Salivary Proteome and Peptidome Profiling in Type 1 Diabetes Mellitus Using a Quantitative Approach

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Supporting Information

ABSTRACT: In the present study, we applied iTRAQ-based quantitative approach to explore the salivary proteome and peptidome profile in selected subjects with type 1 diabetes, with and without microvascular complications, aiming to identify disease-related markers. From a total of 434 distinct proteins, bactericidal/permeability-increasing protein-like 1 and pancreatic adenocarcinoma up-regulated factor were found in higher levels in the saliva of all patients while increased content of other proteins like alpha-2-macroglobulin, defensin alpha 3 neutrophil-specific, leukocyte elastase inhibitor, matrix metalloproteinase-9, neutrophil elastase, plasin-2, protein S100-A8 and protein S100-A9 were related with microvascular complications as retinopathy and nephropathy. Protein–protein interaction network analysis suggests the functional clusters defense, inflammation and response to wounding as the most significantly associated with type 1 diabetes pathogenesis. Peptidome data not only support a diabetes-related higher susceptibility of salivary proteins to proteolysis (mainly of αPRP, βPRP1 and βPRP2), but also evidenced an increased content of some specific protein fragments known to be related with bacterial attachment and the accumulation of phosphopeptides involved in tooth protection. Overall, the salivary protein and peptide profile highlights the importance of the innate immune system in the pathogenesis of type 1 diabetes mellitus and related complications. This study provides an integrated perspective of salivary proteome and peptidome that should be further explored in future studies targeting specific disease markers.

KEYWORDS: quantitation, LC–MS/MS, nephropathy, retinopathy, saliva

1. INTRODUCTION

In recent years, saliva has attracted widespread interest as a diagnostic fluid. Salivary composition generally reflects the health status of an individual or disease susceptibility for oral and systemic pathologies. The advantages of saliva in comparison with other bodily fluids for diagnostic purposes are given by its accessibility and noninvasive and easy collection. The recent advancements in proteomic technologies hold special promise in the use of saliva to explore novel biomarkers and therapeutic targets. Indeed, new potential therapeutic targets. The recent advancements in proteomic technologies are given by its accessibility and noninvasive and easy comparison with other bodily fluids for diagnostic purposes. The advantages of saliva in comparison with other bodily fluids for diagnostic purposes are given by its accessibility and noninvasive and easy collection. The recent advancements in proteomic technologies hold special promise in the use of saliva to explore novel biomarkers and therapeutic targets. Indeed, new potential diagnostic salivary markers of oral and systemic diseases as dental and gingival pathologies, salivary gland disease, Sjögren syndrome, diabetes, head and neck carcinoma, breast and gastric cancers, sclerosis and psychiatric and neurological diseases have been proposed based on proteomic approaches. Nevertheless, little emphasis has been given to salivary proteome analysis in subjects with diabetes, namely, in type 1 diabetes mellitus (DM). No study is known that quantitatively evaluated the saliva proteome and peptidome changes related with this pathological condition.

The importance of quantitative proteomics has been increasingly recognized aiming to provide useful information for clinical applications once it screens nonphysiological levels of certain proteins and/or peptides that may reflect pathological conditions.

To evaluate the effect of more than 12 years of type 1 DM and related complications on their salivary proteome and peptidome, we performed a iTRAQ analysis using pooled saliva samples collected from four groups of individuals (subjects with no diagnosed diabetes-related complications, with nephropathy and retinopathy, with retinopathy, and healthy individuals) to identify potential protein and peptide targets for disease diagnosis. The results obtained highlight the involvement of an inflammatory and immune system response in the pathogenesis of type 1 DM, with higher amounts of proteins like alpha-2-macroglobulin, MMP-9, S100A8 and S100A9 found in the saliva of patients with microvascular complications.

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Salivary peptidome data not only support a DM-related higher susceptibility of salivary proteins to proteolysis but also evidence an increased content of some specific protein fragments known to be related with bacterial attachment and the accumulation of phosphopeptides that seem to be involved in tooth protection against erosion, more frequent in subjects with diabetes.

2. MATERIAL AND METHODS

2.1. Participating Subjects

Subjects enrolled in the present study included 15 type 1 DM patients: 5 with retinopathy and nephropathy (T1D-R+N), 5 with retinopathy (T1D-R) and 5 without chronic complications (T1D) followed-up by the internal medicine service of Hospital Distrital da Figueira da Foz, Portugal. Five healthy volunteers (Ctrl) were also included in the study. All groups were matched by gender and age. Subjects with diabetes presented disease duration of a minimum of 15 years, with HbA1c levels higher than 7.7%, significantly different from healthy individuals (T1D-R+N vs Ctrl group (p < 0.01); T1D-R and T1D vs Ctrl group (p < 0.05)). The patients were all examined by the same clinical team in the management of diabetes. Subjects did not demonstrate evidence of acute and chronic inflammatory disease (normal values of serum C-reactive protein), infectious diseases, cancer and oral diseases. Indeed, subjects did not ever have periodontal surgery, and did not presented signs of oral mucosal inflammatory lesions, such as lichen planus and candidiasis. Smoking subjects were excluded from the present study. All diabetic subjects were under insulin-based treatment. The subjects with nephropathy presented a urinary albumin excretion of more than 300 mg in a 24-h collection and a urinary albumin to creatinine ratio (UACR) higher than 300 mg/g, significantly different from healthy individuals (T1D-R+N vs Ctrl group (p < 0.01); T1D-R and T1D vs Ctrl group (p < 0.05)).

2.2. Sample Collection

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. The sample collection time and volume were registered for each individual. Saliva samples were prepared according to Vitorino et al. Briefly, saliva was mixed with solubilization buffer (7 M urea, 2 M thiourea, 1% w/v CHAPS, 1% Triton X-100) in the proportion of 2:1. The mixture was sonicated (2 cycles of 5 s each) and then centrifuged at 12 000g for 30 min at 4 °C. The supernatant was stored at −70 °C until analysis. The total protein content was estimated using the DC protein assay kit (Bio-Rad, Hercules, CA.).

2.3. Digestion of Salivary Proteins

For iTRAQ analysis, salivary pools were prepared for each experimental group. Each saliva pool was prepared using equal amounts of total protein from each individual per group. The volume of sample pools was normalized for trypsin digestion. Aliquots of 100 μg of protein were analyzed in duplicate. An insulin digestion was performed for iTRAQ labeling according to the protocol provided by the manufacturer (Applied Biosystems, Foster City, CA). Two independent runs were carried out. Briefly, proteins were reduced, alkylated and digested. Samples were mixed with triethyl ammonium bicarbonate buffer (TEAB) (1 M, pH 8.5) and RapiGest (Waters) to a final concentration of 0.5 M and 0.1%, respectively, and then reduced with 50 mM tris(2-carboxyethyl) phosphine (TCEP) for 1 h at 37 °C and alkylated with 10 mM S-methyl methanethiosulfonate (MMTS) for 10 min at room temperature. The aliquots were digested with trypsin (Promega, Madison, WI) at a protein-to-enzyme ratio of 10:1 at 37 °C overnight and then dried in a Speed-Vac (Thermo Savant, NY).

2.4. Peptide Labeling with iTRAs

After protein digestion, the extracted peptides were labeled with iTRAQ reagents (4-plex) according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Briefly, one vial of iTRAQ reagent, previously dissolved in 70 μL of ethanol, was added to each aliquot and incubated for 2 h at room temperature. The reaction was stopped by adding water and the labeled digests corresponding to each of the four 4-plex experiments were combined and dried in the Speed-Vac (Thermo Savant, NY).

2.5. Peptide Separation by Multidimensional LC

The trypsin labeled digests were separated by a multidimensional approach based on a first dimension with high pH reverse-phase and a second dimension with the acidic reverse-phase system as previously described. In the first dimension, sample loading was performed at 200 μL/min with buffers (A) 72 mM TEA, 52 mM acetic acid in H2O, pH 10 and (B) 72 mM TEA, 52 mM acetic acid in acetonitrile (ACN), pH 10 (98% A/2% B). After 5 min of sample loading and washing,
peptide fractionation was performed with linear gradient to 50% B over 35 min followed by a 100% B step. Sixteen fractions were collected, evaporated, and resuspended in 2% ACN and 0.1% trifluoroacetic acid (TFA). Collected fractions were separated using an Ultimate 3000 ( Dionex, LC Packings, Sunnyvale, CA) 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. The mobile phases A and B were 2% ACN 0.1% TFA in water and 95% ACN, 0.045% TFA, respectively. The gradient started at 10 min and ramped to 60% B until reaching 50 min and 100% B at 55 min and retained at 100% B until reaching 65 min. The chromatographic separation was monitored at 214 nm using a UV detector ( Dionex/LC Packings) equipped with a 3 nL flow cell. The peptides eluting from the column were mixed with a continuous flow of matrix solution (270 nL/min, 2 mg/mL α-CHCA in 70% ACN/0.3% TFA and internal standard Glu-Fib at 15 fmol) in a fractions microcollector (Probot, Dionex/LC Packings) and directly deposited onto the LC-MALDI plates at 12 s intervals for each spot (150 nL/fraction). For every chromatographic run, a total of 208 fractions were collected.

2.6. Separation of Endogenous Salivary Peptides

Salivary endogenous peptides were extracted as previously described.14 To 200 µL of supernatant obtained from saliva pretreatment, explained in section 2.2, ice-cold acetone was added drop by drop in the proportion of 9:1. After agitation on ice for 1 h, the mixture was centrifuged at 19 000g for 15 min. The supernatant containing peptides was separated and saved. The pellet was resuspended in 200 µL of ACN/12 mM HCl, incubated during 1 h on ice under agitation and centrifuged again at 19 000g for 15 min. The obtained supernatant was added to the previous one and dried in a Speed Vac for subsequent analysis. Two types of analysis were performed. For qualitative analysis, peptides fractions from individual samples were separated by LC Ultimate 3000 ( Dionex, LC Packings) onto a Pepmap100 capillary analytical C18 column, using the conditions described above. The eluted peptides were directly deposited onto the LC-MALDI plates. For every chromatographic run, a total of 156 fractions were collected. For iTRAQ-based quantitative analysis, pools of extracted peptides were prepared for each experimental group, in a similar way as described for salivary protein analysis. The dried peptide pools were labeled with iTRAQ reagents (4-plex) according to manufacturer’s instructions, as described above in section 2.4. Labeled peptides were separated by LC Ultimate 3000 (Dionex, LC Packings) onto a Pepmap100 capillary analytical C18 column and analyzed by MALDI-TOF/TOF.

2.7. Protein ID and Quantification

MALDI-TOF/TOF MS analysis was performed using a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, CA), as described by Vitorino et al.15 A S/N threshold of 50 was used to select peaks for MS/MS analyses. A fragmentation voltage of 2 kV was used throughout the automated runs. The spectra were processed and analyzed by the ProteinPilot software (v4.0 AB Sciei), which uses paragon algorithm for protein/peptide identification based on MS/MS data. Searches were performed against the SwissProt protein database (release date 01012011) for Homo sapiens, Lactobacilli and Streptococci. Default search parameters were used: specifying trypsin as the digestion enzyme, fixed modification of methionylation on cysteine residue and iTRAQ 4Plex, biological modification with emphasis on phosphorylation and urea denaturation as the variable modification setting. The mass tolerances for precursor and fragments were default values for ProteinPilot. The cutoff score value for protein identification with ProteinPilot was a ProteoScore of 1.3 (95% confidence). A decoy database search strategy was also used to estimate the false discovery rate (FDR), defined as the percentage of decoy proteins identified against the total protein identification. The local FDR was calculated by searching the spectra against SwissProt (H. sapiens + Bacteria) decoy database. The estimated low FDR of 0.9% indicated a high reliability in the proteins identified (four proteins identified in decoy database). Data was normalized for loading error by bias correction, which is an algorithm in ProteinPilot that corrects for unequal mixing when combining the labeled samples of one experiment. It does so by calculating the median protein ratio for all proteins reported in each sample, adjusted to unity and assigning an autobias factor to it. Nevertheless, the quantification results were reviewed manually for all proteins found to be differentially expressed (iTRAQ ratio >1.3 or <0.7 according to Vitorino et al.14).

2.8. Immunoblotting Analysis

Slot blot analyses were performed according to Caseiro et al.16 with slight modifications. In brief, saliva samples were diluted in Tris buffered saline (TBS) to a final protein concentration of 0.01 µg/µL and a volume of 100 µL was slot-blotted into a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The membranes were blocked with 5% (w/v) dry non-fat milk in TBS-Tween (TBS-T) and then incubated overnight at 4 °C with the primary antibodies anti-deleted in malignant brain tumors 1 protein (Rabbit anti-deleted in malignant brain tumors, P4856Rb, Epitomics, Burlingame, CA) and anti-cytostatin S (Rabbit anti-cytostatin S polyclonal antibody, ab58515, Abcam, Cambridge, U.K.), anti-histatin 1 (Rabbit anti-histatin 1 polyclonal antibody, ab81089, Abcam, Cambrige, U.K.), and anti-C-reactive protein (Rabbit anti-C-reactive protein monoclonal antibody, ab32412, Abcam, Cambrige, U.K.) diluted 1:500 in blocking solution and anti-amyase (Rabbit anti-amyase polyclonal antibody, A8273, Sigma, St. Louis, MO) diluted 1:1000 in blocking solution. The membranes were washed three times, 10 min each, with TBS-T and incubated 2 h with secondary antibody (horse-radish-conjugated anti-mouse, GE Healthcare, Buckinghamshire, U.K.) in a dilution of 1:10 000. Detection was carried out with enhanced chemiluminescence according to manufacturer’s instructions (GE Healthcare). Film images were acquired using GelDoc XR system (Bio-Rad, Hercules, CA) and quantitative analysis of optical density (OD) was performed with Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA).

2.9. Statistics

Statistical calculations were performed with the GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego CA). Mean and standard deviation were calculated and a Kruskal–Wallis test was performed to analyze the statistical significance of differences between groups in relation to OD measures, followed by Dunn’s multiple comparison post-hoc test. Differences were considered statistically significant at p values lower than 0.05.
3. RESULTS

3.1. Saliva Proteome Profiling

The merge of two data sets obtained from LC independent runs resulted in the identification of 3834 peptides when based on peptide identifications with ProteinPilot score higher than 1.3 (confidence level of 95%) and a false discovery rate (FDR) lower than 1%. Overall, 443 distinct proteins were identified, most of which were assigned as presenting catalytic activity (22.6%) involved in protein binding (30.9%) and structural molecule activity (17.8%) according to PANTHER classification system (http://www.pantherdb.org). The mean percent peptide coverage for the complete panel of proteins was 33.7% ($\pm$22.9) with a range of 1.3% to 89.2% of coverage. The reproducibility of the experiment was evaluated, and the correlation coefficient was higher than 0.94 in all groups.

Figure 1. Comparison of the log ratio of the relative intensity of the significantly regulated salivary proteins among groups (T1D-R+N/Ctrl; T1D-R/Ctrl and T1D/Ctrl).

Figure 2. Protein interaction network generated with Intact and visualized with Cytoscape (v2.8.3) consisting of 266 proteins connected by 334 protein–protein interactions. Major clusters of interacting proteins include those involved in defense, inflammation and response to wounding. Green nodes represent up-regulated proteins and red nodes down-regulated ones in the saliva of subjects with type 1 diabetes.
Proteins like L-plastin variant (fragment), leukocyte elastase inhibitor, matrix metalloproteinase-9 and alpha-2-macroglobulin, defensin alpha 3 neutrophil-specific were only found overexpressed in T1D-R+N group, such as pancreatic adenocarcinoma up-regulated factor. Other proteins like bactericidal/permeability-increasing protein-like 1 and proteins were detected in high levels in all DM patient groups, complications. As can be depicted from this figure, specific proteins were detected in high levels in all DM patient groups, like bactericidal/permeability-increasing protein-like 1 and pancreatic adenocarcinoma up-regulated factor. Other proteins were only found overexpressed in T1D-R+N group, such as alpha-2-macroglobulin, defensin alpha 3 neutrophil-specific, leukocyte elastase inhibitor, matrix metalloproteinase-9 and neutrophil elastase. Proteins like l-plastin variant (fragment), plasmin-2, protein S100-A8 and protein S100-A9 were detected in higher levels in the saliva of DM patients with chronic complications (T1D-R+N and T1D-R), though in increased amounts in T1D-R+N patients.

Looking to the molecular functions and the associated biological processes of these differentially expressed proteins, according to PANTHER (Supporting Information Figure S2), it can be noticed that the majority are involved in metabolic pathways (23.3%) and in the immune response (23.3%). Proteins related to cellular and developmental processes comprise 16.7% and 3.3%, respectively, of all DM-modulated salivary proteins. Clusters like cell communication (13.3%), cellular component organization (3.3%), response to stimulus (16.7%) and transport (11.1%) were also among the ones responsive to DM.

An integrated analysis of all proteins found in different levels among groups was performed with Cytoscape (v2.8.3). The protein–protein interaction network presented in Figure 2 was imported from Intact (http://www.ebi.ac.uk/intact/) and contains 266 proteins and 334 protein–protein interactions. These proteins are distributed in ten clusters (Supplementary figure S3), one of which including a down-regulated protein, agglutinin (DMBT1), known to interact with proteins involved in proliferation (PARD6B and CDK5RAP3). To identify the relevant biological pathways that were altered in type 1 DM, BiNGO17 was used to find GO biological pathway and molecular function terms that were enriched among the differentially expressed proteins in the network. In total 576 biological pathway terms and 65 molecular function terms are annotated in association with the differentially expressed proteins. Regarding biological pathways, the most significant ones modulated by type 1 DM are defense, inflammation and response to wounding. Protein binding is the most significant molecular functions found altered in diabetics. Considering the disease-related down-regulated proteins, it can be noticed that a majority are involved in binding to bacteria surface.

There are some interactions centered in the overexpressed proteins that are evidenced. For instance, increased levels of alpha-2-macroglobulin seem to be related with alterations in lipid metabolism as evidenced by protein–protein interactions with LCAT, LRPI, leptin and ApoE. This protein is also associated with inflammation as evidenced by its interaction with cytokines as IL-1B and proteins from SERPIN family. The clusters centered in the inflammatory S100 proteins interact with adhesion proteins as VCAM, signaling proteins, namely...
from the NF-kB pathway, MAP kinase or GTPase mediated signal transduction, and with proteins involved in the regulation of proliferation (e.g., RIF1, CDK2, TP53, GDF9). These S100 proteins are also known to interact with proteins from bacteria and virus as for example glycoprotein B from the human herpesvirus S. A clear association of the overexpressed PAUF with Toll-like receptors can also be depicted from Figure 2. Mostly of the salivary proteins are included in a protein–protein interaction cluster centered in Mucins and amylase. MUC7, MUC5B and AMY1A interact with statherin histatins and PRPs. Redox proteins as SOD2 and PRDX3 are also included in this cluster.

For the validation of iTRAQ experiments, immunoblotting analysis of target proteins was performed in individual samples. In this sense, cystatin S, deleted in malignant brain tumors 1 protein and amylase expression were evaluated in triplicate in all saliva samples (from the 15 patients and 5 controls; Figure 3) and data obtained highlight the same tendency observed with iTRAQ analysis. Cystatin S did not present statistical differences between groups as well as in iTRAQ experiment and deleted in malignant brain tumors 1 protein showed DM-related lower values, corroborating iTRAQ data. Increased levels of amylase were observed, in accordance with iTRAQ data, though more significant in T1D-R. C-reactive protein (CRP) was evaluated in serum and saliva samples from the same individuals to screen the inflammatory status. As can be seen in Figure 3, significantly higher levels of this inflammatory protein were observed in diabetics with retinopathy and nephropathy. No significances differences were observed among groups for CRP salivary levels.

3.2. Saliva Peptidome Profiling

Saliva contains several protein species of low molecular weight, comprising around 20–30% of the total secreted proteins, which contributes to the oral cavity homeostasis. From the LC-MS/MS analysis of endogenous salivary peptides, 794 different sequences from the main salivary protein classes (bPRP1, bPRP2, bPRP3, bPRP4, histatin 1, histatin 3, aPRP, MUC7, PlgR, statherin, SMR3B (P-B peptide) and PROL4) were identified (Supporting Information Table S2), and aPRP1, bPRP1 and bPRP2 were the most representative ones. The distribution of the identified peptides by patient groups can be depicted in Figure 4. An average of 270 ± 26 peptides was identified per group, and from these, 182 peptides were common to all groups, 48 were exclusively identified in healthy individuals, 58 in T1D-R+N, 49 in T1D-R and 69 in T1D (Supporting Information Table S3). Comparing the distribution among groups (Table 2), an increase of bPRP2 was observed in T1D-R+N group in relation to Ctrl (51.50 ± 8.49 vs 36.25 ± 5.30, p < 0.05) and a decrease of aPRP peptides in all diabetic patient groups (T1D-R+N, T1D-R and T1D; p < 0.05, p < 0.001 and p < 0.001, respectively). Looking to the unique peptides identified in each group, a predominance of bPRP1 peptides was notorious in diabetics while a higher number of aPRP and statherin unique peptides were noticed in healthy individuals. The alignment of those unique peptides in the main protein sequences evidenced an enrichment of N-terminal peptides from bPRP1 and C-terminal peptides from aPRP in subjects with diabetes (Figure 5). Moreover, the cleavage site motifs were annotated using a homemade software for protease prediction. This analysis highlighted the predominance of Q/G and P/Q motifs in the saliva from diabetics (Supporting Information Table S4). Looking for potential proteases acting on these motifs, MEROPS (http://merops.sanger.ac.uk) search was performed and retrieved cathepsin L and MMP-2/MMP-9 as the most probable ones for Q/G and P/Q motifs, respectively.

The presence of specific post-translational modifications such as phosphorylation, Gln → pyro-Glu at N-terminal, oxidation, glycation, glycosylation, as well as protein variants, was searched in all salivary peptides. From previously identified salivary protein variants, we only observed fragments of PRH2-1, a variant of aPRP, which corresponds to the replacement of Asp by Asn in the position 66 (Supporting Information Figure S4).

The comparative analysis of these PTMs between groups showed a significant 2.2-fold increase of Gln → pyro-Glu in PROL4 peptides from diabetics in comparison to controls (Supporting Information Table S5). HexNac modification in bPRP2 was denoted in diabetics with chronic complications in comparison to Ctrl group (2.4-fold in T1D-R+N and 2.5-fold in T1D-R). The HexNac modification in bPRP3 was exclusively observed in T1D-R+N group. The percentage of phosphorylated peptides originated from bPRP3 and aPRP was higher in subjects with diabetes, accounting for 5-fold increase of bPRP3 phoshopeptides in T1D-R+N and T1D groups in comparison to Ctrl (p < 0.001). The same tendency was observed for aPRP. The higher content of phosphorylated and glycosylated peptides in diabetics reflects the data given by the slot blot analysis of whole saliva using specific staining methods (Diamond ProQ and Emerald ProQ, respectively) for these modifications (Supporting Information Figure S5).

To evaluate the impact of DM in saliva peptidome, we performed a comparative iTRAQ-based analysis of identified peptides between patients with and without chronic complications and controls (Supporting Information Figures S6–S9). From all identified peptides above-referred, 99 presented differences among groups (Supporting Information Table S2). From these, fragments of histatin 1 presented higher levels in T1D and T1D-R. This tendency was also observed by immunoblotting for intact histatin 1 (Figure 3D). Statistical significance was observed for 5 distinct peptides (Table 3). For instance, the peptides originated from bPRP3 (148GPPPQGGRPPQGPPQGQSPQ166 and 297GRHRPPQGQSPQ180) were observed in higher levels in T1D group (p < 0.01), whereas the peptide 297GRHRPPQGQSPQ180 was only found in increased amount in group T1D-R group (p < 0.05). The peptide 148GPPPQGGRPPQGPPQGQSPQ166 from aPRP was detected.
Table 2. Distribution of the Identified Peptides per Group and Protein Class According to Their Relative Abundance

<table>
<thead>
<tr>
<th></th>
<th>T1D-R+N</th>
<th>T1D-R</th>
<th>T1D</th>
<th>Ctrl</th>
</tr>
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<tbody>
<tr>
<td>Basic salivary proline-rich protein 1 (bPRP1)</td>
<td>55.25 ± 11.67</td>
<td>47.50 ± 16.26</td>
<td>55.25 ± 7.42</td>
<td>42.50 ± 4.24</td>
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<tr>
<td>Basic salivary proline-rich protein 2 (bPRP2)</td>
<td>51.50 ± 8.49</td>
<td>35.50 ± 11.31</td>
<td>47.25 ± 7.42</td>
<td>36.25 ± 5.30</td>
</tr>
<tr>
<td>Basic salivary proline-rich protein 3 (bPRP3)</td>
<td>15.75 ± 2.47</td>
<td>14.25 ± 4.60</td>
<td>15.75 ± 1.06</td>
<td>19.50 ± 2.83</td>
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<tr>
<td>Basic salivary proline-rich protein 4 (bPRP4)</td>
<td>25.00 ± 4.24</td>
<td>13.00 ± 6.36</td>
<td>10.75 ± 0.35</td>
<td>18.75 ± 1.77</td>
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<td>Histatin 1</td>
<td>13.75 ± 6.72</td>
<td>19.00 ± 9.90</td>
<td>12.75 ± 0.35</td>
<td>12.75 ± 1.06</td>
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<tr>
<td>Histatin 3</td>
<td>5.75 ± 0.35</td>
<td>7.25 ± 1.77</td>
<td>10.75 ± 5.30</td>
<td>2.00 ± 1.41</td>
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<td>Mucin-7 (MUC7)</td>
<td>8.00 ± 0.71</td>
<td>4.25 ± 1.06</td>
<td>7.50 ± 4.95</td>
<td>7.00 ± 0.71</td>
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<tr>
<td>Polymeric immunoglobulin receptor (PIgR)</td>
<td>1.25 ± 0.35</td>
<td>1.00 ± 0.00</td>
<td>2.75 ± 1.77</td>
<td>1.75 ± 0.35</td>
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<tr>
<td>Proline-rich protein 4 (PRL4)</td>
<td>1.50 ± 0.71</td>
<td>0.75 ± 1.06</td>
<td>0.25 ± 0.33</td>
<td>1.75 ± 1.06</td>
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<tr>
<td>Salivary acidic proline-rich phosphoprotein 1/2 (aPRP)</td>
<td>65.50 ± 13.44</td>
<td>51.25 ± 18.74</td>
<td>56.25 ± 11.67</td>
<td>81.75 ± 10.25</td>
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<td>Statherin</td>
<td>27.25 ± 8.13</td>
<td>19.75 ± 9.53</td>
<td>20.25 ± 3.18</td>
<td>30.25 ± 1.06</td>
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<tr>
<td>Submaxillary gland androgen-regulated protein 3B (SMR3B)</td>
<td>29.25 ± 12.37</td>
<td>26.25 ± 8.84</td>
<td>18.25 ± 7.42</td>
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<td>Total</td>
<td>299.00 ± 69.30</td>
<td>239.75 ± 83.79</td>
<td>257.75 ± 36.42</td>
<td>283.25 ± 21.57</td>
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</tbody>
</table>

“Data is presented as mean ± STD.

Figure 5. bPRP1 and aPRP main sequences evidencing the location of the unique peptides identified in all groups (light blue) and only in diabetics (dark blue).

in higher levels in patients groups T1D-R and T1D (p < 0.001, Figure 5).

4. DISCUSSION

To the best of our knowledge, the present study represents the first attempt to determine salivary protein profile alterations in type 1 diabetes mellitus using iTRAQ experiment. The objective of this approach was to add new insights on type 1 diabetes mellitus pathogenesis and identify in saliva potential markers. The objective of this approach was to add new insights on type 1 diabetes mellitus pathogenesis and identify in saliva potential markers.

From the comparative analysis of saliva proteome performed by LC–MS/MS with iTRAQs, several proteins were identified that could potentially be used to distinguish individuals with type 1 DM as well as the ones with chronic complications associated with this disease. According to PANTHER, a majority of them are involved in metabolic (23.3%) and immune response (23.3%) processes, which corroborates the nature of the pathology, e.g., a metabolic disorder accompanied by inflammation.

This phenotype was further corroborated by protein–protein interaction network analysis of differentially expressed proteins performed with Cytoscape. This analysis highlighted the functional clusters defense, inflammation and response to wounding as the most relevantly modulated by type 1 DM. These findings are in accordance with data retrieved from the integrative analysis of diabetes mellitus-related proteome alterations of biofluids, which suggests the involvement of immune and cellular processes independently of the fluid analyzed.

The significant up-regulation of bactericidal/permeability-increasing protein-like 1 (BPI) and Pancreatic adenocarcinoma factor (PAUF) in the saliva of all diabetics clearly suggest the involvement of immune and cellular processes independently of the fluid analyzed.
activation of the immune system in type 1 DM. BPI is an essential component of the innate immune system with bacteriostatic and bactericidal effects against gram-negative bacteria through lipopolysaccharides binding, whereas PAUF is an endogenous ligand of Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4).29,30 The inflammatory response seems more exacerbated in patients with retinopathy and nephropathy considering the high salivary levels of alpha-2-macroglobulin, defensin alpha 3 neutrophil-specific, leukocyte elastase inhibitor, matrix metalloproteinase-9 and neutrophil elastase. The protease inhibitor alpha-2-macroglobulin and the leukocyte elastase inhibitor were previously reported, based on proteomic data, as being up-regulated in the saliva of diabetics but with type 2 DM.12 Increased levels of alpha-2-macroglobulin were also observed in serum samples from diabetics with complications.31,32 The involvement of the naturally occurring inhibitor of neutrophil proteases identified in this study was previously described in several human inflammatory diseases.33 Chan et al.34 also found an association between the salivary levels of this protein and periodontitis in subjects with type 2 diabetes. The significance of diabetes mellitus as a risk factor for periodontal destruction is believed to be related with the nature and intensity of the inflammatory response in the periodontal tissues.35 Though no clinical evidence of periodontal disease were observed, the DM-related inflammatory status given by proteome data suggest an increased risk for the development of periodontitis, which is a clinical condition difficult to be quantitatively assessed.25,35 Nevertheless, the correlation between salivary alpha-2-macroglobulin and periodontitis was not supported by data obtained from Zhou et al.36 Despite the increased levels of these protease inhibitors detected in the saliva of diabetics, higher content (e.g., MMP-9; Supporting Information Table S2) and activity of MMPs,16,37,38 and of neutrophil elastase, which activity is known to be regulated by leukocyte elastase inhibitor,59 have been reported.

Other molecular players of the inflammatory process were found in higher content in the saliva of diabetics, particularly in the ones with complications. The up-regulation of the actin bundling protein α-plastin was previously described in subjects with type 2 diabetes54 and in nondiabetic individuals with periodontitis.40 The possible association with periodontitis was not corroborated by Grant et al.41 that found lower levels of α-plastin in healthy volunteers with gingivitis. The higher content of S100-A8 and S100-A9 observed in diabetics suggests that in these patients occurs the formation of the heterodimer known as Myeloid-Related Protein-8/14 (MRP8/14), which binds to the receptor for advanced glycation end products and TLR4, mediating intracellular inflammatory signaling transduction.42–44 Elevated MRP8/14 complex plasma levels were already suggested as a prediction factor for increased risk of cardiovascular events55 and were associated with inflammatory diseases.46 Cabras et al.11 also reported higher levels of S100A9 in the saliva of type 1 diabetic children. But more than a relation with type 1 diabetes per se, our data suggest an association between increased salivary content of MRP8/14 and microvascular complications, which were not described before and could be used in the future in the monitoring of diabetes-related chronic complications.

Together with the analysis of salivary proteome, peptidome profiling might give new insights of the effect of type 1 DM and related chronic complications on oral cavity homeostasis and consequent physiological impairment. In this sense, a straightforward analysis of low molecular weight salivary peptides (<5 kDa) was performed using an iTRAQ experiment for quantitative comparison of peptidome profiles. Overall, a higher content of peptide fragments from the main salivary protein classes, mainly from aPRP, bPRP1 and bPRP2, was observed in subjects with diabetes (Table 2), which seems to be related with the higher proteolytic activity previously reported in the saliva of diabetics,16,25,47,48 and the PRP classes were the most susceptible ones.47 Moreover, the predominance of KPQ motif in acidic and basic PRPs47 might be attributed to a glutamine endopeptidase not yet identified, which could be originated from the oral microbiome.50 However, among the proteases modulated by the disease, we identified by iTRAQ analysis MMP-9 as being overexpressed. This data was also corroborated by MEROPS19 search of more prevalent cleavage sites (Supporting Information Table S4). The higher activity of MMP-9 as well as of other MMPs was already reported in the saliva of subjects with type 1 diabetes.9,16 Protein–protein interaction network analysis also highlight a correlation between the higher levels of MUC and salivary peptides, mainly statherin, histatins and PRPs (Figure 2), suggesting an attempt to maintain the oral homeostasis.

The analysis of the unique peptides in each group evidenced an enrichment of N-terminal peptides from bPRP1 and C-terminal peptides from aPRP in diabetics. Although bPRP role in oral cavity are not well characterized, in the case of aPRP, the C-terminal peptide (148GPPQGGRPQGPPQGSPQ160) is known to be associated with bacterial attachment via PQ segments to tooth surface, avoiding bacterial binding.51 The elevated levels of this peptide together with the high content of BPI detected in the saliva of diabetics might be seen as a protective mechanism against bacterial infection that predispose to tooth demineralization.52,53 The elevated content of these peptide fragments was also paralleled by increased proteolytic activity observed in children with diabetes, as previously observed by Cabras et al.11 According to these authors, the low concentration of the intact P-C peptide associated with high levels of its fragments suggests the impairment of oral cavity safeguard given by salivary peptides. A different tendency was observed for histatin 1 in the saliva of adult subjects (with approximately 40-years old), with DM-related higher content of intact and fragments of this salivary peptide.

To gain a deep perspective of the peptidome dynamics, other post-translation modifications besides proteolysis were analyzed. Data obtained points to a functional impact of phosphorylation and glycosylation (Supporting Information Table S5). From those prevalent phosphopeptides, no significant expression differences were detected among groups using iTRAQ analysis; however, a higher number of phosphopeptides was detected in the saliva of diabetics. In fact, the phosphorylation of serine residues in PRPs, statherin and histatin 1 has been associated with the acquired enamel pellicle formation and, consequently, with the participation in the remineralization process, having a protective effect against tooth erosion.54,55 A similar trend was also observed for HexNac modification in bPRPs, which is in agreement with our previous findings for head and neck cancer where a predominance of this modification was detected.20 Furthermore, the higher levels of HexNac modification might also reflect the O-GlcNAcylation observed during diabetes that might act as an autoprotective alarm or stress response.56 This data is in agreement with the increased expression of pro-inflammatory mediators observed in the saliva of subjects with
diabetes, particularly in the ones with chronic complications (Figure 1).

5. CONCLUSION

Data from the present study highlight the potential use of saliva for the monitoring of diabetes-related microvascular complications. Salivary proteome evidenced an overexpression of the endogenous regulators of TLR, MRP8/14 and PAUF, affecting myeloid MyD88-dependent activation of NF-kB and tumor necrosis factor-α expression, in the saliva of subjects with diabetes-related retinopathy and nephropathy, pointing to the importance of the innate immune system in the pathogenesis of DM-related complications.

Peptidome data not only supported the DM-related higher susceptibility of salivary proteins to proteolysis, but also evidenced an increased content of some specific protein fragments known to be related with bacterial attachment and the accumulation of phosphopeptides that seem to be involved in tooth protection against erosion. Particularly, the proteolytic fragments from bPRP1, bPRP2 and aPRP might be seen as a hallmark of the disease pathogenesis with potential use for its monitoring. Future studies should be undertaken to disclose the functional role of these salivary peptides under different pathophysiological conditions aiming to define disease-specific biomarkers.

ASSOCIATED CONTENT

Supporting Information
Additional material as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
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