td>
</tr>
<tr>
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<td>Sa</td>
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<tr>
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<td>52</td>
<td>U</td>
<td>–</td>
</tr>
<tr>
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<td>U1</td>
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<td>–</td>
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<td>–</td>
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<td>P20160</td>
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<tr>
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<td>Sa1</td>
<td>Azurocidin</td>
<td>P20160</td>
<td>27</td>
<td>Sa</td>
<td>↑</td>
</tr>
</tbody>
</table>

All proteases identified by mass spectrometry were validated with at least two peptides with 95% confidence level.

↑↑: statistically up-regulated; ↑: up-regulated; –: no activity differences were observed.

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Fig. 2. (A) Representative zymography for serum samples of T1D patients with nephropathy and retinopathy (A), with retinopathy (B), without chronic complications (C) and controls (D). (B) An overlap of the average whole-gel lane OD traces for A, B, C and D serum samples. (C) Optical density measurements of proteolytic bands S1 and S3. *$p<0.05$; **$p<0.01$.
These results are in agreement with the ones obtained by zymography, corroborating the good correlation between expression and activity levels of proteases.

Discussion

Proteolysis plays a central role in pathophysiological events, being known for its involvement in inflammatory processes where neutrophils are essential for host defense against invading pathogens. Proteases are part of these cells’ array of weapons, for instance, cathepsin G, elastase and proteinase 3 [23]. Although present in low amounts, these enzymes can produce a high number of protein fragments, modifying not only proteins but also their products yielding a characteristic peptide signature. Serum peptidome analysis already allowed distinguishing metastatic thyroid carcinoma from cancer-free controls based only on its profile [24]. More
our results do not corroborate NGAL/MMP-9 as a biomarker for predicting vascular remodeling in type 1 diabetes (Fig. 4, band U5). DM complications-related higher activity levels were also observed for Complement C1r subcomponent-like protein, a serine-type endopeptidase (Fig. 4, band U2) highly expressed in kidney. Its physiological function remains to be determined, but may provide a novel means for the formation of the classical pathway C3/C5 convertase [37]. Although carboxypeptidase cathepsin A was previously identified in the urine of diabetic patients and related to kidney damage [38], no activity differences were found between groups (band U1, Table 1). Kallikrein-1 and azurocidin were identified in urine and also in saliva, with higher activity levels observed in DM patients with retinopathy and/or nephropathy. Previously, azurocidin, a neutrophil granule-derived antibacterial and monocyte- and fibroblast-specific chemotactic glycoprotein, was described as a promising biomarker in gingival crevicular fluid for the development of early diagnosis of periodontitis [39]. Considering that diabetics present a higher risk of periodontitis [39], one might expect to observe increased activity levels of this protease in patient’s saliva. The higher activity differences observed by these authors might be justified, at least partially, by the higher concentration of proteases in gingival crevicular fluid (GCF) than in saliva. Moreover, Choi et al. [39] evaluated azurocidin expression levels instead of activity in GCF, which, owing to its very small sample size, submicroliter volumes, and the hard collection procedure associated, make challenging GCF proteome characterization by classical biochemical methods [40]. It also worth of note that though an overall increased proteolytic activity was evident in diabetes’ saliva with or without related complications, high variability intra- and intergroups were observed, highlighting its proteome dynamic nature with potential clinical applications for diagnosis.

Concluding remarks

Overall, our data emphasize the relevance of proteolytic events underlying diabetes and its related complications, which can be monitored in biofluids like serum, saliva and urine. From these, urine and saliva are the ones that present a more pronounced proteolysis’ modulation by DM, particularly by its complications. Besides a clear disease-related activation of MMPs was noticed in all fluids, mostly for MMP-9 and its isoform, our data suggest the complex MMP-9/Neutrophil gelatinase-associated lipocalin, aminopeptidase N, azurocidin and kallikrein 1, identified by MS in urine and saliva, as potential screening targets for DM complications. Future prospective studies are essential for the recent saliva peptidome profiling of type 1 diabetes evidenced several fragments of type 1 collagen as a result of increased activity of MMPs [18]. Although recognized their involvement in pathogenic events, deciphering the activity of proteases in vivo is complicated by the presence of other proteins from interactive cascades and pathways [16,25]. In an attempt to disclose the proteolytic events underlying type 1 diabetes’ pathogenesis and its related complications, a combined approach with zymography-nano-LC-MS/MS was applied to the bodily fluids serum, saliva and urine. Besides the most used fluids for clinical purposes, saliva was included in the study attending to its increasingly recognized potential for early diagnosis of diseases [26,27], as already tested in oral and systemic pathologies, with some works reporting increased proteolytic activity in periodontitis, Sjögren’s syndrome and acute myocardial infarction [28–30].

For the best of our knowledge this is the first study where a deep characterization of proteases underlying type 1 diabetes and related complications like retinopathy and nephropathy was performed simultaneously in serum, urine and saliva. Interestingly, the most outstanding disease-related proteolytic alterations were detected in urine and saliva. Indeed, in serum, less zymo bands were detected comparing to the other fluids. Among the four bands detected, only the ones assigned by western blotting as MMP-9 and MMP-2 presented significantly variations among groups, being higher in diabetics with retinopathy and nephropathy (Fig. 2), as previously reported [12,13].

Disease-related increase of MMPs activity was also observed in the other fluids (Figs. 3 and 4) with a similar variation as observed by others [15,31]. MMPs are known for their involvement in extra- cellular matrix vascular remodeling and its excretion in urine was already reported in patients with vascular malformation or tumor angiogenesis [15]. McKitterick et al. [15] suggested urinary MMP activities as biomarkers for predicting vascular remodeling in type 1 diabetic renal and vascular complications. Indeed, diabetic nephropathy is the main cause of end-stage renal disease but the mechanisms leading to the development of renal injury are not well defined [32]. More than MMP-9 per se, its complex with neutrophil gelatinase-associated lipocalin (NGAL/MMP-9) was proposed by these authors as an earlier marker of nephropathy, comparing with albuminuria [33]. Our results do not corroborate NGAL/MMP-9 as a specific marker of nephropathy but of type 1 diabetes in general, with and without complications. Thrailkill et al. [34] also described disease- and gender-specific differences in NGAL and MMP-9 concentrations in T1D patients’ urine. Although less studied regarding proteolytic events in diabetes, in the saliva of type 2 diabetic patients were also reported disease-related alterations of MMPs activity, more specifically in MMP-9 and MMP-8 [31]. Our results suggest the contribution of MMP-9 to the previously described salivary peptide signature in type 1 diabetes [18].

While in serum only MMPs were identified, in the other fluids studied more zymo bands were observed and a higher number of proteases (Table 1) seem to contribute to the modulation of biofluids’ proteome. Among those identified by mass spectrometry are proteases related with immune functions and biological events as cell proliferation, secretion, invasion and angiogenesis. One of the proteases identified in urine that presented DM-related activity alterations was aminopeptidase N (APN), a type II metalloprotease existent in a wide variety of human tissues and cell types, namely endothelial, epithelial and leukocyte. APN is a multifunctional enzyme related with immune system [35], which according to Mitic et al. [36] present a significantly higher activity in the urine from type 1 diabetic patients with microalbuminuria. Our results support these findings since significantly higher activity levels of this protease were observed in diabetics with retinopathy and nephropathy (Fig. 4, band U5). DM complications-related higher activity levels were also observed for Complement C1r subcomponent-like protein, a serine-type endopeptidase (Fig. 4, band U2) highly expressed in kidney. Its physiological function remains to be determined, but may provide a novel means for the formation of the classical pathway C3/C5 convertase [37]. Although carboxypeptidase cathepsin A was previously identified in the urine of diabetic patients and related to kidney damage [38], no activity differences were found between groups (band U1, Table 1). Kallikrein-1 and azurocidin were identified in urine and also in saliva, with higher activity levels observed in DM patients with retinopathy and/or nephropathy. Previously, azurocidin, a neutrophil granule-derived antibacterial and monocyte- and fibroblast-specific chemotactic glycoprotein, was described as a promising biomarker in gingival crevicular fluid for the development of early diagnosis of periodontitis [39]. Considering that diabetics present a higher risk of periodontitis [39], one might expect to observe increased activity levels of this protease in patient’s saliva. The higher activity differences observed by these authors might be justified, at least partially, by the higher concentration of proteases in gingival crevicular fluid (GCF) than in saliva. Moreover, Choi et al. [39] evaluated azurocidin expression levels instead of activity in GCF, which, owing to its very small sample size, submicroliter volumes, and the hard collection procedure associated, make challenging GCF proteome characterization by classical biochemical methods [40]. It also worth of note that though an overall increased proteolytic activity was evident in diabetes’ saliva with or without related complications, high variability intra- and intergroups were observed, highlighting its proteome dynamic nature with potential clinical applications for diagnosis.
early predictive value of these potential biomarkers for type 1 diabetes diagnosis and prognosis.

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Conflict of interest

The authors have declared no conflict of interest.

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