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RESEARCH

Salivary proteomic analysis in patients with type 2 diabetes mellitus and periodontitis

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Abstract

Objective This study aimed to compare the salivary protein profile in individuals with Type 2 Diabetes Mellitus (DM2) and periodontitis and their respective controls.

Methods Eighty participants were included in the study. The four groups were formed by individuals with DM2 and periodontitis (DM2 + P, $n = 20$), DM2 without periodontitis (DM2, $n = 20$), periodontitis without DM2 (P, $n = 20$) and individuals without periodontitis and without DM2 (H, $n = 20$). Periodontal clinical examinations were performed and unstimulated saliva was collected. Proteomic analysis was performed by *shotgun* mass spectrometry. The results were obtained by searching the Homo sapiens database of the UniProt catalog.

Results A total of 220 proteins were identified in saliva samples. In the comparison between DM2 + P and DM2 groups, 27 proteins were up-regulated [e.g. *S100-A8* was 6 times up-regulated (humoral immune response pathway)]. The DM2 + P and P groups had 26 up-regulated proteins [e.g. *Immunoglobulin lambda constant 7* more than 2 times up-regulated (complement activation pathway)]. The non-DM2 groups (P and H) presented 22 up-regulated proteins [e.g. *Glyceraldehyde-3-phosphate dehydrogenase* more than 2 times up-regulated (Peptidyl-cysteine S-nitrosylation pathway)]. The groups without periodontitis (DM2 and H) showed 23 were up-regulated proteins [e.g. *Hemoglobin subunit alpha* that was more than 10 times up-regulated (cellular oxidant detoxification pathway)].

Conclusion The presence of DM2 and periodontitis significantly impacts the salivary proteome. Our proteomic analysis demonstrated that changes in the S100 family proteins (S100A8 and S100 A9) are highly related to the presence of DM2 and periodontitis.

Clinical relevance Diabetes Mellitus (DM) and periodontitis are highly prevalent chronic diseases that present a wide variety of signs and symptoms. They present a bidirectional relationship, where patients with DM have a higher prevalence and severity of periodontitis, and patients with periodontitis have a higher prevalence of DM, worse glycemic control, and more diabetic complications. Diagnosing periodontitis requires specific clinical examinations, which require a highly trained operator. In this study, we used high throughput proteomics in order to evaluate non-invasive biomarkers for periodontitis in type 2 DM subjects. The results can contribute to earlier, more accurate, and less costly diagnosis of periodontitis in diabetic subjects, enabling better diabetes control.

Keywords Periodontitis · Saliva · Proteomics · Diabetes Mellitus

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Introduction

Periodontitis is a chronic multifactorial inflammatory disease that results in the destruction of tooth support tissues and tooth loss [1]. Epidemiological studies have shown an increase in prevalence, affecting about half of the adult population [2]. Regarding its etiopathogenesis, periodontitis is no longer considered a simple bacterial infection; this disease represents a complex interaction involving the host's inflammatory response and immune system, subgingival microbiota, and systemic and environmental factors [3].

Diabetes Mellitus (DM), a metabolic disorder that leads to elevated blood glucose, represents one of the main systemic modifying factors for periodontitis [4]. This chronic disease is associated with several systemic complications, such as retinopathy, nephropathy, neuropathy, impaired wound healing, and reduced host resistance [5]. Periodontal disease has been considered the sixth major complication of DM. In fact, several epidemiological studies have shown that diabetic patients have a higher prevalence and severity of periodontitis when compared to non-diabetic patients [6, 7]. In addition, longitudinal studies have found a greater progression of alveolar bone loss and attachment loss in type 2 diabetic mellitus (DM2) individuals than in non-diabetics [8, 9]. The main mechanisms linking periodontitis to DM involve elevations in interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-6, nuclear factor-kappa B receptor activator ratio (RANKL) / osteoprotegerin (OPG), oxidative stress, increased Toll-like receptor (TLR) 2/4 expression, formation of advanced glycation end products (AGEs) and alteration in neutrophil function and chemotaxis [4].

The literature indicates that the relationship between periodontitis and DM is bidirectional, as studies have shown that periodontitis can result in poorer glycemic control and increased risk for DM complications [10]. Thus, methods for monitoring periodontal status can contribute to periodontal health, maintenance of teeth, better quality of life, and better glycemic control in DM individuals. Besides clinical parameters for diagnosing and monitoring periodontal conditions, saliva has been proposed as a non-invasive fluid for analyzing biomarkers in systemic and oral diseases, including DM and periodontitis [11–13].

Salivary biomarkers have been investigated in patients with periodontitis, such as interleukin IL-1β, IL-6, IL-17, TNF-α, and MMPs [14, 15]. However, it is essential to point out that within the complex inflammatory context of periodontal disease, which can be intensified with DM, it seems unlikely that choosing just one protein, or even a small group of them, could be sufficient. In this context, proteomics can allow global salivary analysis of proteins, representing a tool capable of dissecting complex immuno-inflammatory interrelationships such as periodontitis and DM [16].

Mass spectrometry used in proteome is the basic technology for large-scale identification of salivary proteins, with high robustness, sensitivity, speed, and analysis yield [17–19]. Salivary proteomic analyses have been used in studies in the field of periodontics to obtain a better understanding of the disease, as well as the possible detection of biomarkers for early diagnosis and monitoring of periodontal disease activity [16, 18, 20, 21]. However, there is still a lack of studies to elucidate the salivary protein profile of patients with DM2 and periodontitis, which could provide relevant information for a better understanding of this interrelationship, as well as potential biomarkers for the disease in this population. Thus, with this detailed information, we could begin to make more detailed diagnoses and direct personalized treatments for patients with these conditions.

Given the need for a better understanding of the salivary protein profile of DM2 patients with periodontitis and possibly the detection of biomarkers for periodontitis in this population, this study aimed to compare the salivary protein profile of individuals with DM2 and Periodontitis with systemically and periodontally healthy controls.

Materials and methods

Study design and patients

This observational clinical study was approved by the University of Taubaté ethics committee (CAAE: 19319219.8.0000.5501). The study was conducted with patients who sought treatment from 2019 to 2022 at the Dental School of the University of Taubaté, São Paulo, Brazil. Patients with type 2 DM were enrolled at the Municipal University Hospital of Taubate (HMUT) Endocrinology Division and referred to the Dental School of the University of Taubate for oral examination and dental treatment.

Inclusion criteria for the DM2 population were: (1) DM2 diagnosed for at least five years, (2) Glycated hemoglobin (HbA1C) 7 to 10%, 40 years old or more, and 15 natural teeth. Exclusion criteria were: Smokers and former smokers (less than five years), pregnancy and lactating, periodontal treatment in the past 12 months, antibiotic therapy three months prior to the inclusion, and individuals who had any other systemic disease (blood dyscrasias, immunodeficiency, autoimmune diseases).

The eligibility criteria of the systemically healthy control groups were the same for the DM2 group, except for the presence of DM2. DM2 participants were matched with controls according to sex and age (± 5 years old). Eligible patients willing to participate signed an informed consent form after receiving oral and written information.

Periodontitis definition: For periodontitis, groups included patients with stages III-IV, Grades B-C [22]. Stage III-IV periodontitis case definition: having at least 1 tooth with at least one interproximal site with PS ≥ 6 mm, attachment loss ≥ 5 mm, and bone loss exceeding the middle third of the root, with attachment loss affecting 30% or more of the teeth.

Considering a mean difference test of independent groups, an α of 5%, 75% power, and a large effect size (Cohen's d = 0.80), 20 participants per group were required. Therefore, a total of 80 participants were included as follows:

i. Participants with DM2 and Periodontitis (DM2 + P) ($n = 20$);
ii. Participants with DM2 without Periodontitis (DM2) ($n = 20$);
iii. Participants without DM2 with Periodontitis (P) ($n = 20$);
iv. Participants without DM2 and without Periodontitis (H) ($n = 20$).

Interview

A calibrated investigator (MVF) applied a structured questionnaire in order to record demographic variables such as sex, age, systemic diseases, and DM2 condition (diagnosis, medications, and date of diagnosis).

Periodontal clinical examination

The periodontal evaluation was performed by a single trained and calibrated periodontist (ESR) with a manual periodontal probe (PCPUNC 15, Hu Friedy, Mfg. Co. Inc., Chicago, IL., USA.) at 6 sites per tooth (mesio-buccal, buccal, distobuccal, mesiolingual, lingual and disto-lingual) and consisted of measuring the following parameters:

1. Plaque Index (PI), determined dichotomously by the presence (+) or absence (–) of plaque [23].
2. Probing depth (PD), measured by the distance between the gingival margin and the bottom of the gingival sulcus/pocket.
3. Gingival recession (GR), measured by the distance between the enamel-cement junction and the gingival margin.
4. Clinical Attachment Level (CAL), measured by the distance of the enamel-cement junction to the bottom of the gingival sulcus/pocket.
5. Bleeding on probing (BOP), determined dichotomously by the presence (+) or absence (–) of bleeding observed 30 s after the first insertion of the probe into the gingival sulcus.

Periodontist calibration was performed by examining five patients, two quadrants per patient, and measurements repeated two times, with 1-week intervals between them. The intraclass correlation coefficients between duplicate measures were then calculated. The reproducibility of parameters was verified for PD and GR, considering a difference of ± 1 mm between measurements. The intraclass correlation coefficient for PD was 0.89 ($p = 0.001$), while GR was 0.94 ($p = 0.001$), indicating substantial agreement.

Laboratory tests

From DM participants, HB1Ac, HDL, LDL, and C-reactive protein were collected from the patient's electronic health records during their periodontal clinical examination. All tests were performed at HMUT.

Saliva collection

Unstimulated saliva was collected with the patient fasting for 2 h using the spitting method [24]. The exam was performed in the morning between 8 and 12 h, respecting the circadian cycle. Patients were informed not to eat, drink, or brush their teeth 1 h before salivary sample collection. A rinse of 20 mL of distilled water was performed, and participants rested for another 15 min. Patients were instructed to spit for 10 min into a 50 ml Falcon tube immersed in ice. At least 2 mL of saliva was supposed to be collected; if one participant could not deposit 2 mL of saliva in 10 min, the collection time was increased until reaching this amount. Then, the saliva vials were centrifuged at 2600 x g for 15 min at 4 °C. Then, the supernatant was collected in 1 ml and transferred to a 1.5 ml Eppendorf tube. The vials of salivary aliquots were then stored at -80 °C.

Preparation of salivary samples

Salivary samples were prepared according to a previous study from our group [24]. Each collected sample

was divided into 1 mL aliquots and separated into different tubes. Aliquots were diluted 1:1 with the extraction solution containing 6 M Urea, 2 M Thiourea, and 50 mM NH_4HCO_3, pH 7.8. After dilution, the samples were vortexed for 10 min at 4 °C, sonicated for 5 min, and then centrifuged at 20,817 x g at the same temperature for 10 min. This step was repeated twice. Samples were concentrated to 150 µL in Amicon® Ultra 4 mL Centrifugal Filters (Merck, Rahway, NJ, USA). Protein quantification by the Bradford Method (Bio-Rad®, Hercules, CA, USA). was performed and 50 ug of protein of which sample was used for analysis. For the reduction of protein 5 mM dithiothreitol (DTT) was used for 40 min at 37 °C and subsequently added 10 mM iodoacetamide was used for 30 min in the dark room. The digestion of proteins was used 2% (w/w w) trypsin (Thermo Fisher Scientific®, Waltham, MA, USA) for 14 h at 37 °C. Then, 10 µL 5% formic acid were added. Samples were purified and desalted using Spin C18 columns. The samples were resuspended in the solution containing 3% acetonitrile and 0.1% formic acid and finally submitted to Mass Spectrometry.

Quantitative proteomic analysis

Analysis of tryptic peptides was performed on the nanoACQUITY UPLC (System Waters, Milford, MA, USA) coupled to the Xevo Q-TOF G2 mass spectrometer (System Waters, Milford, MA, USA). Data were processed using ProteinLynx GlobalServer (PLGS) software version 3.03 (System Waters, Milford, MA, USA). Identification of salivary proteins was performed using the ion counting algorithm built into the software.

The results were obtained by searching the Homo sapiens database, the UniProt catalog. The statistical analysis used was the t-test, considering $p<0.05$. The software CYTOSCAPE version 3.9.0 (JAVA) was used to build networks of molecular interactions between the identified proteins with the aid of the ClueGo and String applications. The proteins were classified by molecular function, cellular component, biological and immunological processes according to the terms described in the Mapper of Generic Terms of the University of Princeton (Gene Generic Ontology - GO - Term Finder). The search was performed using the GOA database for Homo sapiens [25].

Statistical analysis

Differences in the salivary protein profile between groups were considered our primary outcome. The statistical analysis of the sociodemographic, periodontal clinical parameters and blood test was performed using statistical software (GraphPad Prism 5.01, San Diego, CA, USA). The chi-square test analyzed dichotomous variables. Quantitative data were expressed as mean and standard deviation, and submitted to the Kolmogorov-Smirnov normality test to determine the data distribution. The ANOVA test with Tukey's post-hoc was used for multiple groups, while the t-student test was used for the analysis of two groups. Analyzes were performed with a significance level of 5%.

Results

From 2019 to 2022, 92 patients with DM2 within the inclusion criteria were referred from HMUT Endocrinology Division for oral evaluation at the Dental School of the University of Taubate. Of these, 40 (20 DM2 + P and 20 DM2– age and sex-matched) were included. During the same period, 210 controls without DM2 were interviewed, and 40 participants age and sex-matched were included (20 P and 20 H).

Men represented 45% of the population. There was no statistical difference in age among groups ($p>0.05$). The DM2 + P presented higher LDL and C-Reactive Protein values than the DM2 group ($p<0.05$). There were no differences between groups of patients with type 2 DM (DM2 + P and DM2) for the time of DM2 diagnosis, HB1Ac, total and HDL cholesterol ($p>0.05$) (Table S1).

The salivary flow of the DM2 + P group was significantly lower than those without DM2 ($p<0.05$).

The groups with Periodontitis (DM2 + P and P) showed increased values of PD, CAL, BOP, PI, and number of pockets of 5 mm or more ($p<0.05$). There was no difference between the DM + P and P groups for the parameters PD, CAL, PI, and number of pockets of 5 mm or more ($p>0.05$). However, the DM2 + P group presented an increased BOP compared to the P group ($p<0.05$). (Table S2)

Both groups without periodontitis (DM2 and H) showed similar PD, CAL, and BOP values ($p>0.05$); however, the DM2 group had a higher PI when compared to the H group ($p<0.05$).

Salivary proteomic profile in the presence of DM2 and periodontitis

A total of 220 proteins were identified in the saliva of the four groups. The Venn diagram (Figure S1) revealed 15 common proteins across all comparisons. In addition, exclusive proteins were identified in each comparison. Overall, the number of proteins identified in the groups

Table 1 Up and downregulated proteins identified in the saliva of the DM2 + P and DM2 groups and their expression differences

Dif. Exp.	Accession	Description	GENE	Score	Ratio	*p* value
↑	**P05109**	**Protein S100-A8**	**S100A8**	**196**	**6.11**	**<0.01**
↑	**P07737**	**Profilin-1**	**PFN1**	**278**	**2.66**	**<0.01**
↑	**Q562R1**	**Beta-actin-like protein 2**	**ACTBL2**	**375**	**2.61**	**<0.01**
↑	**Q5VSP4**	**Putative lipocalin 1-like protein 1**	**LCN1P1**	**466**	**2.39**	**<0.01**
↑	**P80188**	**Neutrophil gelatinase-associated lipocalin**	**LCN2**	**330**	**2.36**	**<0.01**
↑	**P01037**	**Cystatin-SN**	**CST1**	**16,564**	**2.25**	**<0.01**
↑	**B9A064**	**Immunoglobulin lambda-like polypeptide 5**	**IGLL5**	**1095**	**2.16**	**<0.01**
↑	**P06702**	**Protein S100-A9**	**S100A9**	**309**	**2.16**	**0.03**
↑	**P0CG04**	**Immunoglobulin lambda constant 1**	**IGLC1**	**1095**	**2.12**	**<0.01**
↑	**P0DOX8**	**Immunoglobulin lambda-1 light chain**		**1095**	**2.1**	**<0.01**
↑	**P0CF74**	**Immunoglobulin lambda constant 6**	**IGLC6**	**347**	**2.03**	**<0.01**
↑	P25311	Zinc-alpha-2-glycoprotein	AZGP1	383	1.75	<0.01
↑	P0DOY2	Immunoglobulin lambda constant 2	IGLC2	1095	1.72	<0.01
↑	P0CG39	POTE ankyrin domain family member J	POTEJ	58	1.58	<0.01
↑	P59665	Neutrophil defensin 1	DEFA1B	601	1.58	0.04
↑	P0CG38	POTE ankyrin domain family member I	POTEI	58	1.42	<0.01
↑	P12273	Prolactin-inducible protein	PIP	7860	1.38	<0.01
↑	P06733	Alpha-enolase	ENO1	46	1.32	0.01
↑	P60709	Actin_ cytoplasmic 1	ACTB	601	1.28	<0.01
↑	P63261	Actin_ cytoplasmic 2	ACTG1	601	1.28	<0.01
↑	P68133	Actin_ alpha skeletal muscle	ACTA1	467	1.26	<0.01
↑	P68032	Actin_ alpha cardiac muscle 1	ACTC1	467	1.25	<0.01
↑	P62736	Actin_ aortic smooth muscle	ACTA2	438	1.25	<0.01
↑	P63267	Actin_ gamma-enteric smooth muscle	ACTG2	438	1.22	<0.01
↑	A5A3E0	POTE ankyrin domain family member F	POTEF	292	1.19	<0.01
↑	P02768	Albumin	ALB	29,149	1.17	<0.01
↑	Q6S8J3	POTE ankyrin domain family member E	POTEE	292	1.16	<0.01
↓	P01876	Immunoglobulin heavy constant alpha 1	IGHA1	14,660	0.9	<0.01
↓	P02787	Serotransferrin	TF	3275	0.86	<0.01
↓	P0DOX5	Immunoglobulin gamma-1 heavy chain	1 SV	2943	0.84	0.03
↓	P0DUB6	Alpha-amylase 1 A	AMY1A	41,471	0.83	<0.01
↓	P0DTE7	Alpha-amylase 1B	AMY1B	41,475	0.83	<0.01
↓	P01036	Cystatin-S	CST4	24,245	0.83	<0.01
↓	P0DOX2	Immunoglobulin alpha-2 heavy chain		11,038	0.83	<0.01
↓	P0DTE8	Alpha-amylase 1 C	AMY1C	41,471	0.81	<0.01
↓	P19961	Alpha-amylase 2B	AMY2B	33,595	0.81	<0.01
↓	P01877	Immunoglobulin heavy constant alpha 2	IGHA2	11,973	0.8	<0.01
↓	P04746	Pancreatic alpha-amylase	AMY2A	30,589	0.79	<0.01
↓	P01833	Polymeric immunoglobulin receptor	PIGR	801	0.77	<0.01
↓	P01860	Immunoglobulin heavy constant gamma 3	IGHG3	1303	0.75	<0.01
↓	Q8TAX7	Mucin-7	MUC7	289	0.73	0.02
↓	P68871	Hemoglobin subunit beta	HBB	6355	0.7	<0.01
↓	P02647	Apolipoprotein A-I	APOA1	3747	0.7	<0.01
↓	Q96DR5	BPI fold-containing family A member 2	BPIFA2	282	0.68	<0.01
↓	P69891	Hemoglobin subunit gamma-1	HBG1	2159	0.66	<0.01
↓	P04080	Cystatin-B	CSTB	706	0.64	<0.01
↓	P02100	Hemoglobin subunit epsilon	HBE1	2159	0.64	<0.01
↓	P09228	Cystatin-SA	CST2	5807	0.64	<0.01
↓	P69892	Hemoglobin subunit gamma-2	HBG2	2159	0.63	<0.01
↓	P02008	Hemoglobin subunit zeta	HBZ	328	0.62	0.03
↓	P01591	Immunoglobulin J chain	JCHAIN	621	0.62	<0.01
↓	P02790	Hemopexin	HPX	607	0.57	<0.01
↓	P02042	Hemoglobin subunit delta	HBD	2542	0.55	<0.01
↓	P01859	Immunoglobulin heavy constant gamma 2	IGHG2	347	0.53	<0.01
↓	**P01023**	**Alpha-2-macroglobulin**	**A2M**	**923**	**0.50**	**<0.01**

Table 1 (continued)

Dif. Exp.	Accession	Description	GENE	Score	Ratio	*p* value
↓	**Q96DA0**	**Zymogen granule protein 16 homolog B**	**ZG16B**	**5478**	**0.45**	**<0.01**
↓	**P02808**	**Statherin**	**STATH**	**11,119**	**0.43**	**<0.01**
↓	**P01024**	**Complement C3**	**C3**	**225**	**0.37**	**<0.01**
↓	**P61626**	**Lysozyme C**	**LYZ**	**2839**	**0.36**	**<0.01**
↓	**P23280**	**Carbonic anhydrase 6**	**CA6**	**156**	**0.30**	**<0.01**
↓	**P28325**	**Cystatin-D**	**CST5**	**856**	**0.24**	**<0.01**
↓	**P31025**	**Lipocalin-1**	**LCN1**	**601**	**0.22**	**<0.01**
↓	**O95196**	**Chondroitin sulfate proteoglycan 5**	**CSPG5**	**66**	**0.18**	**<0.01**
↓	**P69905**	**Hemoglobin subunit alpha**	**HBA2**	**501**	**0.17**	**<0.01**
↓	**A0M8Q6**	**Immunoglobulin lambda constant 7**	**IGLC7**	**347**	**0.17**	**<0.01**
↓	**P01871**	**Immunoglobulin heavy constant mu**	**IGHM**	**282**	**0.16**	**<0.01**
↓	**P0DOX6**	**Immunoglobulin mu heavy chain**		**263**	**0.15**	**<0.01**
↓	**P02810**	**Salivary acidic proline-rich phosphoprotein 1/2**	**PRH2**	**1202**	**0.10**	**0.02**

↑ = up-regulated; ↓ = down-regulated ($p<0.05$); Ratio: Differences in protein expressions in the saliva of the DM2 + P vs. DM2 groups

Bold: Means that the difference between groups was at least two times fold

with DM2 and periodontitis was higher. Comparing the groups DM2 + P and DM2, 163 common proteins were initially found and classified according to their expression. Twenty-seven were up-regulated (e.g. Protein S100-A8 was 6-fold up-regulated), and 42 were down-regulated (e.g. Salivary acidic proline-rich phosphoprotein 1/2, which was 10-fold down-regulated). More information on the proteins found and their expressions are shown in Table 1. Figure 1a shows the molecular function, cell component, and biological processes involved according to Gene Ontology. The network (Fig. 2a) illustrates the protein interaction using the up and downregulated proteins, as shown in Table 1.

Comparing the groups affected by Periodontitis (DM2 + P and P), 87 common proteins were found and classified according to expression. Twenty-six were up-regulated (e.g. Immunoglobulin lambda constant 7, Cystatin-SA and S100A8, which were about 2 times up-regulated), and 15 were down-regulated (e.g. Immunoglobulin mu heavy chain, which was 7 times down-regulated). More information on the proteins found and their expressions in this comparison are shown in Table 2. Figure 1b shows the molecular function, cell component, and biological processes involved according to Gene Ontology in the comparison of DM2 + P vs. P. The network (Fig. 2b) illustrates the protein interaction using the up and downregulated proteins, as shown in Table 2.

The groups without DM2 (P and H) had 115 common proteins. Twenty-two were up-regulated (e.g. Glyceraldehyde-3-phosphate dehydrogenase, S100A8, A9 and Carbonic anhydrase 6 were about 2 times up-regulated), and 25 were down-regulated (e.g. Alpha-amylase 1 C which was about 3 times down-regulated). More information on the proteins found and their expressions in the above comparison are shown in Table 3. Figure 1c shows the molecular function, cell component, and biological processes involved in comparing P vs. H according to Gene Ontology. The network (Fig. 2C) illustrates the protein interaction using the up and downregulated proteins, as shown in Table 3.

The groups without periodontitis (DM2 vs. H) presented 119 common proteins and were classified according to expression. Twenty-three were up-regulated (e.g. Hemoglobin subunit alpha, which was more than 10 times up-regulated), and 25 were down-regulated (e.g. Protein S100-A9, which was 5-fold down-regulated). More information on the proteins found and their expressions in this comparison are shown in Table 4. Figure 1d shows the molecular function, cell component, and biological processes involved in comparing DM2 vs. H according to Gene Ontology. The network (Fig. 2d) illustrates the protein interaction using the up and downregulated proteins, as shown in Table 4.

Figure S2 shows the Reactome analysis, highlighting the interactions among specific proteins.

Discussion

Detecting the salivary protein profile of patients with DM2 and periodontitis can help find possible biomarkers for the diseases and also provide a better understanding of the relationship between both conditions, which can serve as an important tool to guide diagnosis, prognosis, and treatment of the affected population. Under the author's knowledge, this is the first study that used mass spectrometry to compare the salivary proteomic profile of individuals with DM2 and periodontitis in relation to their respective controls. The primary findings of this

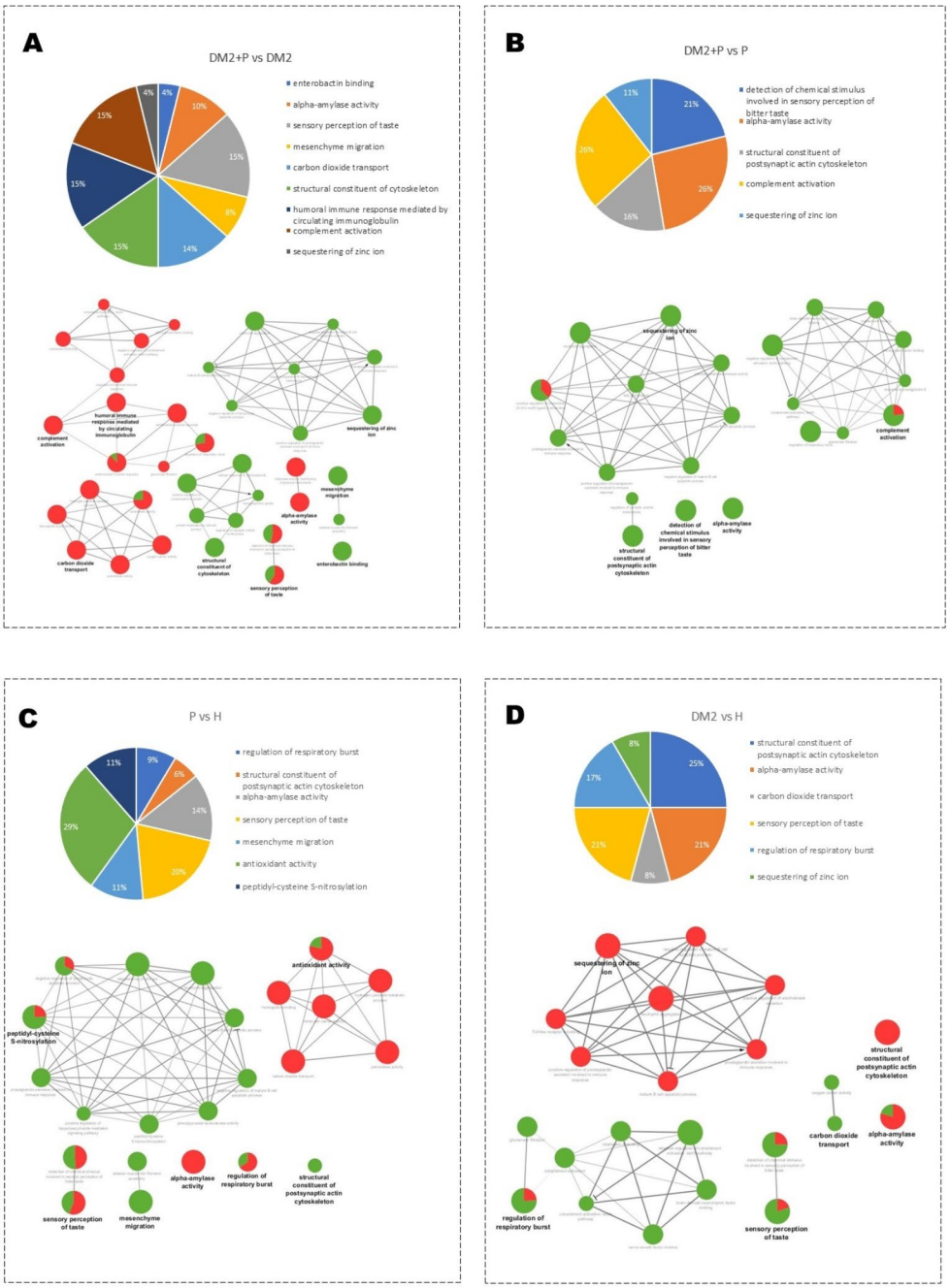

Fig. 1 ClueGO® analysis showing the percentage distribution of molecular functions, immunological processes, and biological processes involved in the comparisons: DM2 + P vs. DM2 (**A**), DM2 + P vs. P (**B**), P vs. H (**C**), and DM2 vs. H (**D**). Additionally, the network layouts represent the proportion of upregulated (green) and downregulated (red) proteins within each highlighted process. The size of the nodes reflects the significance of the process, with larger nodes indicating a lower False Discovery Rate (FDR) and thus greater significance. All processes presented in the figure are statistically significant. The process labeled in black represents the cluster's leading pathway, as identified by the software

study demonstrate that uncontrolled DM2 and periodontitis induce alterations in the salivary protein profile.

Our results showed a large number of proteins present in the salivary samples, with more diversity found in the diseased groups with DM2 and periodontitis. Proteomic analysis revealed 15 common proteins across all comparisons, with exclusive proteins identified in each comparison, indicating that the presence of periodontitis or diabetes led to a distinct profile.

When evaluating the DM2 + P and DM2 groups, 163 common proteins were identified. Bioinformatics analysis revealed that the main processes involved were related to alpha-amylase activity (down-regulated), mesenchymal migration (up-regulated), and various immune-inflammatory processes, including the humoral immune response and complement system activation. The alterations in these processes, particularly those related to the immune response, can be explained by the presence of periodontitis and its infection-inflammatory characteristics. Furthermore, it was possible to observe a high ratio of S100 family proteins within the processes involved. Protein S100-A8 was 6-fold up-regulated in the group

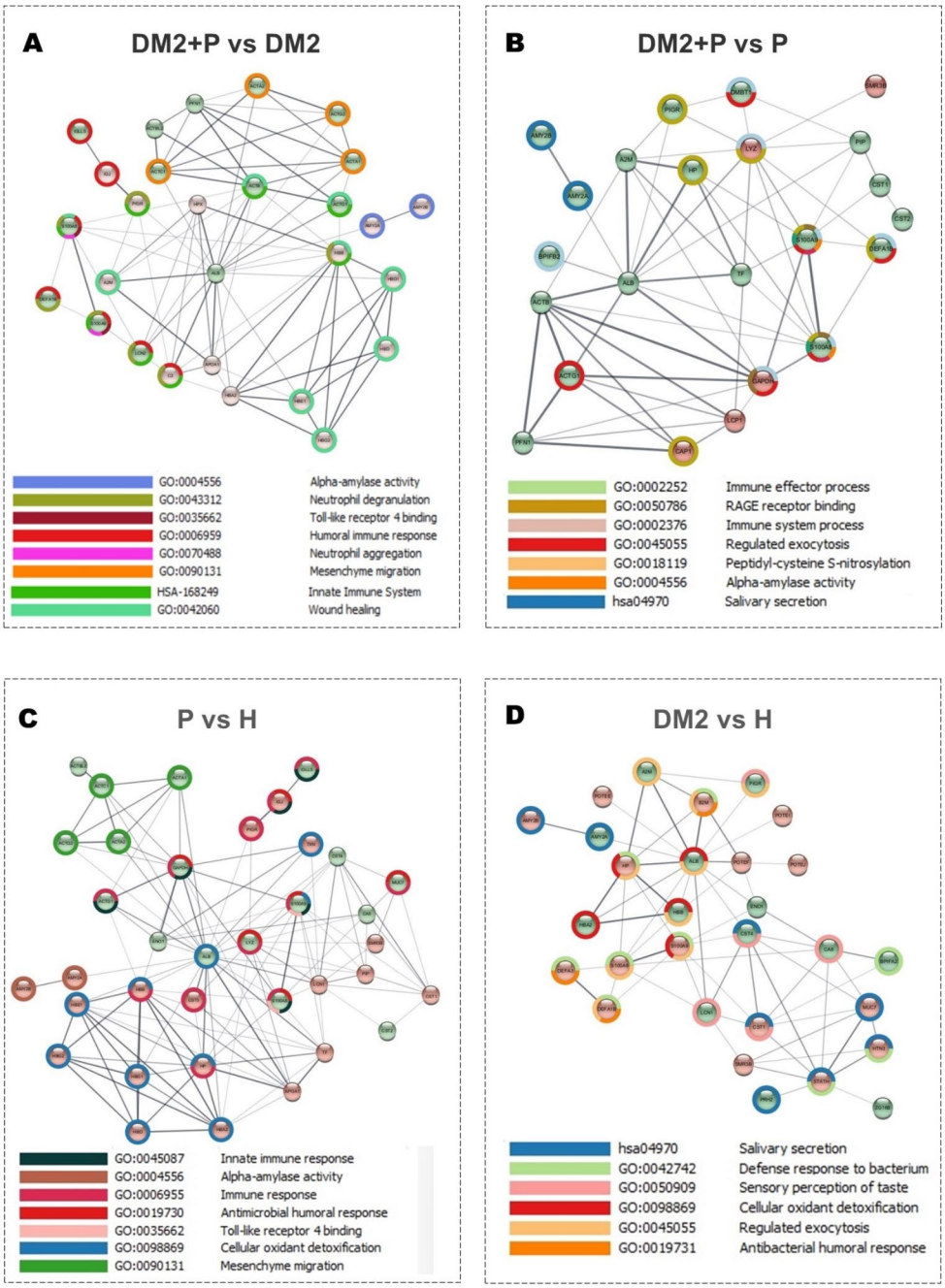

Fig. 2 The network illustrates the results performed by the CYTOSCAPE® software and String Applicative using the up and downregulated proteins. The positively regulated proteins are in green and the negatively regulated proteins are in red. Figure legend demonstrates the different processes involved for each protein in the analysis of DM2 + P vs. DM2 (**A**), DM2 + P vs. P (**B**), P vs. H (**C**) and DM2 vs. H (**D**)

DM2 + P when compared to DM2. Other studies have related S100 family proteins as biomarkers of inflammation in patients with periodontal disease [26, 27]. In addition, S100 proteins are characterized by two calcium binding sites and exert diverse regulatory activities on monocytes/macrophages, neutrophils, lymphocytes, mast cells, endothelial cells, and epithelial cells, participating in the regulation of proliferation, differentiation, apoptosis, Ca^{+2} homeostasis, specific and non-specific inflammatory responses and inflammation [26].

The S100A8 protein is related to regulating inflammation, and its primary sources are monocytes and macrophages. S100A8 is reported to induce TNF-α and IL-1β production in bone marrow cells via TLR-4 activation [28]. Further, in vitro studies have shown that S100A8 can directly induce osteoclast activity, suggesting a possible mechanism by which the immune system can regulate bone resorption [29]. Thus, our results indicate that the S100 A8 protein may play an important role in the relationship between periodontal disease and DM2, suggesting that it may serve as a possible biomarker

Table 2 Up and downregulated proteins identified in the saliva of the DM2 + P and P groups and their expression differences

Dif. Exp	Accession	Description	GENE	Score	Ratio	*p* value
↑	**A0M8Q6**	**Immunoglobulin lambda constant 7**	**IGLC7**	**869**	**2.83**	**<0.01**
↑	**P09228**	**Cystatin-SA**	**CST2**	**395**	**2.08**	**<0.01**
↑	P01023	Alpha-2-macroglobulin	A2M	76	1.95	<0.01
↑	P05109	Protein S100-A8	S100A8	863	1.82	<0.01
↑	P0DUB6	Alpha-amylase 1 A	AMY1A	4898	1.8	<0.01
↑	P0DTE7	Alpha-amylase 1B	AMY1B	4898	1.75	<0.01
↑	P01037	Cystatin-SN	CST1	2742	1.72	<0.01
↑	P07737	Profilin-1	PFN1	1760	1.7	0.01
↑	P04746	Pancreatic alpha-amylase	AMY2A	2890	1.68	<0.01
↑	P0DTE8	Alpha-amylase 1 C	AMY1C	4898	1.65	<0.01
↑	P02768	Albumin	ALB	3294	1.63	<0.01
↑	P19961	Alpha-amylase 2B	AMY2B	4242	1.63	<0.01
↑	P00738	Haptoglobin	HP	62	1.63	<0.01
↑	P06702	Protein S100-A9	S100A9	1192	1.62	<0.01
↑	Q8N4F0	BPI fold-containing family B member 2	BPIFB2	166	1.57	<0.01
↑	P59665	Neutrophil defensin 1	DEFA1B	699	1.39	<0.01
↑	Q96DA0	Zymogen granule protein 16 homolog B	ZG16B	818	1.34	<0.01
↑	P63261	Actin_ cytoplasmic 2	ACTG1	6181	1.3	<0.01
↑	Q9UGM3	Deleted in malignant brain tumors 1 protein	DMBT1	161	1.3	0.01
↑	P60709	Actin_ cytoplasmic 1	ACTB	6183	1.28	<0.01
↑	P0CG38	POTE ankyrin domain family member I	POTEI	499	1.27	0.02
↑	P12273	Prolactin-inducible protein	PIP	1727	1.26	<0.01
↑	P02787	Serotransferrin	TF	291	1.26	<0.01
↑	P01833	Polymeric immunoglobulin receptor	PIGR	674	1.17	<0.01
↑	P01877	Immunoglobulin heavy constant alpha 2	IGHA2	2313	1.07	0.02
↑	P01876	Immunoglobulin heavy constant alpha 1	IGHA1	2092	1.06	0.01
↓	P0CF74	Immunoglobulin lambda constant 6	IGLC6	869	0.66	<0.01
↓	P0DOY2	Immunoglobulin lambda constant 2	IGLC2	956	0.64	<0.01
↓	P0DOY3	Immunoglobulin lambda constant 3	IGLC3	956	0.64	<0.01
↓	P0DOX8	Immunoglobulin lambda-1 light chain		830	0.53	<0.01
↓	B9A064	Immunoglobulin lambda-like polypeptide 5	IGLL5	830	0.53	<0.01
↓	P0CG04	Immunoglobulin lambda constant 1	IGLC1	830	0.51	<0.01
↓	**P02814**	**Submaxillary gland androgen-regulated protein 3B**	**SMR3B**	**2587**	**0.47**	**<0.01**
↓	**P28325**	**Cystatin-D**	**CST5**	**1757**	**0.44**	**<0.01**
↓	**P61626**	**Lysozyme C**	**LYZ**	**237**	**0.41**	**<0.01**
↓	**Q5VSP4**	**Putative lipocalin 1-like protein 1**	**LCN1P1**	**158**	**0.37**	**<0.01**
↓	**Q01518**	**Adenylyl cyclase-associated protein 1**	**CAP1**	**171**	**0.35**	**0.01**
↓	**P04406**	**Glyceraldehyde-3-phosphate dehydrogenase**	**GAPDH**	**366**	**0.32**	**<0.01**
↓	**P13796**	**Plastin-2**	**LCP1**	**92**	**0.31**	**<0.01**
↓	**P01871**	**Immunoglobulin heavy constant mu**	**IGHM**	**195**	**0.16**	**<0.01**
↓	**P0DOX6**	**Immunoglobulin mu heavy chain**		**181**	**0.14**	**<0.01**

↑ = up-regulated; ↓ = down-regulated ($p < 0.05$); Ratio: Differences in protein expressions in the saliva of the DM2 + P vs. P groups

Bold: Means that the difference between groups was at least two times fold

for periodontal disease in this population. Concerning S100A9, it can induce the production of IL-6, IL-8, and RANKL in human periodontal ligament fibroblasts and osteocyte cells, respectively, acting in inflammation and destruction of periodontal tissue [30–32]. Also, in agreement with our results, studies have shown significantly higher levels of S100A8 and A9 in the saliva of systemically healthy patients with periodontitis [33–35]. Noteworthy, the S100 family proteins can bind to TLR-4 and RAGEs, activating a signaling cascade and inducing a proinflammatory response [36], which provides an additional biological link between periodontitis and DM2.

When comparing the groups DM2 + P and P, 87 common proteins were found. The main process involved was the up-regulation of complement activation. In fact, hyperglycemia and AGEs can amplify complement system activation, representing an important mechanism underlying the interplay between DM and periodontitis.

Table 3 Up and downregulated proteins identified in the saliva of the P and H groups and their expression differences

Dif. Exp.	Accession	Description	GENE	Score	Ratio	*p* value
↑	**P04406**	**Glyceraldehyde-3-phosphate dehydrogenase**	**GAPDH**	**135**	**2.48**	**<0.01**
↑	**P23280**	**Carbonic anhydrase 6**	**CA6**	**288**	**2.16**	**<0.01**
↑	P01859	Immunoglobulin heavy constant gamma 2	IGHG2	15	1.97	0.01
↑	P06702	Protein S100-A9	S100A9	858	1.9	<0.01
↑	P06733	Alpha-enolase	ENO1	39	1.75	<0.01
↑	P0CG04	Immunoglobulin lambda constant 1	IGLC1	761	1.65	<0.01
↑	P05109	Protein S100-A8	S100A8	1235	1.65	0.02
↑	Q8TAX7	Mucin-7	MUC7	104	1.62	<0.01
↑	B9A064	Immunoglobulin lambda-like polypeptide 5	IGLL5	718	1.6	<0.01
↑	Q562R1	Beta-actin-like protein 2	ACTBL2	102	1.58	<0.01
↑	P0DOX8	Immunoglobulin lambda-1 light chain		718	1.57	<0.01
↑	P0DOY3	Immunoglobulin lambda constant 3	IGLC3	718	1.55	<0.01
↑	P0DOY2	Immunoglobulin lambda constant 2	IGLC2	718	1.54	<0.01
↑	P61626	Lysozyme C	LYZ	222	1.46	<0.01
↑	P09228	Cystatin-SA	CST2	5455	1.32	<0.01
↑	P68032	Actin_ alpha cardiac muscle 1	ACTC1	144	1.31	<0.01
↑	P62736	Actin_ aortic smooth muscle	ACTA2	144	1.31	<0.01
↑	P63267	Actin_ gamma-enteric smooth muscle	ACTG2	144	1.31	<0.01
↑	P68133	Actin_ alpha skeletal muscle	ACTA1	144	1.3	<0.01
↑	P01036	Cystatin-S	CST4	10,315	1.21	<0.01
↑	P02768	Albumin	ALB	859	1.17	<0.01
↑	P63261	Actin_ cytoplasmic 2	ACTG1	297	1.13	0.02
↓	P01876	Immunoglobulin heavy constant alpha 1	IGHA1	1723	0.85	<0.01
↓	P01591	Immunoglobulin J chain	JCHAIN	730	0.82	<0.01
↓	P01833	Polymeric immunoglobulin receptor	PIGR	1306	0.81	<0.01
↓	P69905	Hemoglobin subunit alpha	HBA2	1368	0.76	<0.01
↓	P68871	Hemoglobin subunit beta	HBB	2369	0.75	<0.01
↓	P31025	Lipocalin-1	LCN1	2021	0.74	<0.01
↓	Q96DA0	Zymogen granule protein 16 homolog B	ZG16B	802	0.74	<0.01
↓	P01034	Cystatin-C	CST3	189	0.7	0.02
↓	P02042	Hemoglobin subunit delta	HBD	659	0.64	<0.01
↓	P02100	Hemoglobin subunit epsilon	HBE1	395	0.63	<0.01
↓	P12273	Prolactin-inducible protein	PIP	3177	0.63	<0.01
↓	P69892	Hemoglobin subunit gamma-2	HBG2	395	0.62	<0.01
↓	P02787	Serotransferrin	TF	153	0.6	<0.01
↓	P69891	Hemoglobin subunit gamma-1	HBG1	395	0.59	<0.01
↓	P10599	Thioredoxin	TXN	164	0.58	0.03
↓	P02647	Apolipoprotein A-I	APOA1	845	0.58	<0.01
↓	**P01037**	**Cystatin-SN**	**CST1**	**7229**	**0.49**	**<0.01**
↓	**P02814**	**Submaxillary gland androgen-regulated protein 3B**	**SMR3B**	**2094**	**0.46**	**<0.01**
↓	**P0DTE7**	**Alpha-amylase 1B**	**AMY1B**	**10,498**	**0.45**	**<0.01**
↓	**P00738**	**Haptoglobin**	**HP**	**140**	**0.45**	**<0.01**
↓	**Q14508**	**WAP four-disulfide core domain protein 2**	**WFDC2**	**279**	**0.39**	**<0.01**
↓	**P0DUB6**	**Alpha-amylase 1 A**	**AMY1A**	**10,498**	**0.34**	**<0.01**
↓	**P19961**	**Alpha-amylase 2B**	**AMY2B**	**7930**	**0.34**	**<0.01**
↓	**P04746**	**Pancreatic alpha-amylase**	**AMY2A**	**6667**	**0.34**	**<0.01**
↓	**P0DTE8**	**Alpha-amylase 1 C**	**AMY1C**	**10,498**	**0.33**	**<0.01**

↑ = up-regulated; ↓ = down-regulated ($p<0.05$); Ratio: Differences in protein expressions in the saliva of the P vs. H groups

Bold: Means that the difference between groups was at least two times fold

The protein-protein network interaction showed a high involvement of Immunoglobins, S100 family proteins, and defensins. Immunoglobulin lambda constant 7 was more than 2 times fold up-regulated in participants with DM2 and periodontitis compared to periodontitis patients without DM2. Immunoglobulin lambda constant 7 is an antibody released by B lymphocytes and is part of the specific humoral immune response [37]. NF-κB plays an

Table 4 Up and downregulated proteins identified in the saliva of the DM2 and H groups and their expression differences

Dif. Exp.	Accession	Description	GENE	Score	Ratio	*p* value	
↑		**P69905**	**Hemoglobin subunit alpha**	**HBA2**	**1368**	**10.38**	**<0.01**
↑		**Q5VSP4**	**Putative lipocalin 1-like protein 1**	**LCN1P1**	**1852**	**8.41**	**<0.01**
↑		**P02810**	**Salivary acidic proline-rich phosphoprotein 1/2**	**PRH2**	**294**	**7.32**	**<0.01**
↑		**P31025**	**Lipocalin-1**	**LCN1**	**2021**	**6.75**	**<0.01**
↑		**P68871**	**Hemoglobin subunit beta**	**HBB**	**2369**	**5.16**	**<0.01**
↑		**P01859**	**Immunoglobulin heavy constant gamma 2**	**IGHG2**	**15**	**2.34**	**<0.01**
↑		P02768	Albumin	ALB	859	1.84	<0.01
↑		P0DOY3	Immunoglobulin lambda constant 3	IGLC3	718	1.6	<0.01
↑		P0CG04	Immunoglobulin lambda constant 1	IGLC1	761	1.58	<0.01
↑		P0DOY2	Immunoglobulin lambda constant 2	IGLC2	718	1.58	<0.01
↑		B9A064	Immunoglobulin lambda-like polypeptide 5	IGLL5	718	1.57	<0.01
↑		P01023	Alpha-2-macroglobulin	A2M	113	1.54	<0.01
↑		P23280	Carbonic anhydrase 6	CA6	288	1.54	<0.01
↑		P06733	Alpha-enolase	ENO1	39	1.54	0.04
↑		Q96DA0	Zymogen granule protein 16 homolog B	ZG16B	802	1.38	<0.01
↑		P01833	Polymeric immunoglobulin receptor	PIGR	1306	1.23	<0.01
↑		Q96DR5	BPI fold-containing family A member 2	BPIFA2	97	1.22	<0.01
↑		P01591	Immunoglobulin J chain	JCHAIN	730	1.22	<0.01
↑		P01877	Immunoglobulin heavy constant alpha 2	IGHA2	995	1.21	<0.01
↑		P01876	Immunoglobulin heavy constant alpha 1	IGHA1	1723	1.19	<0.01
↑		P0DOX2	Immunoglobulin alpha-2 heavy chain		980	1.17	<0.01
↑		P04746	Pancreatic alpha-amylase	AMY2A	6667	1.17	<0.01
↑		P01036	Cystatin-S	CST4	10,315	1.13	<0.01
↓		P0DTE7	Alpha-amylase 1B	AMY1B	10,498	0.94	0.02
↓		P19961	Alpha-amylase 2B	AMY2B	7930	0.91	<0.01
↓		P0DTE8	Alpha-amylase 1 C	AMY1C	10,498	0.9	<0.01
↓		P0DUB6	Alpha-amylase 1 A	AMY1A	10,498	0.89	<0.01
↓		P01834	Immunoglobulin kappa constant	IGKC	171	0.88	0.03
↓		P0DOX7	Immunoglobulin kappa light chain		150	0.82	<0.01
↓		Q562R1	Beta-actin-like protein 2	ACTBL2	102	0.81	0.03
↓		P15516	Histatin-3	HTN3	1859	0.79	0.04
↓		P02808	Statherin	STATH	10,814	0.76	0.02
↓		A5A3E0	POTE ankyrin domain family member F	POTEF	71	0.76	<0.01
↓		P00738	Haptoglobin	HP	140	0.76	<0.01
↓		Q8TAX7	Mucin-7	MUC7	104	0.76	<0.01
↓		Q6S8J3	POTE ankyrin domain family member E	POTEE	71	0.76	<0.01
↓		Q9BYX7	Putative beta-actin-like protein 3	POTEKP	71	0.69	0.01
↓		P59665	Neutrophil defensin 1	DEFA1B	233	0.66	<0.01
↓		P59666	Neutrophil defensin 3	DEFA3	233	0.66	0.02
↓		P0CG38	POTE ankyrin domain family member I	POTEI	23	0.64	<0.01
↓		P61769	Beta-2-microglobulin	B2M	247	0.51	<0.01
↓		P02814	Submaxillary gland androgen-regulated protein 3B	SMR3B	2094	0.51	<0.01
↓		**P0CG39**	**POTE ankyrin domain family member J**	**POTEJ**	**23**	**0.5**	**<0.01**
↓		**P19013**	**Keratin_ type II cytoskeletal 4**	**KRT4**	**86**	**0.45**	**0.01**
↓		**Q9UBG3**	**Cornulin**	**CRNN**	**177**	**0.35**	**<0.01**
↓		**P01037**	**Cystatin-SN**	**CST1**	**7229**	**0.34**	**<0.01**
↓		**P05109**	**Protein S100-A8**	**S100A8**	**1235**	**0.3**	**0.01**
↓		**P06702**	**Protein S100-A9**	**S100A9**	**858**	**0.2**	**<0.01**

↑ = up-regulated; ↓ = down-regulated ($p<0.05$); Ratio: Differences in protein expressions in the saliva of the DM2 vs. H groups

Bold: Means that the difference between groups was at least two times fold

essential role in B cell activation and development, and NF-κB activation is critical in inflammation and DM2 development [38]. In this context, the increased B lymphocyte response is related to a greater Th2 response over the Th1, which is associated with the development and progression of periodontitis [39, 40].

In evaluating the groups without DM2 (P and H), out of the 115 common proteins, 22 proteins were up-regulated. The processes involved were mesenchyme migration, antioxidant activity, and S-nitrosylation of peptide cysteine. Further, the inflammatory condition of periodontitis led to an increase in proteins associated with immune processes, including neutrophil aggregation, antioxidant activity, prostaglandin secretion involved in the immune response, and B cell apoptosis. Network protein analysis showed the involvement of S100 family proteins (S100 A8 and A9) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH was more than 2-fold up-regulated in participants with periodontitis. GAPDH represents one of the main receptors for the fimbriae of *Porphyromonas gingivalis*, one of the main pathogens related to periodontitis [41]. Indeed, the literature has suggested that GAPDH likely plays a role in adhesion and colonization of the oral cavity by *P. gingivalis*, as well as triggering host cellular processes involved in the pathogenesis of periodontal diseases [42, 43]. Furthermore, our results showed that S100 family proteins were also up-regulated in participants without DM2 but with periodontitis. In 2019, a cross-sectional study observed increased S100A8 and A9 proteins in the saliva of patients with periodontitis but without DM2 in proteomic analysis, thus corroborating our results [33].

Similarly to our results, a clinical study found a significant increase in the levels of S100A8, S100A9, and immunoglobulins proteins in the diseased groups after analyzing the gingival crevicular fluid proteome of 20 DM2 and periodontitis individuals and controls [44]. S100A8/A9 levels in stimulated saliva were evaluated in a cross-sectional study, where the levels of these biomarkers were correlated with worse BOP values, PD ≥ 4 mm, and participants with a high periodontal inflammatory burden [45]. However, certain methodological differences should be considered when comparing with our study, such as sample heterogeneity, including participants with various systemic conditions and/or smokers, and the use of ELISA assays to identify salivary proteins.

Noteworthy, a recent systematic review of salivary biomarkers in proteomic analyses identified several potential biomarkers for periodontitis, such as alpha-amylase, serum albumin, complement C3, neutrophil defensin, profilin-1, and S100-P [46]. However, the participants in the included studies were systemically healthy, and those findings may not be directly applicable to patients with both periodontitis and DM. Other clinical studies have attempted to identify potential salivary biomarkers for periodontitis and diabetes, including MMP-8 and IL-1β [47], e-cadherin [48], and plasminogen activator inhibitor-1 [49]. Nevertheless, these above-mentioned studies employed immunoassays, in which researchers often arbitrarily choose selected biomarkers.

In participants without periodontitis (DM2 and H groups), of the 119 common proteins found, 23 proteins were up-regulated. The main processes identified included the downregulation of zinc ion sequestration and alpha-amylase activity, alongside the upregulation of carbon dioxide transport, regulation of respiratory burst, and sensory perception of taste. Additionally, there was an upregulation of proteins associated with complement system activity, neutrophil aggregation, and the regulation of prostaglandin involved in immune responses. These alterations can be attributed to the inflammatory state induced by the diabetic (DM) condition. The protein network analysis showed a great interaction among up-regulated proteins of the hemoglobin family. Hemoglobin subunit alpha is a hemoglobin protein that is encoded by the HBA1 gene in humans [50]. An increase in hemoglobin has been reported in patients with insulin resistance and DM2 in serum proteomic analysis studies [50]. Thus, our data suggest this salivary protein as a possible biomarker for DM2. In addition, several inflammatory markers were altered in the DM2 group, corroborating this population's increased risk for systemic and periodontal diseases.

Our study identified the salivary proteomic profile and some potential biomarkers in patients with both periodontitis and DM. Considering that DM exacerbates both the risk and severity of periodontitis, while periodontitis can impair glycemic control and increase the likelihood of DM-related complications [4], our findings offer critical insights for future screening efforts. Such biomarkers may aid in the early diagnosis, treatment, and monitoring of periodontal disease in this vulnerable population.

Some limitations can be highlighted in our study. Although gender and age-matched provided some bias control, other factors may have influenced our results. The study's inclusion criteria, such as type 2 DM with glycated hemoglobin of 7–10% and stage III-IV periodontitis, limit the validity of the results to only this population. The use of metformin as an antidiabetic agent may have influenced the results in comparisons with non-diabetic groups, as this medication modulate the inflammatory pathways. Further, we did not assess obesity status, physical activity, diet, and other oral diseases, which can also change the salivary proteomic profile of patients. In

addition, the cross-section study design does not allow for further conclusions. Thus, future prospective studies should assess the influence of DM2 and periodontitis on the salivary proteomic profile in order to confirm our findings. Salivary proteomic profile of individuals with other stages of periodontitis and different DM statuses of the included herein remain uncertain.

Conclusion

In conclusion, the presence of DM2 and periodontitis significantly alters the salivary proteome of patients. Our proteomic analysis demonstrated that changes in the S100 family proteins (S100A8 and S100 A9) may be highly related to the presence of DM2 and periodontitis. These alterations are associated with the immunoinflammatory process, increasing periodontitis and DM2 severity.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00784-025-06171-1.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate This observational clinical study was approved by the University of Taubaté ethics committee (CAAE: 19319219.8.0000.5501). Eligible patients willing to participate signed an informed consent form after receiving oral and written information.

Competing interests The authors declare no competing interests.

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