Journal of Dentistry 81 (2019) 64–69

Contents lists available at ScienceDirect

Journal of Dentistry

journal homepage: www.elsevier.com/locate/jdent

Proteomics of acquired pellicle in gastroesophageal reflux disease patients with or without erosive tooth wear

Tatiana Martini[a], Daniela Rios[a], Luiza Paula Silva Cassiano[a], Cíntia Maria de Souza Silva[a], Even Akemi Taira[a], Talita Mendes Silva Ventura[a], Heloísa Aparecida Barbosa Silva Pereira[a], Ana Carolina Magalhães[a], Thiago Saads Carvalho[b], Tommy Baumann[b], Adrian Lussi[b], Ricardo Brandt Oliveira[c], Regina Guenka Palma-Dibb[d], Marília Afonso Rabelo Buzalaf[a,*]

[a] Bauru School of Dentistry, University of São Paulo, Al. Octávio Pinheiro Brisolla, 9-75, 17012-901, Bauru, São Paulo, Brazil
[b] Department of Restorative, Preventive and Pediatric Dentistry, University of Bern, Freiburgstrasse 7, CH-3010, Bern, Switzerland
[c] Ribeirão Preto School of Medicine, University of São Paulo, Av. Bandeirantes, 3900, 14048-900, Ribeirão Preto, São Paulo, Brazil
[d] Ribeirão Preto School of Dentistry, University of São Paulo, Av. Bandeirantes, 3900, 14048-900, Ribeirão Preto, São Paulo, Brazil

ARTICLE INFO

Keywords:
Acquired enamel pellicle
Erosive tooth wear
Gastroesophageal reflux
Proteomics

ABSTRACT

Objectives: This *in vivo* study compared the protein profile of the acquired enamel pellicle (AEP) in volunteers 1) with gastroesophageal reflux disease (GERD) and erosive tooth wear (ETW) (BEWE ≥ 9; GE group); 2) with GERD without ETW (BEWE = 0; GNE group) and 3) control (without GERD and BEWE = 0; C group).
Materials and methods: Twenty-four subjects (8/group) participated. AEP was formed during 120 min and collected. After protein extraction, the samples were submitted to reverse phase liquid chromatography coupled to mass spectrometry. Label-free proteomic quantification was performed using Protein Lynx Global Service software.
Results: In total, 458 proteins were identified. Seventy-six proteins were common to all the groups. The proteomic profile of the AEP was quite different among the distinct groups. The numbers of proteins exclusively found in the C, GE and GNE groups were 113, 110 and 81, respectively. Most of the proteins exclusively identified in the C and GNE groups bind metals, while those in the GE group are mainly membrane proteins. Many proteins were found exclusively in the reflux groups. In the quantitative analyses, when the GNE group was compared with the GE group, the proteins with the highest decreases were *Lysozyme C, Antileukoproteinase, Cathepsin G,* Neutrophil defensins and Basic salivary proline-rich proteins, while those with the highest increases were subunits of Hemoglobin, *Albumin* and isoforms of Cystatin.
Conclusion: Profound alterations in the proteomic profile of the AEP were seen in GNE compared with GE volunteers, which might play a role in the resistance to ETW seen in the first.
Clinical significance: This pioneer study compared the proteomic profile of the AEP of patients with GERD with or without ETW. Increased proteins in those without ETW might be protective and are good candidates to be added to dental products to protect against erosion caused by intrinsic acids.

1. Introduction

Erosive tooth wear (ETW) is caused by the interplay between non-bacterial intrinsic or extrinsic acids and mechanical abrasive forces [1]. Intrinsic acids come from the stomach, when gastric juice travels up through the esophagus and enters the mouth [2]. This typically occurs in gastroesophageal reflux disease (GERD) that affects around 10–20% of the population [3]. The pH of the gastric acids is lower than that of dietary acids. In addition, the titratability of the former is greater than that of the latter, leading usually to more severe destruction of the tooth structure [2]. Thus, preventive measures against intrinsic erosion, comprising new prophylactic approaches and development of new dental products are highly desirable.

Around 24–48% of patients with GERD have ETW [4–7] due to the very low pH (1–3) of regurgitated gastric contents [8]. Because of this low pH, greater prevalence of ETW among patients with GERD would

* Corresponding author at: Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Al. Octávio Pinheiro Brisolla, 9-75, 17012-901, Bauru, São Paulo, Brazil.
E-mail address: mbuzalaf@fob.usp.br (M.A.R. Buzalaf).

https://doi.org/10.1016/j.jdent.2018.12.007
Received 15 August 2018; Received in revised form 11 December 2018; Accepted 17 December 2018

be expected. Thus, patients with GERD who do not have ETW may have some protective factor. It was shown that the proteomic profiles of the esophageal mucosa of patients with non-erosive esophageal reflux disease (NERD) and erosive esophageal reflux disease (ERD) are different [9], and patients who develop ERD have reduced ability to respond to insults caused by acid and pepsin. These patients have weaker capacity of the esophageal mucosa, such as reduction in cell proliferation, cell migration, glucose metabolism, stress responses and probably esophageal keratinization [10].

One of the most important preventive factors against dental erosion is the acquired enamel pellicle (AEP), an organic bacteria-free film composed mainly by proteins and glycoproteins that covers enamel. Due to its composition, the AEP is capable of protecting the underlying tooth structure, reducing the degree of acid dissolution [11,12]. Therefore, it is plausible that the protein composition of the AEP from patients with GERD without ETW is different from that of patients with the same disease, but with ETW. Thus, the aim of the present study was to compare the protein composition of the AEP of GERD patients with ETW with that of GERD patients without ETW. Control volunteers (without GERD or ETW) were also evaluated for comparison. The null hypotheses tested were: 1) There is no difference in the protein composition of the AEP from patients with GERD without ETW compared with that of patients with the same disease, but with ETW; 2) GERD does not alter the protein composition of the AEP.

2. Material and methods

2.1. Ethical aspects and subjects

The protocol of this study was approved by the Ethics Committees of Bauru and Ribeirão Preto School of Dentistry, University of São Paulo, (#CAAE 44,007,415.1.0000.5417 and 44,007,415.1.3001.5419, respectively). Prior to the beginning of the study the subjects signed an informed consent document. The sample size (n = 8/group) was chosen based on previous studies that compared the proteomic profile of the AEP formed under different conditions or on distinct locations [13–16].

The volunteers from both genders (20–60 years of age) who participated in this *in vivo* study were non-smokers, had good general and oral health (without active caries lesions, gingivitis, periodontitis or any other oral condition affecting the composition of the oral fluids) and presented normal salivary flow (stimulated flow > 1 mL/min). Volunteers presenting conditions associated with vomiting (*e.g.* anorexia or bulimia), bruxism as well as occupations associated with increased risk of ETW, such as swimmers and battery, charging and galvanizing workers, were not included in the study. The clinical examination was conducted by a calibrated examiner (CMSS). Calibration was assessed before the beginning of the study, by the repeated examination of 30 photographs. Reproducibility was monitored by means of repeated examinations conducted on 5% of the volunteers. The intra-examiner kappa value was 0.92. Clinical examinations were performed at a dental clinic with the volunteers in a supine position using artificial light, air compressor, suction, clinical mirror, and probe. After a professional prophylaxis, the examiner classified all teeth according to the Basic Erosive Wear Examination (BEWE) index.

The volunteers also answered questions on GERD-related symptoms. The inclusion criteria for GERD were heartburn and/or regurgitation for at least 1 year (frequency more than 2 times/week) and abnormal pH parameters of 24 h [(distal oesophageal acid exposure time (AET) > 6%)] [17]. Exclusion criteria were patients with malignant lesions in the esophagus or stomach, Barrett's esophagus, gastric or duodenal ulcer, previous gastric or esophageal surgery, and patients taking antisecretory or prokinetic drugs at least 15–30 days prior to the AEP collection [9]. These volunteers and all the others underwent esophageal pH-metry and endoscopy exams. They were divided into 3 groups, as follows:

a) Volunteers with GERD-related symptoms and ETW (GE; n = 8): The inclusion criteria for ETW were BEWE ≥ 9 or grade 3 in the upper anterior sextant (with all incisors affected) [18].
b) Volunteers with GERD-related symptoms without ETW (GNE; n = 8): Patients without ETW were included in this group (BEWE = 0) [18].
c) Control group (C; n = 8): volunteers in this group did not have GERD-related symptoms, which was confirmed by esophageal pH-metry and endoscopy and they did not have ETW (BEWE = 0) [18].

2.2. In vivo experiment

The procedures were conducted during the morning in order to avoid circadian effects on the composition of the pellicle [19]. The volunteers were submitted to professional dental prophylaxis with coarse pumice and a rubber cup and were instructed not to eat or drink during the AEP formation. After 120 min, the dental surfaces were thoroughly rinsed with deionized water and dried with a jet of air. The AEP was collected from the buccal surface of the upper and lower teeth (both anterior and posterior teeth), from the middle and incisal/occlusal third of each tooth, using an electrode filter paper (Bio-Rad, Hercules, CA) of 5 X 10 mm pre-dipped in 3% citric acid [20]. One filter paper was used for each quadrant and they were stored at −80 °C until analysis.

2.3. Proteomic analysis

The papers collected from all volunteers of the same group were cut into small pieces and grouped to constitute a *pool*. The procedures of preparation of AEP samples and shotgun proteomic analysis were performed exactly as described by Ventura et al. [15]. The equipment used was a nanoACQUITY UPLC-Xevo QTof MS system (Waters, Manchester, UK), equipped with nanoACQUITY HSS T3, analytical reverse phase column (75μmX150 mm, 1.8 μm particle size, Waters). ProteinLynx Global Server (PLGS) version 3.0 (Waters Co., Manchester, UK) was used to process and search the hucontinuum LC-MSE data. Proteins were identified with the embedded ion accounting algorithm in the software and a search of the *Homo sapiens* database (reviewed only, UniProtKB/Swiss-Prot) downloaded on February 2017 from UniProtKB (http://www.uniprot.org/).

For label-free quantitative proteome, three MS raw files from each pooled group were analysed using the PLGS software. All the proteins identified with confidence greater than 95% were included in the quantitative analysis. Identical peptides from each triplicate by sample were grouped based on mass accuracy (< 10 ppm) and on time of retention tolerance < 0.25 min, using the clustering software embedded in the PLGS software. Difference in expression among the groups was expressed as $p < 0.05$ for down-regulated proteins and $1\text{-}p > 0.95$ for up-regulated proteins. The relevant comparisons were GE *vs.* C, GNE *vs.* C and GNE *vs.* GE.

3. Results

Data were collected between February and November 2017. All the volunteers completed the study. Table 1 shows the characterisation of the volunteers according to gender, age, % time of esophageal pH < 4 and BEWE score. Groups C and GE had both male and female volunteers, while GNE had only female volunteers. The mean age of the volunteers was quite similar among the groups. C volunteers had mean % time of esophageal pH < 4 of 1%, while the corresponding percentage for GE and GNE volunteers was 10%. Volunteers in GE group had mean BEWE score around 16.

The identified proteins, classified according to their function, molecular interaction and origin are displayed in Table S1. In total, 458 proteins were identified. The highest and lowest numbers of proteins were identified in the C (260) and GNE group (193), while the GE group

Table 1
Characterization of the volunteers according to gender, age, percentage time of esophageal pH < 4 and BEWE score.

Group[a]	Gender	Median age (95% CI)	Mean (± SD) % time esophageal pH < 4	Median BEWE (95% CI)
C	5F, 3M	27.0 (23.5 - 39.3)	1.0 ± 0.3	0
GE	7F, 1M	30.0 (25.0 – 39.8)	10.0 ± 1.2	17.0 (19.9 – 42.6)
GNE	8F, 0M	25.0 (19.9 – 42.6)	10.0 ± 1.4	0

[a] C - no GERD, BEWE = 0; GE - GERD, BEWE ≥ 9 or grade 3 in the upper anterior sextant; GNE - GERD, BEWE = 0. F – female; M – male. n = 8.

presented 235 proteins. Figure S1 shows the number of proteins common to the groups, as well as the numbers of proteins found in only one of the groups. Seventy-six proteins were common to all the groups (Figure S1, Table S1). Among them are proteins typically found in AEP, such as isoforms of cystatin, cytoskeletal keratin, neutrophil defensin, actin, protein S100-A, proline-rich protein (PRP), albumin, Ig A, Ig G, besides *Lactotransferrin*, *Serotransferrin* and *Lysozyme C*.

The proteomic profile of the AEP pellicle was considerably different among the groups. The numbers of proteins exclusively found in the C, GE and GNE groups were 113, 110 and 81, respectively. Regarding the proteins identified exclusively in one or two of the groups, some findings must be highlighted: a) Heat-shock proteins were not found in GE; b) Histatins were not found in GNE; c) Most of the identified isoforms of 14-3-3 protein were only present in C and GE groups; d) Serine/threonine-protein kinase (various isoforms) were only identified in GNE; e) Many of the proteins identified exclusively in the groups with no ETW (C and GNE) are metal-binding proteins, while many of those exclusive to the GE group are membrane proteins and proteins related to neutrophil degranulation (Tables S1 and S2).

Regarding quantitative analysis (Table 2), for the comparison GE *vs.* C group, 32 proteins were significantly increased and 14 proteins were significantly decreased in the first. Among the increased proteins are various isoforms of cytoskeletal keratin, neutrophil defensin and actin, besides *Cathepsin G*, *Lysozyme C, Antileukoproteinase*, *Myeloperoxidase*, *Mucin-7* and *Lactotransferrin.* On the other hand, various PRPs, isoforms of hemoglobin, cystatin and albumin, as well as *Statherin* were reduced in the GE group compared with the C group. When the GNE group was compared with the C group, 13 and 20 proteins were increased and decreased, respectively, in the first. The protein with the highest increase (> 6-fold) was *Microtubule-associated protein*. Other proteins increased in a lesser extent include *Hemoglobin subunit beta*, isoforms of actin and immunoglobulins. Proteins with the greatest decreases were isoforms of PRPs, neutrophil defensins and cystatins, as well as *Lysozyme C* and *Cathepsin G*. The most relevant comparison is GNE *vs.* GE. In this case, 8 proteins were increased and 22 were decreased in the first. Remarkably, the proteins with the highest rates of increase (close to or higher than 3-fold) were 2 isoforms of hemoglobin. Other increased proteins, despite in lower rates, were isoforms of albumin and of cystatin. The proteins with the highest decreases (close to or higher than 2-fold) were *Lysozyme C, Cathepsin G, Proline-rich protein 27*, and various isoforms of cytoskeletal keratin, neutrophil defensins and basic salivary PRPs. Proteins decreased in lower rates were *Lactotransferrin, Cystatin-B* and *Protein S100-A9*.

4. Discussion

This is the first study to compare the proteomic profile of the AEP of GERD patients with ETW with that of GERD patients without ETW. A control group constituted of volunteers with no GERD and no ETW was also included, to allow the detection of changes in the proteomic profile of the AEP in function of GERD. The results revealed many alterations in the protein profile of the AEP from GERD volunteers with and without ETW, as well as between GERD and control volunteers, which led us to reject both null hypotheses of the study. The characteristics of the volunteers included in the study were quite similar in terms of age. Regarding gender, since this was a convenience sample, there was a higher number of female volunteers in all the groups and GNE group had only females (Table 1). However, there is no reason to suspect that this would influence in the results of the proteomic analysis of the AEP, since the majority of the GE group was made up of females. The mean BEWE score of the volunteers of GE group was around 16, which denotes severe ETW [18]. The inclusion criteria for volunteers with GERD assured that all of them had symptoms for at least 1 year (frequency > 2 times/week) [9], *i.e.*, there was enough time for ETW to occur. Moreover, the mean % time of esophageal pH < 4 was similar for GE and GNE groups and much higher than that found in C group (Table 1).

The protocol of protein extraction and proteomic analysis followed a recently developed methodology that increases the identification of proteins in the AEP samples [15]. Accordingly, the number of identified proteins was 458, which is the highest number ever reported in studies involving *in vivo* analysis of AEP. The proteomic profiles of the AEPs collected in the 3 groups was quite different, as can be depicted from the high numbers of unique proteins in each of the groups (close to or higher than 100). Most of the proteins identified in all the groups are proteins typically found in the AEP (Fig. S1, Table S1) that presented differences in expression among the groups (Table 2). This means that GERD has a great impact on the proteomic profile of the AEP that also changes remarkably in patients with GERD presenting ETW or not.

Many of the proteins identified exclusively in the GE group are membrane proteins, suggesting a higher degree of epithelial cell lysis by the gastric acids, consistent with the higher incidence of lesions in the oral mucosa of patients with GERD [21,22]. Proteins related to neutrophil degranulation were also exclusive of GE group, in-line with findings of neutrophil infiltrates in eroded areas of the mucosa [23]. Some of these proteins are secreted as active proteases and could change the structure of the AEP, reducing its protective ability against demineralization.

Among the proteins exclusively identified in the GNE group are those with sites of phosphorylation in serine, as well as isoforms of serine/threonine-protein kinases. Phosphorylation in serine confers negative charge to this amino acid. Hydroxyapatite binds proteins through both calcium and phosphate sites [24,25]. Phosphorylated and negatively charged proteins, such as acidic PRPs, *Histatin 1* and *Statherin*, have strong affinity to hydroxyapatite and are included among the pellicle precursor proteins, constituting the basal layer of this integument [26] that confers most of the protection against demineralization, since it is not removed after erosive challenges [27]. Thus, it is possible that the greater number of serine-phosphorylated proteins in the AEP of the GNE group might be responsible, at least in part, for the protection against ETW.

Other interesting findings related to the exclusive proteins were the fact that heat-shock proteins were not identified in the GE group and that most of the identified isoforms of 14-3-3 protein were present in C and GE groups. These findings are consistent with a study that evaluated the proteomic profile of the esophagus mucosa in patients with erosive and non-erosive GERD. The authors found higher expression of *Heat shock cognate 71 kDa protein* in patients with non-erosive GERD when compared to those with erosive GERD, as well as higher expression of 14-3-3 proteins in patients with reflux when compared to the healthy ones [9]. In addition, many proteins were exclusive of the GERD groups, regardless the presence of ETW. Thus, the occurrence of

Table 2
Classification and relative quantification of proteins identified in the acquired enamel pellicle collected from volunteers with gastro-esophageal reflux disease (GERD) and erosive tooth wear (GE), GERD and no erosive tooth wear (GNE) or controls (no GERD, no erosive tooth wear; C).

Accession number	Protein name	Ratio GNE/C	*P*
E7EVA0	Microtubule-associated protein[m,n,q,u,]	**6.05**	1.00
P68871	Hemoglobin subunit beta[b, c, m, n, o, u, w]	1.49	1.00
P01859	Ig gamma-2 chain C region[b, j, o, u]	1.46	1.00
P68133	Actin_ alpha skeletal muscle[d, m, n, q, u, w]	1.45	1.00
P68032	Actin_ alpha cardiac muscle 1[b, m, n, q, u w]	1.43	1.00
P62736	Actin, aortic smooth muscle[b, d, m, n, q, u]	1.40	1.00
P63267	Actin, gamma-enteric smooth muscle[b, m, n, q, u, w]	1.39	1.00
A5A3E0	POTE ankyrin domain family member F[f, m, n, u]	1.35	0.98
P0CG04	Ig lambda-1 chain C regions[b, m, o, s, u, w]	1.34	0.97
P0CG05	Ig lambda-2 chain C regions[b, m, o, s, u, w]	1.31	0.97
P01857	Ig gamma-1 chain C region[b, m, o, u, w]	1.28	0.99
P63261	Actin, cytoplasmic 2[a, d, g, j, n, q, u, w]	1.21	0.97
P60709	Actin_ cytoplasmic 1[b, m, n, q, u, w]	1.19	0.99
P02768	Serum albumin[a, b, c, g, o, u, w]	0.90	0.02
C9JKR2	Albumin, isoform CRA_k[c,g,o,i,u]	0.90	0.04
P02788	Lactotransferrin[b,c,i,j,n,o,p,u,w]	0.87	0.03
P09228	Cystatin-SA[a, b, g, o, u]	0.83	0.01
P01036	Cystatin-S[a, b, g, o, u]	0.81	0.00
P04080	Cystatin-B[a, g, n, p, u]	0.80	0.04
P01037	Cystatin-SN[a, b, g, o, u]	0.79	0.00
P02810	Salivary acidic proline-rich phosphoprotein ½[b, d, h, l, o, u, v]	0.77	0.00
A0A0A0MT31	Proline-rich protein 4[b, l, p, u]	0.76	0.00
A0A087WZY1	Uncharacterized protein[m,t,x]	0.75	0.00
P08311	Cathepsin G[a, b, g, i, j, o, p, u]	0.51	0.00
P61626	Lysozyme C[a, b, g, i, j, o, u, w]	**0.41**	0.00
P59665	Neutrophil defensin 1[b, i, j, o, u]	**0.35**	0.00
P59666	Neutrophil defensin 3[b, i, j, o, u]	**0.35**	0.00
Q6MZM9	Proline-rich protein 27[b, l, o, x]	**0.32**	0.00
P03973	Antileukoproteinase[a, b, g, i, j, o, u]	**0.31**	0.00
P08493	Matrix Gla protein[b, m, o, u]	**0.30**	0.00
P02812	Basic salivary proline-rich protein 2[b, l, o, u]	**0.27**	0.00
Q9HCE3	Zinc finger protein 532[b, m, p, u]	**0.14**	0.00
P04280	Basic salivary proline-rich protein 1[b, l, o, u]	**0.13**	0.00

Accession number	Protein name	Ratio GE/C	*P*
Q01546	Keratin_ type II cytoskeletal 2 oral[d, m, p, o, u, w]	**3.13**	0.98
P08311	Cathepsin G[a, b, g, i, j, o, p, u]	**2.72**	1.00
P61626	Lysozyme C[a, b, g, i, j, o, u, w]	**2.66**	1.00
P0CG39	POTE ankyrin domain family member J[b, m, o, u]	**2.25**	1.00
P04259	Keratin, type II cytoskeletal 6B[b, i, o, u, w]	1.97	1.00
P02538	Keratin_ type II cytoskeletal 6A[b, d, m, o, u, w]	1.93	1.00
P48668	Keratin_ type II cytoskeletal 6C[d, m, o, u]	1.88	1.00
P02533	Keratin, type I cytoskeletal 14[d, m, o, u, w]	1.84	1.00
P08779	Keratin, type I cytoskeletal 16[b, m, o, u, w]	1.84	1.00
P13647	Keratin, type II cytoskeletal 5[d, m, n, o, q, u]	1.84	0.99
P03973	Antileukoproteinase[a, b, g, i, j, o, u]	1.80	1.00
P13646	Keratin, type I cytoskeletal 13[d, m, o, p, q, u]	1.72	1.00
P05164	Myeloperoxidase[a, b, g, j, r, u]	1.67	1.00
P0CG38	POTE ankyrin domain family member I[b, m, o, u]	1.67	0.99
P19012	Keratin, type I cytoskeletal 15[b, m, o, u, w]	1.65	1.00
A5A3E0	POTE ankyrin domain family member F[f, m, n, u]	1.52	0.99
Q6S8J3	POTE ankyrin domain family member E[b, m, o, u]	1.52	0.98
P59665	Neutrophil defensin 1[b, i, j, o, u]	1.51	1.00
P68133	Actin, alpha skeletal muscle[b, d, m, n, q, u, w]	1.49	1.00
Q8TAX7	Mucin-7[b, i, k, o, u]	1.48	1.00
P62736	Actin, aortic smooth muscle(b, d, m, n, q, u)	1.46	1.00
P59666	Neutrophil defensin 3[b, i, j, o, u]	1.46	1.00
P68032	Actin, alpha cardiac muscle 1[d, m, n, q, u, w]	1.43	1.00
P63267	Actin, gamma-enteric smooth muscle[d, m, n, q, u]	1.40	1.00
P02788	Lactotransferrin[f, g, h, i, j, n, o, p, u, w]	1.40	1.00
Q5T3N1	Annexin (Fragment)[b, l, n, p, s, u]	1.31	0.99
P01859	Ig gamma-2 chain C region[b, j, o, u]	1.28	1.00
P60709	Actin_ cytoplasmic 1[b, m, n, q, u, w]	1.22	1.00
P63261	Actin, cytoplasmic 2[a, d, g, j, n, q, u, w]	1.22	1.00
P06702	Protein S100-A9[a, b, g, i, j, n, o, q, s, u, w]	1.20	1.00
P01876	Ig alpha-1 chain C region[b, e, i, j, o, u]	1.14	0.98
P01877	Ig alpha-2 chain C region[b, e, i, j, o, u]	1.14	0.97
P02808	Statherin[b, e, i, l, o, u]	0.71	0.00
A0A0A0MT31	Proline-rich protein 4[b, l, p, u]	0.70	0.00
P02810	Salivary acidic proline-rich phosphoprotein ½[b, d, h, l, o, u, v]	0.70	0.00
A0A087WZY1	Uncharacterized protein[m,t,x]	0.70	0.00
P02812	Basic salivary proline-rich protein 2[b, l, o, u]	0.70	0.00

(*continued on next page*)

Table 2 (*continued*)

Accession number	Protein name	Ratio GE/C	*P*
C9JKR2	Albumin, isoform CRA_k[c, g, o, i, u]	0.68	0.00
P09228	Cystatin-SA[a, b, g, o, u]	0.66	0.00
P02768	Serum albumin[a, b, c, g, o, u, w]	0.66	0.00
P01037	Cystatin-SN[a, b, g, o, u]	0.65	0.00
P01036	Cystatin-S[a, b, g, o, u]	0.64	0.00
P68871	Hemoglobin subunit beta[b, c, m, n, o, u, w]	**0.43**	0.00
P69905	Hemoglobin subunit alpha[b, c, m, n, o, s, u]	**0.41**	0.00
G3V1N2	HCG1745306_ isoform CRA_a[b, c, m, r, u]	**0.34**	0.00
P04280	Basic salivary proline-rich protein 1[b, l, o, u]	**0.27**	0.00
Accession number	**Protein name**	**Ratio GNE/GE**	***P***
P68871	Hemoglobin subunit beta[b, c, m, n, o, u, w]	**3.49**	1.00
P69905	Hemoglobin subunit alpha[b, c, m, n, o, s, u]	**2.94**	1.00
P02768	Serum albumin[a, b, c, g, o, u, w]	1.38	1.00
C9JKR2	Albumin, isoform CRA_k[c,g,o,i,u]	1.30	1.00
P01036	Cystatin-S[a, b, g, o, u]	1.26	1.00
P09228	Cystatin-SA[a, b, g, o, u]	1.26	0.98
P01037	Cystatin-SN[a, b, g, o, u]	1.21	1.00
P01857	Ig gamma-1 chain C region[b,j,o,u,w]	1.19	0.96
P06702	Protein S100-A9[a, b, g, i, j, n, o, q, s, u, w]	0.89	0.00
P04080	Cystatin-B[a, g, n, p, u]	0.76	0.02
P13647	Keratin, type II cytoskeletal 5[d, m, n, o, q, u]	0.64	0.03
P02788	Lactotransferrin[f, g, h, i, j, n, o, p, u, w]	0.62	0.00
P19012	Keratin, type I cytoskeletal 15[b, m, o, u, w]	0.61	0.01
P02533	Keratin, type I cytoskeletal 14[d, m, o, u, w]	0.55	0.00
P48668	Keratin, type II cytoskeletal 6C[d, m, o, u]	0.54	0.00
P13646	Keratin, type I cytoskeletal 13[d, m, o, p, q, u]	0.53	0.00
P08779	Keratin, type I cytoskeletal 16[b, m, o, u, w]	0.53	0.00
P19013	Keratin_ type II cytoskeletal 4[d, m, q, u]	0.51	0.00
P02538	Keratin, type II cytoskeletal 6A[b, d, m, o, u, w]	0.51	0.00
P04280	Basic salivary proline-rich protein 1[b, l, o, u]	**0.47**	0.00
P0CG39	POTE ankyrin domain family member J[b, m, o, u]	**0.44**	0.01
P04259	Keratin, type II cytoskeletal 6B[b, i, o, u, w]	**0.43**	0.00
P02812	Basic salivary proline-rich protein 2[b, l, o, u]	**0.38**	0.00
Q6MZM9	Proline-rich protein 27[b, l, o, x]	**0.36**	0.00
P08493	Matrix Gla protein[b, m, o, u]	**0.25**	0.00
P59666	Neutrophil defensin 3[b, i, j, o, u]	**0.24**	0.00
P59665	Neutrophil defensin 1[b, i, j, o, u]	**0.23**	0.00
P08311	Cathepsin G[a, b, g, i, j, o, p, u]	**0.19**	0.00
P03973	Antileukoproteinase[a, b, g, i, j, o, u]	**0.18**	0.00
P61626	Lysozyme C[a, b, g, i, j, o, u, w]	**0.15**	0.00

Proteins were classified according to: **General Function:** [a] metabolism; [b] biological process; [c] transport; [d] structure and structural organization; [e] information pathways; [f] miscellanea; **Function in AEP:** [g] metabolism; [h] tissue regeneration; [i] antimicrobial; [j] immune response; [k] lubrication; [l] biomineralization; [m] unknown biological function; **Origin:** [n] cytoplasm origin; [o] extracellular origin; [p] nucleus origin; [q] cytoskeleton origin; [r] intracellular origin; [s] membrane origin; [t] unknown protein origin; **Interaction:** [u] protein/protein interaction; [v] calcium/phosphate binding; [w] other molecular interaction; [x] unknown molecular interaction. Proteins with difference in expression higher than 2-fold are highlighted in bold.

these proteins might be associated with the disease itself.

Regarding the expression analyses, the first two compare the reflux groups with the control one. These comparisons likely reflect the proteins that have their rates of expression changed in function of the reflux. It is noteworthy that proteins lower in GE when compared with C were higher when GNE was compared with C, such as isoforms of hemoglobin. The opposite was also found, *i.e.*, proteins higher in GE compared with C were lower in GNE compared with C, such as *Lysozyme C* and *Cathepsin G* as well as isoforms of neutrophil defensin. The GE group also had higher levels of various isoforms of cytoskeletal keratin. Higher levels of these proteins in the AEP of the GE group suggest higher degree of epithelial cell lysis [21,22] and neutrophil degranulation [23]. It is also noteworthy that *Statherin*, a calcium-binding protein, was around 30% lower in the GE group when compared with control, which is consistent with recent reports of reduction of this protein in patients with erosion [28,29]. It is also interesting to highlight the increase in *Mucin-7* in the GE group as compared with C. Mucins are included among the acquired enamel pellicle precursors [30] and are mainly associated with lubrication [31] but also with protection against demineralization [32]. Increased expression of mucins in the acquired pellicle has been reported after exposure to lactic and citric acids [13], which might explain the increase of this protein in the GE group.

The most interesting comparison, considering the main aim of this study that is to find proteins in the GNE group that might be associated with protection against dental erosion, is between GNE and GE groups. Among the proteins with the lowest rates of expression (more than 2-fold reduction) in GNE group compared with GE group are *Lysozyme C* and *Cathepsin G*. Increases in lysozyme and cathepsins have been reported in patients with Barrett´s esophagus and esophageal adenocarcinoma induced by GERD [33]. In addition, *Lysozyme C* was recently reported as an acid-resistant protein, since it was higher in the AEP after challenge with 1% citric acid [34]. The lower expression of *Cathepsin G* in GNE volunteers might be associated with lower rates of erosion and caries progression in dentin, since proteases degrade the demineralized organic matrix, increasing the rate of erosion and caries progression [35,36]. In the present study, volunteers in GNE group presented slightly higher levels of cystatins when compared to their GE counterparts, which must contribute to protection against ETW, since cystatins bind hydroxyapatite [37]. Two isoforms of albumin were also increased in GNE volunteers when compared to GE volunteers. Albumin binds calcium [38] and reduces the dissolution of hydroxyapatite [39,40].

An interesting finding of the present study was the higher level of

distinct subunits of hemoglobin in the GNE group compared with the GE group. Hemoglobins were recently identified in the AEP collected from the posterior region [15]. Hemoglobin has strong affinity to hydroxyapatite. Hydroxyapatite columns are used to purify hemoglobin [41] and nanostructured hydroxyapatite polyhedral [42] were developed to deliver this protein in a controlled manner. Interestingly, the adsorption rate of hemoglobin to hydroxyapatite increases as pH decreases [42]. GERD patients have an oral pH typically lower than that found in healthy people [43], which might increase the chance of hemoglobin adsorption onto the dental surfaces. However, this relationship does not seem to be so simplistic because GE volunteers had lower levels of hemoglobin than controls. The reason why the hemoglobin levels in the AEP of GNE volunteers was around 3-fold higher than that of GE volunteers is not clear. Some possible explanations could be: 1) the GNE volunteers had higher concentrations of hemoglobin in saliva; 2) other proteins found only in the AEP of GNE patients might have stabilized hemoglobin adsorbed to hydroxyapatite. Both hypotheses deserve investigation in future studies. Anyway, the fact that hemoglobin was present in higher levels in GNE volunteers when compared with GE volunteers indicates that this protein might have protective role against dental erosion caused by intrinsic acids, which needs to be investigated in future studies.

Conflict of interest statement

The authors have declared no conflict of interest.

Acknowledgments

This study is based on a dissertation submitted to the first author to Bauru School of Dentistry, University of São Paulo, to obtain the title of Master in Applied Dental Sciences. The authors are grateful to Ms. Aline de Lima Leite for technical support with proteomic analysis. The authors thank FAPESP for the concession of a Master scholarship to the first author [Proc. 2014/26606-5]. The funder had no role on study design, data collection and analysis, decision to publish or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdent.2018.12.007.

References

[1] R.P. Shellis, C. Ganss, Y. Ren, D.T. Zero, A. Lussi, Methodology and models in erosion research: discussion and conclusions, Caries Res. 45 (Suppl 1) (2011) 69–77.
[2] R. Moazzez, D. Bartlett, Intrinsic causes of erosion, Monogr. Oral Sci. 25 (2014) 180–196.
[3] J. Dent, H.B. El-Serag, M.A. Wallander, S. Johansson, Epidemiology of gastro-oesophageal reflux disease: a systematic review, Gut 54 (5) (2005) 710–717.
[4] J.H. Meurman, J. Toskala, P. Nuutinen, E. Klemetti, Oral and dental manifestations in gastroesophageal reflux disease, Oral Surg. Oral Med. Oral Pathol. 78 (5) (1994) 583–589.
[5] R. Moazzez, D. Bartlett, A. Anggiansah, Dental erosion, gastro-oesophageal reflux disease and saliva: how are they related? J. Dent. 32 (6) (2004) 489–494.
[6] J.V. Munoz, B. Herreros, V. Sanchiz, C. Amoros, V. Hernandez, I. Pascual, F. Mora, M. Minguez, J.V. Bagan, A. Benages, Dental and periodontal lesions in patients with gastro-oesophageal reflux disease, Dig. Liver Dis. 35 (7) (2003) 461–467.
[7] P.L. Schroeder, S.J. Filler, B. Ramirez, D.A. Lazarchik, M.F. Vaezi, J.E. Richter, Dental erosion and acid reflux disease, Ann. Intern. Med. 122 (11) (1995) 809–815.
[8] A. Milosevic, D.A. Brodie, P.D. Slade, Dental erosion, oral hygiene, and nutrition in eating disorders, Int. J. Eat. Disord. 21 (2) (1997) 195–199.
[9] C. Calabrese, V. Marzano, A. Urbani, G. Lazzarini, M.C. Valerii, G. Liguori, S. Di Molfetta, F. Rizzello, P. Gionchetti, M. Campieri, E. Spisni, Distinct proteomic profiles characterise non-erosive from erosive reflux disease, Aliment. Pharmacol. Ther. 34 (8) (2011) 982–993.
[10] C. Calabrese, D. Trere, G. Liguori, V. Gabusi, M. Vici, G. Cenacchi, M. Derenzini, G. Di Febo, Esophageal cell proliferation in gastroesophageal reflux disease: clinical-morphological data before and after pantoprazole, World J. Gastroenterol. 15 (8) (2009) 936–941.
[11] M.A.R. Buzalaf, A.R. Hannas, M.T. Kato, Saliva and dental erosion, J. Appl. Oral Sci. 20 (5) (2012) 493–502.
[12] D. Vukosavljevic, W. Custodio, M.A.R. Buzalaf, A.T. Hara, W.L. Siqueira, Acquired pellicle as a modulator for dental erosion, Arch. Oral Biol. 59 (6) (2014) 631–638.
[13] T.R. Delecrode, W.L. Siqueira, F.C. Zaidan, M.R. Bellini, A.L. Leite, Y. Xiao, D. Rios, A.C. Magalhaes, M.A. Buzalaf, Exposure to acids changes the proteomic of acquired dentine pellicle, J. Dent. 43 (5) (2015) 583–588.
[14] Y.H. Lee, J.N. Zimmerman, W. Custodio, Y. Xiao, T. Basiri, S. Hatibovic-Kofman, W.L. Siqueira, Proteomic evaluation of acquired enamel pellicle during in vivo formation, PLoS One 8 (7) (2013) e67919.
[15] T.M. Ventura, L.P. Cassiano, E.S.C.M. Souza, E.A. Taira, A.L. Leite, D. Rios, M.A. Buzalaf, The proteomic profile of the acquired enamel pellicle according to its location in the dental arches, Arch. Oral Biol. 79 (2017) 20–29.
[16] E.A. Taira, T.M.S. Ventura, L.P.S. Cassiano, C.M.S. Silva, T. Martini, A.L. Leite, D. Rios, A.C. Magalhaes, M.A.R. Buzalaf, Changes in the proteomic profile of acquired enamel pellicles as a function of their time of formation and hydrochloric acid exposure, Caries Res. 52 (5) (2018) 367–377.
[17] E. Savarino, A.J. Bredenoord, M. Fox, J.E. Pandolfino, S. Roman, C.P. Gyawali, M. International Working Group for Disorders of Gastrointestinal, Function, Expert consensus document: advances in the physiological assessment and diagnosis of GERD, Nat. Rev. Gastroenterol. Hepatol. 14 (11) (2017) 665–676.
[18] D. Bartlett, C. Ganss, A. Lussi, Basic Erosive wear Examination (BEWE): a new scoring system for scientific and clinical needs, Clin. Oral Investig. 12 (Suppl 1) (2008) S65–8.
[19] J.N. Zimmerman, W. Custodio, S. Hatibovic-Kofman, Y.H. Lee, Y. Xiao, W.L. Siqueira, Proteome and peptidome of human acquired enamel pellicle on deciduous teeth, Int. J. Mol. Sci. 14 (1) (2013) 920–934.
[20] W.L. Siqueira, W. Zhang, E.J. Helmerhorst, S.P. Gygi, F.G. Oppenheim, Identification of protein components in in vivo human acquired enamel pellicle using LC-ESI-MS/MS, J. Proteome Res. 6 (6) (2007) 2152–2160.
[21] S. Sujatha, U. Jalihal, Y. Devi, N. Rakesh, P. Chauhan, S. Sharma, Oral pH in gastroesophageal reflux disease, Indian J. Gastroenterol. 35 (3) (2016) 186–189.
[22] A. Preetha, D. Sujatha, B.A. Patil, S. Hegde, Oral manifestations in gastroesophageal reflux disease, Gen. Dent. 63 (3) (2015) e27–31.
[23] G. Leoni, P.A. Neumann, R. Sumagin, T.L. Denning, A. Nusrat, Wound repair: role of immune-epithelial interactions, Mucosal Immunol. 8 (5) (2015) 959–968.
[24] T. Kawasaki, K. Ikeda, S. Takahashi, Y. Kuboki, Further study of hydroxyapatite high-performance liquid chromatography using both proteins and nucleic acids, and a new technique to increase chromatographic efficiency, Eur. J. Biochem. 155 (1986) 249–257.
[25] T. Kawasaki, M. Niikura, S. Takahashi, W. Kobayashi, Strontium-phosphate hydroxyapatite high-performance liquid chromatography, Biochem. Int. 15 (6) (1987) 1137–1149.
[26] U. Lendenmann, J. Grogan, F.G. Oppenheim, Saliva and dental pellicle–a review, Adv. Dent. Res. 14 (2000) 22–28.
[27] C. Hannig, D. Berndt, W. Hoth-Hannig, M. Hannig, The effect of acidic beverages on the ultrastructure of the acquired pellicle–an in situ study, Arch. Oral Biol. 54 (6) (2009) 518–526.
[28] G. Carpenter, E. Cotroneo, R. Moazzez, M. Rojas-Serrano, N. Donaldson, R. Austin, L. Zaidel, D. Bartlett, G. Proctor, Composition of enamel pellicle from dental erosion patients, Caries Res. 48 (5) (2014) 361–367.
[29] M. Mutahar, S. O'Toole, G. Carpenter, D. Bartlett, M. Andiappan, R. Moazzez, Reduced statherin in acquired enamel pellicle on eroded teeth compared to healthy teeth in the same subjects: an in-vivo study, PLoS One 12 (8) (2017) e0183660.
[30] W.L. Siqueira, W. Custodio, E.E. McDonald, New insights into the composition and functions of the acquired enamel pellicle, J. Dent. Res. 91 (12) (2012) 1110–1118.
[31] M. Hannig, A. Joiner, The structure, function and properties of the acquired pellicle, Monogr. Oral Sci. 19 (2006) 29–64.
[32] A.V. Nieuw Amerongen, C.H. Oderkerk, A.A. Driessen, Role of mucins from human whole saliva in the protection of tooth enamel against demineralization in vitro, Caries Res. 21 (4) (1987) 297–309.
[33] P. Cheng, J. Gong, T. Wang, J. Chen, G.S. Liu, R. Zhang, Gene expression in rats with Barrett's esophagus and esophageal adenocarcinoma induced by gastro-duodenoesophageal reflux, World J. Gastroenterol. 11 (33) (2005) 5117–5122.
[34] T.R. Delecrode, W.L. Siqueira, F.C. Zaidan, M.R. Bellini, E.B. Moffa, M.C. Mussi, Y. Xiao, M.A. Buzalaf, Identification of acid-resistant proteins in acquired enamel pellicle, J. Dent. 43 (12) (2015) 1470–1475.
[35] M.A. Buzalaf, S. Charone, L. Tjaderhane, Role of host-derived proteinases in dentine caries and erosion, Caries Res. 49 (Suppl 1) (2015) 30–37.
[36] L. Tjaderhane, M.A. Buzalaf, M. Carrilho, C. Chaussain, Matrix metalloproteinases and other matrix proteinases in relation to cariology: the era of' dentin degradomics', Caries Res. 49 (3) (2015) 193–208.
[37] A.C. Santiago, Z.N. Khan, M.C. Miguel, C.C. Gironda, A. Soares-Costa, V.T. Pela, A.L. Leite, J.M. Edwardson, M.A.R. Buzalaf, F. Henrique-Silva, A new sugarcane cystatin strongly binds to dental enamel and reduces Erosion, J. Dent. Res. 96 (9) (2017) 1051–1057.
[38] H. Schweigel, M. Wicht, F. Schwendicke, Salivary and pellicle proteome: a datamining analysis, Sci. Rep. 6 (2016) 38882.
[39] C.A. Hemingway, R.P. Shellis, D.M. Parker, M. Addy, M.E. Barbour, Inhibition of hydroxyapatite dissolution by ovalbumin as a function of pH, calcium concentration, protein concentration and acid type, Caries Res. 42 (5) (2008) 348–353.
[40] J. Kosoric, M.P. Hector, P. Anderson, The influence of proteins on demineralization kinetics of hydroxyapatite aggregates, J. Biomed. Mater. Res. Part A 94 (3) (2010) 972–977.
[41] T. Kawasaki, S. Takahashi, K. Ikeda, Hydroxyapatite high-performance liquid chromatography: column performance for proteins, Eur. J. Biochem. 152 (2) (1985) 361–371.
[42] Y.D. Yu, Y.J. Zhu, C. Qi, Y.Y. Jiang, H. Li, J. Wu, Hydroxyapatite nanorod-assembled porous hollow polyhedra as drug/protein carriers, J. Colloid Interface Sci. 496 (2017) 416–424.
[43] D.W. Bartlett, D.F. Evans, A. Anggiansah, B.G. Smith, A study of the association between gastro-oesophageal reflux and palatal dental erosion, Br. Dent. J. 181 (4) (1996) 125–131.