Original Research
Cariology

Acquired enamel pellicle protects gastroesophageal reflux disease patients against erosive tooth wear

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Abstract: The objective of this study was to compare the protein profile of the acquired enamel pellicle (AEP) formed *in vivo* in patients with or without gastroesophageal reflux disease (GERD), and with or without erosive tooth wear (ETW). Twenty-four volunteers were divided into 3 groups: 1) GERD and ETW; 2) GERD without ETW; and 3) control (without GERD). The AEP formed 120 min after prophylaxis was collected from the lingual/palatal surfaces. The samples were subjected to mass spectrometry (nLC-ESI-MS/MS) and label-free quantification by Protein Lynx Global Service software. A total of 213 proteins were identified, or 119, 92 and 106 from each group, respectively. Group 2 showed a high number of phosphorylated and calcium-binding proteins. Twenty-three proteins were found in all the groups, including 14-3-3 protein zeta/delta and 1-phosphatidylinositol. Several intracellular proteins that join saliva after the exfoliation of oral mucosa cells might have the potential to bind hydroxyapatite, or participate in forming supramolecular aggregates that bind to precursor proteins in the AEP. Proteins might play a central role in protecting the dental surface against acid dissolution.

Keywords: Tooth Erosion; Dental Pellicle; Proteomics.

Introduction

Erosive ooth wear (ETW) is characterized by the cumulative loss of mineralized tooth substances, resulting from exposure to non-bacterial acids of intrinsic and/or extrinsic origin, where erosion is the primary causative factor.[1] In recent years, several studies have sought to investigate the biochemical process involving ETW, prompted by an increase in the average global prevalence from 30% to 50%.[2,3] To this end, it is important to identify what individuals are likely to develop ETW, so that early diagnosis and preventive measures can be established.[2]

An important clinical condition associated with ETW is gastroesophageal reflux disease (GERD), typical manifestations being regurgitation, dysphagia, and vomiting. It affects 15% to 25% of the high-income and 10% of the low-income population.[4] However, the prevalence of ETW in patients with GERD is 5-47%,[3] caused by direct contact of regurgitated gastric contents (pH between 1 and 2) with the

tooth surface.[2,5] The low pH of the gastric content seems to suggest that GERD patients would have a higher expected prevalence of ETW. This means that GERD patients without ETW might have some protective factor. The acquired enamel pellicle (AEP) is considered one of the most important factors guarding against ETW.[6] Previously, our team compared the proteomic profile of AEP of GERD patients with and without ETW, in an effort to find what proteins in the AEP would resist removal by intrinsic acids. Among the acid-resistant proteins, hemoglobin was found to have increased more than threefold in the AEP of GERD volunteers without ETW, compared with those presenting ETW.[7]

In our previous study,[7] AEP was collected from the buccal surface of the upper and lower teeth. However, AEP composition varies depending on its location in the dental arches.[8] Notably, the severity of the reflux above the upper esophageal sphincter is correlated with the severity of tooth erosion, especially on the palatal/lingual surfaces,[9] which are directly exposed to gastric acids.

Therefore, the aim of this in vivo study was to compare the proteomic profile of the AEP of GERD patients with and without ETW, in order to investigate what proteins in the palatal/lingual AEP would resist removal by intrinsic acids, for the purpose of their use in future AEP engineering procedures.

Methodology

Ethical aspects and subjects

This study was approved by the Ethics Committees of the Bauru and Ribeirão Preto School of Dentistry, (#CAAE 44.007.415.1.0000.5417 and 44.007.415.1.3001.5419, respectively). Twenty-four volunteers signed a consent form to take part in the study (n = 8 per group). These patients were the same as those who participated in our previous study, in which AEP was collected from the buccal surface of the teeth.[7] They were of both genders, and between 20 and 60 years of age. The criteria for inclusion and exclusion, as well as the clinical examination, have been cited elsewhere.[7] The patients were divided into 3 groups,[10] as follows:

a. Patients with GERD-related symptoms and ETW (GE; n = 8): The inclusion criteria for ETW were BEWE (basic erosive wear examination) ≥ 9, or grade 3 in the upper anterior sextant (with all incisors affected);
b. Patients with GERD-related symptoms without ETW (GNE; n = 8): Patients without ETW were included in this group (BEWE = 0);
c. Control group (C; n = 8): Patients in this group did not have GERD-related symptoms or ETW (BEWE = 0).

In vivo experiment

The AEP collection procedures were conducted exactly as described in our previous study.[7] The patients were submitted to prophylaxis, and the AEP was collected from the lingual/palatal surfaces of the upper and lower teeth after 120 min, using a 5 X 10 mm electrode filter paper (Bio-Rad, Hercules, USA) pre-dipped in 3% citric acid.[11] One filter paper was used for each quadrant. The batches of paper were stored at −80 °C until the analysis was performed.

Proteomic analysis

Proteomic analysis of the AEP was conducted as previously reported.[7] The protein was extracted by cutting strips of paper collected from all the patients in the same group into small pieces, and then grouping these pieces to form a pool in a single microtube. The processes for AEP sample preparation and shotgun proteomic analysis were performed as previously described.[8] The equipment used was a nanoACQUITY UPLC-Xevo QT-MS system (Waters, Manchester, UK). ProteinLynx Global Server (PLGS) version 3.0 (Waters, Manchester, UK) was used to process and search the continuum LC-MSE data. The proteins were identified using the ion accounting algorithm embedded in the software and the Homo Sapiens database (only reviewed, UniProtKB/Swiss-Prot) downloaded in February 2020 from UniProtKB (http://www.uniprot.org/). The label-free quantitative proteomic analysis was performed by analyzing three MS raw files from each pool using PLGS software. All the identified proteins with a confidence score > 95% were included

in the quantitative analysis. Identical peptides from each triplicate by sample were grouped based on mass accuracy (< 10 ppm), and on a retention time tolerance of <0.25 min, using the clustering software embedded in the PLGS. Search results were filtered for a false discovery rate (FDR) of 1%. The difference in expression between the groups was analyzed by the t-test ($p < 0.05$), using PLGS software. The comparisons made were GE vs. C, GNE vs. C, and GNE vs. GE.

Results

The characterization of the patients as to age, gender, BEWE score, and % time esophageal pH < 4 was reported elsewhere (Martini et al., 2019), and the proteins were identified (Table 1).

In all, 213 proteins were identified (Table 2), or 106, 119, and 92 from groups C, GE, and GNE, respectively. Figure 1 shows the number of proteins common to the groups, as well as the number of proteins found in just one of the groups. Twenty-three proteins were found in all groups, including 14-3-3 protein zeta/delta, 1-phosphatidylinositol 4_5-bisphosphate phosphodiesterase beta-4, actin isoforms, alpha-internexin, ankyrin-3, annexin A1, apolipoprotein A-II, Ig lambda isoforms, myeloblastin, and myosin light polypeptide 6. Some proteins typically described as existing in the AEP were also common to all groups, such as protein S100-A8, serotransferrin, and serum albumin. The number of proteins found exclusively in groups C, GE and GNE groups was 51, 41 and 40, respectively (Figure, Table 2). Regarding the proteins found exclusively in one or two of the groups, some factors should be highlighted: a) histone H3 isoforms were found only in group C, while histone H2B isoforms were found only in the reflux groups (GE and GNE); b) group GNE had a high number of phosphorylated and calcium-binding proteins; c) isoforms of the spectrin beta-chain were found exclusively in GNE.

As for the quantitative analyses (Table 3), a comparison of GE vs. C showed that the number of GE proteins increased significantly by 4, and that of C decreased significantly by 14. The proteins that increased included the zinc finger and BTB domain–containing protein 21, negative elongation factor E, serotransferrin, and protein S100-A8. On the other hand, the proteins that decreased in the GE vs. C group included myeloblastin, RNA-binding protein 25, 1-phosphatidylinositol 4_5-bisphosphate phosphodiesterase beta-4, basic salivary proline-rich protein 1, breast cancer type 1 susceptibility protein, centrosomal protein of 170 kDa, neurofilament medium polypeptide, neutrophil defensin 1, alpha-internexin, and actin isoforms. As for GNE vs. C, actin_cytoplasmic 1 increased, while protein S100-A8, alpha-internexin, and annexin A1 decreased. The most important comparison (GNE vs. GE) showed that two isoforms of actin as well as myeloblastin were higher, while protein PRR14L, annexin A1, and protein S100-A8 decreased.

Table 1. Characterization of the volunteers according to age, gender and BEWE score.

	Gender	Median Age ± DP	BEWE
C	5F 3M	31.37 ± 8.81	0
GE	7F 1M	32.37 ± 8.27	16.1 2± 2.08
GNE	8F	31.25 ± 12.72	0

Discussion

In the previous study by our team, we compared the proteomic profile of the AEP of GERD patients with ETW vs. without ETW for the first time, in an effort to find what proteins in the AEP would help protect against ETW. However, the AEP was collected from the vestibular surface of the teeth.[7] In cases of intrinsic erosion, the route of the gastric acids impacts the palatal and lingual surfaces more.[10-12] That is why we decided to collected AEP from the palatal and lingual surfaces of the patients in the present study. Notably, the site of AEP collection is an important factor that should be taken into account in proteomic studies of this integument, because deep changes in the proteomic profile of AEPs occur according to its location in the dental arches.[8] The profile of the proteins found herein was very different from that observed in our previous

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

Accession number	Protein name	C	GE	GNE
P63104	14-3-3 protein zeta/delta[d, e, m, t, u]	Yes	Yes	Yes
Q15147	1-phosphatidylinositol 4_5-bisphosphate phosphodiesterase beta-4[b, m, r, v]	Yes	Yes	Yes
P68032	Actin_ alpha cardiac muscle 1[b, m, n, q, u, w]	Yes	Yes	Yes
P68133	Actin_ alpha skeletal muscle[b, d, m, n, q, u, w]	Yes	Yes	Yes
P62736	Actin_ aortic smooth muscle[b, d, m, n, q, u]	Yes	Yes	Yes
P60709	Actin_ cytoplasmic 1[b, m, n, q, u, w]	Yes	Yes	Yes
P63261	Actin_ cytoplasmic 2[a, d, g, j, n, q, u, w]	Yes	Yes	Yes
Q8NC06	Acyl-CoA-binding domain-containing protein 4[b, e, m, t, w]	-	-	Yes
Q08AH3	Acyl-coenzyme A synthetase ACSM2A_ mitochondrial[b, m, t, w]	-	Yes	-
Q8N6G6	ADAMTS-like protein 1[b, m, t, w]	Yes	Yes	-
Q9UIF7	Adenine DNA glycosylase[b, m, t, u]	Yes	-	-
Q8N142	Adenylosuccinate synthetase isozyme 1[b, m, r, w]	-	-	Yes
Q01518	Adenylyl cyclase-associated protein 1[b, m, t, w]	Yes	Yes	-
Q9UHX3	Adhesion G protein-coupled receptor E2[b, d, m, t, u, w]	Yes	-	-
Q16352	Alpha-internexin[b, d, m, t, u, w]	Yes	Yes	Yes
P15144	Aminopeptidase N[b, c, m, t, u, w]	-	-	Yes
O00213	Amyloid beta A4 precursor protein-binding family B member 1[b, m, t, w]	Yes	-	-
Q6UB98	Ankyrin repeat domain-containing protein 12[b, m, r, w]	-	Yes	Yes
Q12955	Ankyrin-3[a, b , m, x, w]	Yes	Yes	Yes
P04083	Annexin A1[b, m, r, w]	Yes	Yes	Yes
P03973	Antileukoproteinase[b, f, m, t, u, w]	-	Yes	Yes
P02652	Apolipoprotein A-II[a, m, t, w]	Yes	Yes	Yes
P02656	Apolipoprotein C-III[a, m, t, u, w]	-	-	Yes
Q5T2S8	Armadillo repeat-containing protein 4[b, m, t, u, w]	-	-	Yes
Q7L311	Armadillo repeat-containing X-linked protein 2[b, m, t, u, w]	Yes	-	-
P51689	Arylsulfatase D[b, m, t, v]	Yes	-	-
Q8IUA7	ATP-binding cassette sub-family A member 9[b, c, m, r, u, w]	-	Yes	-
Q9H4G0	Band 4.1-like protein 1[b, m, s, w]	-	Yes	-
O43491	Band 4.1-like protein 2[b, m, s, w]	-	-	Yes
Q9Y6E2	Basic leucine zipper and W2 domain-containing protein 2[b, m, q, w]	-	Yes	-
P04280	Basic salivary proline-rich protein 1[b, l, o, u]	Yes	Yes	-
P02812	Basic salivary proline-rich protein 2[b, l, o, u]	Yes	Yes	-
O95342	Bile salt export pump[c, m, r, w]	Yes	-	-
Q96IK1	Biorientation of chromosomes in cell division protein 1[b, m, t, w]	-	-	Yes
P38398	Breast cancer type 1 susceptibility protein[b, m, r, w]	Yes	Yes	-
Q9ULB4	Cadherin-9[b, m, t, u, v]	Yes		-
Q5SW79	Centrosomal protein of 170 kDa[d, m, t, x]	Yes	Yes	-
Q96L14	Cep170-like protein(d, m, r, w)	-	-	Yes
Q96G23	Ceramide synthase 2[e, m, t, w]	Yes	-	-

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Q8TBZ0	Coiled-coil domain-containing protein 110[b, m, p, x]	Yes	-	-
Q86WR0	Coiled-coil domain-containing protein 25[b, c, e, m, t, w]	-	Yes	-
Q9UBG3	Cornulin[b, e, m, t, w]	Yes	Yes	-
Q96NY9	Crossover junction endonuclease MUS81[b, c, d, m, t, w]	Yes	-	-
Q13363	C-terminal-binding protein 1[b, m, t, w]		Yes	-
P32320	Cytidine deaminase[b, d, m, t, w]	Yes	Yes	-
O75462	Cytokine receptor-like factor 1[b, m, t, w]	-	-	Yes
Q5M775	Cytospin-B[b, m, p, w]	-	Yes	-
Q13443	Disintegrin and metalloproteinase domain-containing protein 9[b, m, t, u, w]	Yes	-	-
Q9Y485	DmX-like protein 1[b, m, t, w]	-	Yes	-
Q5T890	DNA excision repair protein ERCC-6-like 2[b, e, m, t, u, w]	Yes	-	-
Q9UBZ9	DNA repair protein REV1[b, m, t, u, w]	-	Yes	-
P48382	DNA-binding protein RFX5[b, m, t, w]	-	-	Yes
Q8N7B9	EF-hand calcium-binding domain-containing protein 3[b, m, t, w]	Yes	-	
Q12805	EGF-containing fibulin-like extracellular matrix protein 1[b, f, m, t, w]	-	-	Yes
Q6PCB8	Embigin[b, m, t, w]	Yes	-	-
Q969X5	Endoplasmic reticulum-Golgi intermediate compartment protein 1[c, m, t, w]	Yes	-	-
Q14152	Eukaryotic translation initiation factor 3 subunit A[b, m, t, w]	-	Yes	-
O15360	Fanconi anemia group A protein[b, m, t, w]	Yes	-	-
O60907	F-box-like/WD repeat-containing protein TBL1X[b, m, t, w]	-	Yes	-
Q9BZK7	F-box-like/WD repeat-containing protein TBL1XR1[b, m, t, w]	Yes	-	-
P23142	Fibulin-1[b, m, t, u, w]	Yes	-	-
P09958	Furin[b, e, m, t, u, w]	-	Yes	-
P09104	Gamma-enolase[b, m, t, v, w]	Yes	Yes	-
Q8WUA4	General transcription factor 3C polypeptide 2[b, m, t, w]	-	Yes	-
P04406	Glyceraldehyde-3-phosphate dehydrogenase[a, b, c, m, t, w]	Yes	Yes	-
Q68CQ7	Glycosyltransferase 8 domain-containing protein 1[b, m, t, x]	-	-	Yes
Q08378	Golgin subfamily A member 3[b, d, m, t, x]	-	Yes	-
Q02643	Growth hormone-releasing hormone receptor[b, m, t, x]	-	-	Yes
P61978	Heterogeneous nuclear ribonucleoprotein K[b, m, t, u, w]	Yes	-	-
P16104	Histone H2AX[b, m, t, u, w]	-	Yes	Yes
P33778	Histone H2B type 1-B[b, m, t, w]	-	Yes	Yes
P62807	Histone H2B type 1-C/E/F/G/I[b, m, t, w]	-	Yes	Yes
P58876	Histone H2B type 1-D[b, m, t, w]	-	Yes	Yes
Q93079	Histone H2B type 1-H[b, m, t, w]	-	Yes	Yes
P06899	Histone H2B type 1-J[b, m, t, w]	-	Yes	Yes
O60814	Histone H2B type 1-K[b, m, t, w]	-	Yes	Yes
Q99880	Histone H2B type 1-L[b, m, t, w]	-	Yes	Yes
Q99879	Histone H2B type 1-M[b, m, t, w]	-	Yes	Yes
Q99877	Histone H2B type 1-N[b, m, t, w]	-	Yes	Yes

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P23527	Histone H2B type 1-O[b, m, t, w]	-	Yes	Yes
Q16778	Histone H2B type 2-E[b, m, t, w]	-	Yes	Yes
Q5QNW6	Histone H2B type 2-F[b, m, t, w]	-	Yes	Yes
Q8N257	Histone H2B type 3-B[b, m, t, w]	-	Yes	Yes
P68431	Histone H3.1[b, f, m, t, w]	Yes	-	-
Q16695	Histone H3.1t[b, f, m, t, w]	Yes	-	-
Q71DI3	Histone H3.2[b, f, m, t, w]	Yes	-	-
P84243	Histone H3.3[b, f, m, t, w]	Yes	-	-
Q6NXT2	Histone H3.3C[b, f, m, t, w]	Yes	-	-
P62805	Histone H4[b, m, t, w]	-	Yes	Yes
O43719	HIV Tat-specific factor 1[b, m, t, w]	-	-	Yes
Q4G0P3	Hydrocephalus-inducing protein homolog[e, m, t, w]	Yes	Yes	-
Q9Y4L1	Hypoxia up-regulated protein 1[b, m, t, u, w]	-	Yes	-
P01876	Ig alpha-1 chain C region[b, e, m, t, u, w]	Yes	Yes	-
P01834	Ig kappa chain C region[b, m, r, w]	Yes	Yes	Yes
P0CG04	Ig lambda-1 chain C regions[f, m, t, w]	Yes	Yes	Yes
P0DOY2	Ig lambda-2 chain C regions[b, m, o, w]	Yes	Yes	Yes
P0DOY3	Ig lambda-3 chain C regions[f, m, t, w]	Yes	Yes	Yes
P0CF74	Ig lambda-6 chain C region[b, m, t, w]	Yes	Yes	Yes
P55899	IgG receptor FcRn large subunit p51[b, m, t, w]	-	-	Yes
P0C7H9	Inactive ubiquitin carboxyl-terminal hydrolase 17-like protein 7[b, m, t, u]	-	Yes	-
P14735	Insulin-degrading enzyme[b, m, t, u, w]	Yes	-	-
Q86VS3	IQ domain-containing protein H[b, m, t, w]	Yes	-	-
P13645	Keratin_ type I cytoskeletal 10[b, m, t, w]	Yes	Yes	-
P48668	Keratin_ type II cytoskeletal 6C[d, m, o, u]	Yes	Yes	-
Q7Z4W3	Keratin-associated protein 19-3[b, m, t, u]	Yes	-	-
C9JBD0	KRAB domain-containing protein 1[b, m, t, x]	Yes	-	-
Q38SD2	Leucine-rich repeat serine/threonine-protein kinase 1[b, d, m, t, x]	Yes	-	Yes
Q8N653	Leucine-zipper-like transcriptional regulator 1[b, m, s, u]	Yes	-	
Q8WWI1	LIM domain only protein 7[b, d, m, t, u]	-	-	Yes
Q86W92	Liprin-beta-1[b, d, m, t, u, v]	Yes	Yes	-
O95711	Lymphocyte antigen 86[b, m, t, w]	Yes	-	-
P40925	Malate dehydrogenase_ cytoplasmic[b, m, t, w]	Yes	-	-
P08493	Matrix Gla protein[b, d, m, t, v]	-	Yes	Yes
Q9Y2H9	Microtubule-associated serine/threonine-protein kinase 1[b, d, m, t, u]	Yes	Yes	-
P15941	Mucin-1[b, i, k, o, u]	Yes	-	-
P24158	Myeloblastin[b, c, m, t, w]	Yes	Yes	Yes
P60660	Myosin light polypeptide 6[b, m, r, w]	Yes	Yes	Yes
Q86WG5	Myotubularin-related protein 13[b, m, t, u]	-	Yes	-
P18615	Negative elongation factor E[b, m, p, w]	Yes	Yes	-

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Q9P2S2	Neurexin-2[b, m, t, w]	-	-	Yes
P58401	Neurexin-2-beta[b, m, t, w]	-	-	Yes
P07197	Neurofilament medium polypeptide[b, c, m, t, w]	Yes	Yes	-
Q04721	Neurogenic locus notch homolog protein 2[b, d, m, t, w]	Yes	-	-
Q92823	Neuronal cell adhesion molecule[b, d, e, m, e, w]	-	Yes	-
P59665	Neutrophil defensin 1[b, i, j, o, u]	Yes	Yes	-
Q16288	NT-3 growth factor receptor[b, m, r, w]	-	-	Yes
Q8NGQ1	Olfactory receptor 9G4[b, m, t, u, w]	-	-	Yes
P30044	Peroxiredoxin-5_ mitochondrial[b, m, s, u, w]	Yes	Yes	-
O14832	Phytanoyl-CoA dioxygenase_ peroxisomal[b, m, t, w]	Yes	-	-
Q92508	Piezo-type mechanosensitive ion channel component 1[b, e, m, s, w]	-	-	Yes
Q99569	Plakophilin-4[b, d, m, t, u]	Yes	Yes	-
P11940	Polyadenylate-binding protein 1[a, b, f, m, t, u, w]	-	Yes	-
Q13310	Polyadenylate-binding protein 4[a, b, f, m, t, u, w]	Yes	-	-
P98161	Polycystin-1[b, m, s, u]	-	Yes	-
P01833	Polymeric immunoglobulin receptor[b, m, s, w]	Yes	Yes	-
Q6S8J3	POTE ankyrin domain family member E[b, m, o, w]	Yes	Yes	-
A5A3E0	POTE ankyrin domain family member F[b, m, o, w]	Yes	Yes	-
P0CG38	POTE ankyrin domain family member I[b, m, o, w]	Yes	Yes	-
Q6UN15	Pre-mRNA 3'-end-processing factor FIP1[b, m, t, w]	-	Yes	-
Q5VTL8	Pre-mRNA-splicing factor 38B[b, m, q, w]	-	Yes	-
O43143	Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15[b, m, t, w]	-	Yes	-
Q5GLZ8	Probable E3 ubiquitin-protein ligase HERC4[b, d, m, t, u]	-	-	Yes
Q9H4B0	Probable tRNA N6-adenosine threonylcarbamoyltransferase_ mitochondrial[b, d, m, t, w]	Yes	-	-
Q6MZM9	Proline-rich protein 27[b, l, o, x]		Yes	Yes
Q3B820	Protein FAM161A[b, m, r, w]	Yes	-	-
Q9H0Q0	Protein FAM49A[b, c, r, w]	Yes	-	-
P78504	Protein jagged-1[e, m, r, w]	-	Yes	-
Q5THK1	Protein PRR14L[b, l, o, x]	-	Yes	Yes
Q9Y520	Protein PRRC2C[b, l, o, x]	Yes	-	Yes
P05109	Protein S100-A8[a, b, c, d, f, j, r, u, v]	Yes	Yes	Yes
Q96ER3	Protein SAAL1[b, m, r, u]	-	-	Yes
Q8NB66	Protein unc-13 homolog C[b, m, r, w]	-	Yes	Yes
Q9Y5H0	Protocadherin gamma-A3[b, m, t, v]	-	Yes	-
Q9UBK7	Rab-like protein 2A[c, m, t, u]	Yes	-	-
Q2PPJ7	Ral GTPase-activating protein subunit alpha-2[b, m, t, w]	Yes	-	-
P35251	Replication factor C subunit 1[b, m, t, w]	Yes	-	-
P50120	Retinol-binding protein 2[c, m, t, w]	-	-	Yes
Q8N392	Rho GTPase-activating protein 18[c, m, t, u]	-	Yes	-
Q96QB1	Rho GTPase-activating protein 7[b, m, t, u]	-	Yes	-

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P49756	RNA-binding protein 25[b, m, t, u]	Yes	Yes	-
B4DTS2	Serine/threonine-protein kinase[d, m, t, u]	Yes	Yes	Yes
Q6ZWH5	Serine/threonine-protein kinase Nek10[d, m, t, w]	-	-	Yes
P02787	Serotransferrin[c, m, t, w]	Yes	Yes	Yes
P02768	Serum albumin[a, b, c, g, o, u, w]	Yes	Yes	Yes
O43166	Signal-induced proliferation-associated 1-like protein 1[b, d, m, t, w]	Yes	-	-
Q9Y5Y9	Sodium channel protein type 10 subunit alpha[b, m, t, w]	-	Yes	-
P35499	Sodium channel protein type 4 subunit alpha[b, m, t, w]	-	Yes	-
Q9Y6X4	Soluble lamin-associated protein of 75 kDa[e, m, t, w]	-		Yes
Q5M8T2	Solute carrier family 35 member D3[b, m, t, w]	-	Yes	-
Q96GZ6	Solute carrier family 41 member 3[c, m, r, w]	-	-	Yes
P11277	Spectrin beta chain_ erythrocytic[d, f, m, q, w]	-	-	Yes
Q01082	Spectrin beta chain_ non-erythrocytic 1[d, m, t, w]	-	-	Yes
O15020	Spectrin beta chain_ non-erythrocytic 2[d, m, t, w]	-	-	Yes
Q9H254	Spectrin beta chain_ non-erythrocytic 4[d, m, t, w]	-	-	Yes
Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1[b, m, t, w]	-	Yes	Yes
P02808	Statherin[b, e, i, l, o, u]	-	Yes	Yes
Q9NTJ3	Structural maintenance of chromosomes protein 4[b, m, q, w]	-	Yes	-
Q9Y2K9	Syntaxin-binding protein 5-like[b, c, m, t, w]	Yes	-	-
Q92609	TBC1 domain family member 5[b, c, m, t, w]	-	Yes	-
Q495A1	T-cell immunoreceptor with Ig and ITIM domains[b, m, r, w]	-	-	Yes
Q8IVF5	T-lymphoma invasion and metastasis-inducing protein 2[b, m, r, w]	Yes	Yes	-
P19484	Transcription factor EB[b, m, s, w]	-	Yes	Yes
P46100	Transcriptional regulator ATRX[b, m, r, w]	Yes	Yes	-
Q9Y4A5	Transformation/transcription domain-associated protein[b, m, t, u, w]	-	Yes	-
Q8NFB2	Transmembrane protein 185A[b, m, t, w]	Yes	-	-
Q9H7F4	Transmembrane protein 185B[b, m, t, w]	Yes	-	-
Q8NDV7	Trinucleotide repeat-containing gene 6A protein[b, f, m, t, u, w]	-	Yes	-
Q96LD4	Tripartite motif-containing protein 47[b, m, o, w]	-	Yes	-
Q6EMB2	Tubulin polyglutamylase TTLL5[b, m, t, w]	Yes	-	-
Q9UBB9	Tuftelin-interacting protein 11[b, d, m, t, w]	-	-	Yes
O15327	Type II inositol 3_4-bisphosphate 4-phosphatase[b, m, t, w]	-	Yes	Yes
P17706	Tyrosine-protein phosphatase non-receptor type 2[b, d, e, m, t, w]	-	-	Yes
Q9P2H5	Ubiquitin carboxyl-terminal hydrolase 35[b, m, t, u]	Yes	-	-
P62256	Ubiquitin-conjugating enzyme E2 H[b, m, t, u]	-	-	Yes
B2RTY4	Unconventional myosin-IXa[c, m, t, w]	-	Yes	Yes
Q13459	Unconventional myosin-IXb[c, m, t, w]	-	Yes	-
Q9UID3	Vacuolar protein sorting-associated protein 51 homolog[b, c, m, s, w]	-	Yes	-
Q9H0V9	VIP36-like protein[b, c, m, t, w]	-	-	Yes
P54289	Voltage-dependent calcium channel subunit alpha-2/delta-1[b, f, m, t, w]	-	Yes	-

Continue

Continuation

Q9ULJ3	Zinc finger and BTB domain-containing protein 21[b, m, p, u]	Yes	Yes	Yes
Q8IWY8	Zinc finger and SCAN domain-containing protein 29[b, m, p, u]	-	Yes	-
Q86VM9	Zinc finger CCCH domain-containing protein 18[b, m, p, u]	Yes	Yes	-
P37275	Zinc finger E-box-binding homeobox 1[b, m, p, u]	Yes	-	-
O60315	Zinc finger E-box-binding homeobox 2[b, m, p, u]	Yes	-	Yes
O43296	Zinc finger protein 264[b, m, p, u]	Yes	-	-
Q9HBT7	Zinc finger protein 287[b, m, p, u]	-	Yes	-
Q8IZ26	Zinc finger protein 34[b, m, p, u]	-	-	Yes
Q8N8E2	Zinc finger protein 513[b, m, p, u]	-	-	Yes
Q9C0D4	Zinc finger protein 518B[b, m, p, u]	Yes	-	-
Q3ZCX4	Zinc finger protein 568[b, m, p, u]	-	Yes	-
Q96N77	Zinc finger protein 641[b, m, p, u]	-	-	Yes
O75290	Zinc finger protein 780A[b, m, p, u]	-	-	Yes
Q6ZQV5	Zinc finger protein 788[b, m, p, u]	Yes	-	-

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.

study, in which the AEP was collected from the vestibular surfaces.[7]

One interesting finding of the present study was the number of different types of histones. These proteins are very rich in lysine and arginine residues. This makes it easier to identify these proteins in proteomic studies, since tryptic peptides are generated upon the polypeptide chain cleavage of these residues. However, one finding was quite notable: H3 histones were found solely in the C group, while histone H2B isoforms were found only in the reflux groups (GE and GNE). The reason is not clear, but we should consider that H2B histones have a serine residue that can be phosphorylated in the N-terminus region. On the other hand, histone H3 has 2 serine residues that can be phosphorylated at the N-terminus. However, this region also contains at least 4 lysine residues that can be methylated.[13,14] This can make access of the phosphate ion to the tooth surface difficult, thus reducing the binding of this type of histone to the calcium in the hydroxyapatite. The increase in after-radiotherapy histones is related to the DNA methylation process, which acts concomitantly with histone acetyltransferases.

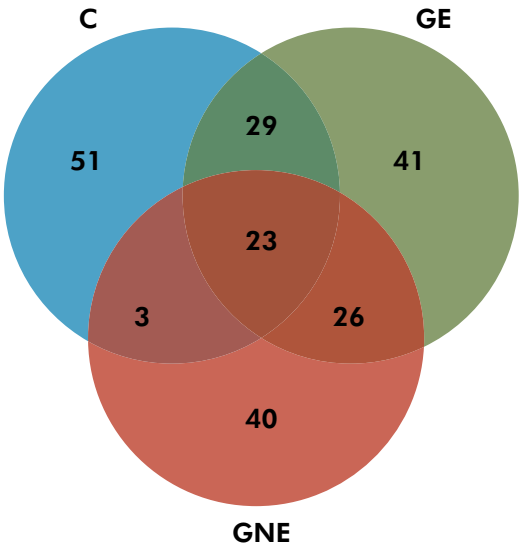

Figure. Venn diagram showing the number of proteins identified in the control (no GERD, no dental erosion), GE (GERD and dental erosion), and GNE (GERD but no dental erosion) groups.

This process is based on the epigenetic mechanisms associated with cellular memory and identity, which influence the cell environment and control

Table 3. Proteins expressed differentially in the acquired enamel pellicle collected from patients with gastroesophageal reflux disease (GERD) and erosive tooth wear (GE), GERD but no erosive tooth wear (GNE), or controls (no GERD, no erosive tooth wear; C).

[a]Accession Number	Protein name	PLGS Score	[b]Ratio: GE:CL
Q9ULJ3	Zinc finger and BTB domain-containing protein 21	225	4.85
P18615	Negative elongation factor E	236	3.94
P02787	Serotransferrin	1819	1.40
P05109	Protein S100-A8	11183	1.25
P24158	Myeloblastin	1361	0.84
P62736	Actin_ aortic smooth muscle	4078	0.73
P60709	Actin_ cytoplasmic 1	4078	0.72
P49756	RNA-binding protein 25	8013	0.70
Q15147	1-phosphatidylinositol 45-bisphosphate phosphodiesterase beta-4	4085	0.64
P63261	Actin_ cytoplasmic 2	4078	0.63
P68032	Actin_ alpha cardiac muscle 1	4078	0.63
P68133	Actin_ alpha skeletal muscle	4078	0.63
P04280	Basic salivary proline-rich protein 1	1868	0.46
P38398	Breast cancer type 1 susceptibility protein	2136	0.38
Q5SW79	Centrosomal protein of 170 kDa	377	0.33
P07197	Neurofilament medium polypeptide	7061	0.27
P59665	Neutrophil defensin 1	7061	0.27
Q16352	Alpha-internexin	327	0.09
[a]Accession Number	Protein name	PLGS Score	[b]Ratio: GNE:CL
P60709	Actin_ cytoplasmic 1	4078	1.58
P05109	Protein S100-A8	11183	0.21
Q16352	Alpha-internexin	327	0.20
P04083	Annexin A1	2838	0.19
[a]Accession Number	Protein name	PLGS Score	[b]Ratio: GNE:GE
P60709	Actin_ cytoplasmic 1	4078	2.20
P62736	Actin_ aortic smooth muscle	4078	2.16
P24158	Myeloblastin	1361	1.40
Q5THK1	Protein PRR14L	158	0.21
P04083	Annexin A1	2838	0.20
P05109	Protein S100-A8	11183	0.17
Q9UIF7	Adenine DNA glycosylase	338	C[c]
Q9UHX3	Adhesion G protein-coupled receptor E2	161	C
O00213	Amyloid beta A4 precursor protein-binding family B member 1	247	C
Q7L311	Armadillo repeat-containing X-linked protein 2	230	C
P51689	Arylsulfatase D	272	C
O95342	Bile salt export pump	201	C

Continue

Continuation

Q9ULB4	Cadherin-9	237	C
Q96G23	Ceramide synthase 2	263	C
Q8TBZ0	Coiled-coil domain-containing protein 110	248	C
Q96NY9	Crossover junction endonuclease MUS81	175	C
Q13443	Disintegrin and metalloproteinase domain-containing protein 9	190	C
Q5T890	DNA excision repair protein ERCC-6-like 2	51	C
Q8N7B9	EF-hand calcium-binding domain-containing protein 3	212	C
Q6PCB8	Embigin	208	C
Q969X5	Endoplasmic reticulum-Golgi intermediate compartment protein 1	215	C
O15360	Fanconi anemia group A protein	188	C
Q9BZK7	F-box-like/WD repeat-containing protein TBL1XR1	224	C
P23142	Fibulin-1	318	C
P61978	Heterogeneous nuclear ribonucleoprotein K	159	C
P68431	Histone H3.1	268	C
Q16695	Histone H3.1t	268	C
Q71DI3	Histone H3.2	668	C
P84243	Histone H3.3	268	C
Q6NXT2	Histone H3.3C	268	C
P14735	Insulin-degrading enzyme	301	C
Q86VS3	IQ domain-containing protein H	68	C
Q7Z4W3	Keratin-associated protein 19-3	343	C
C9JBD0	KRAB domain-containing protein 1	531	C
Q8N653	Leucine-zipper-like transcriptional regulator 1	304	C
O95711	Lymphocyte antigen 86	222	C
P40925	Malate dehydrogenase_ cytoplasmic	119	C
P15941	Mucin-1	202	C
Q04721	Neurogenic locus notch homolog protein 2	112	C
O14832	Phytanoyl-CoA dioxygenase_ peroxisomal	269	C
Q13310	Polyadenylate-binding protein 4	43	C
Q9H4B0	Probable tRNA N6-adenosine threonylcarbamoyltransferase_ mitochondrial	127	C
Q3B820	Protein FAM161A	316	C
Q9H0Q0	Protein FAM49A	293	C
Q9UBK7	Rab-like protein 2A	151	C
Q2PPJ7	Ral GTPase-activating protein subunit alpha-2	156	C
P35251	Replication factor C subunit 1	167	C
O43166	Signal-induced proliferation-associated 1-like protein 1	251	C
Q9Y2K9	Syntaxin-binding protein 5-like	191	C
Q8NFB2	Transmembrane protein 185A	213	C
Q9H7F4	Transmembrane protein 185B	223	C
Q6EMB2	Tubulin polyglutamylase TTLL5	172	C

Continue

Continuation

Q9P2H5	Ubiquitin carboxyl-terminal hydrolase 35	267	C
P37275	Zinc finger E-box-binding homeobox 1	234	C
O43296	Zinc finger protein 264	187	C
Q9C0D4	Zinc finger protein 518B	227	C
Q6ZQV5	Zinc finger protein 788	226	C
Q08AH3	Acyl-coenzyme A synthetase ACSM2A_ mitochondrial	209	GE[d]
Q8IUA7	ATP-binding cassette sub-family A member 9	238	GE
Q9H4G0	Band 4.1-like protein 1	581	GE
Q9Y6E2	Basic leucine zipper and W2 domain-containing protein 2	147	GE
Q86WR0	Coiled-coil domain-containing protein 25	591	GE
Q13363	C-terminal-binding protein 1	566	GE
Q5M775	Cytospin-B	402	GE
Q9Y485	DmX-like protein 1	117	GE
Q9UBZ9	DNA repair protein REV1	309	GE
Q14152	Eukaryotic translation initiation factor 3 subunit A	101	GE
O60907	F-box-like/WD repeat-containing protein TBL1X	415	GE
P09958	Furin	121	GE
Q8WUA4	General transcription factor 3C polypeptide 2	388	GE
Q08378	Golgin subfamily A member 3	360	GE
Q9Y4L1	Hypoxia up-regulated protein 1	270	GE
P0C7H9	Inactive ubiquitin carboxyl-terminal hydrolase 17-like protein 7	154	GE
Q86WG5	Myotubularin-related protein 13	297	GE
Q92823	Neuronal cell adhesion molecule	275	GE
P11940	Polyadenylate-binding protein 1	244	GE
P98161	Polycystin-1	90	GE
Q6UN15	Pre-mRNA 3′-end-processing factor FIP1	175	GE
Q5VTL8	Pre-mRNA-splicing factor 38B	286	GE
O43143	Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	126	GE
P78504	Protein jagged-1	92	GE
Q9Y5H0	Protocadherin gamma-A3	193	GE
Q8N392	Rho GTPase-activating protein 18	185	GE
Q96QB1	Rho GTPase-activating protein 7	131	GE
Q9Y5Y9	Sodium channel protein type 10 subunit alpha	165	GE
P35499	Sodium channel protein type 4 subunit alpha	129	GE
Q5M8T2	Solute carrier family 35 member D3	133	GE
Q9NTJ3	Structural maintenance of chromosomes protein 4	194	GE
Q92609	TBC1 domain family member 5	255	GE
Q9Y4A5	Transformation/transcription domain-associated protein	287	GE
Q8NDV7	Trinucleotide repeat-containing gene 6A protein	171	GE
Q96LD4	Tripartite motif-containing protein 47	211	GE

Continue

Continuation

Q13459	Unconventional myosin-IXb	268	GE
Q9UID3	Vacuolar protein sorting-associated protein 51 homolog	369	GE
P54289	Voltage-dependent calcium channel subunit alpha-2/delta-1	147	GE
Q8IWY8	Zinc finger and SCAN domain-containing protein 29	174	GE
Q9HBT7	Zinc finger protein 287	283	GE
Q3ZCX4	Zinc finger protein 568	220	GE
Q8NC06	Acyl-CoA-binding domain-containing protein 4	263	GNE[e]
Q8N142	Adenylosuccinate synthetase isozyme 1	102	GNE
P15144	Aminopeptidase N	251	GNE
P02656	Apolipoprotein C-III	141	GNE
Q5T2S8	Armadillo repeat-containing protein 4	170	GNE
O43491	Band 4.1-like protein 2	263	GNE
Q96IK1	Biorientation of chromosomes in cell division protein 1	263	GNE
Q96L14	Cep170-like protein	263	GNE
O75462	Cytokine receptor-like factor 1	327	GNE
P48382	DNA-binding protein RFX5	327	GNE
Q12805	EGF-containing fibulin-like extracellular matrix protein 1	513	GNE
Q68CQ7	Glycosyltransferase 8 domain-containing protein 1	454	GNE
Q02643	Growth hormone-releasing hormone receptor	144	GNE
O43719	HIV Tat-specific factor 1	144	GNE
P55899	IgG receptor FcRn large subunit p51	279	GNE
Q8WWI1	LIM domain only protein 7	215	GNE
Q9P2S2	Neurexin-2	215	GNE
P58401	Neurexin-2-beta	220	GNE
Q16288	NT-3 growth factor receptor	174	GNE
Q8NGQ1	Olfactory receptor 9G4	150	GNE
Q92508	Piezo-type mechanosensitive ion channel component 1	169	GNE
Q5GLZ8	Probable E3 ubiquitin-protein ligase HERC4	140	GNE
Q96ER3	Protein SAAL1	152	GNE
P50120	Retinol-binding protein 2	161	GNE
Q6ZWH5	Serine/threonine-protein kinase Nek10	161	GNE
Q9Y6X4	Soluble lamin-associated protein of 75 kDa	45	GNE
Q96GZ6	Solute carrier family 41 member 3	605	GNE
P11277	Spectrin beta chain_ erythrocytic	645	GNE
Q01082	Spectrin beta chain_ non-erythrocytic 1	184	GNE
O15020	Spectrin beta chain_ non-erythrocytic 2	186	GNE
Q9H254	Spectrin beta chain_ non-erythrocytic 4	210	GNE
Q495A1	T-cell immunoreceptor with Ig and ITIM domains	193	GNE
Q9UBB9	Tuftelin-interacting protein 11	203	GNE
P17706	Tyrosine-protein phosphatase non-receptor type 2	169	GNE

Continue

Continuation

P62256	Ubiquitin-conjugating enzyme E2 H	384	GNE
Q9H0V9	VIP36-like protein	410	GNE
Q8IZ26	Zinc finger protein 34	473	GNE
Q8N8E2	Zinc finger protein 513	473	GNE
Q96N77	Zinc finger protein 641	473	GNE
O75290	Zinc finger protein 780A	176	GNE

[a]Identification is based on ID proteins from Uniprot protein database, only reviewed (http://www.uniprot.org/); [b]Proteins with significantly altered expression are organized according to the ratio; [c]Indicates proteins found exclusively in the acquired enamel pellicle (AEP) of C patients (in alphabetical order); [d]Indicates proteins found exclusively in the acquired enamel pellicle (AEP) of GE patients (in alphabetical order); [e]Indicates proteins found exclusively in the acquired enamel pellicle (AEP) of GNE patients (in alphabetical order). Ratios highlighted in bold indicate proteins with higher increase or decrease in GNE compared with GE patients.

epigenetic regulation. Furthermore, non-coding RNA action and histone modification also interact in epigenetic regulation.[15] Acetylation is a type of histone alteration that plays an important role in modulating gene expression and cell cycles, and in neoplasm diffusion.[16] The acetylation process starts with the action of acetyltransferases, prompted by the binding of acetyl radicals and lysine residues of histone proteins, and results in the decompression of chromatin and transcriptional activity. Histones H2A and H2B are examples of proteins whose function is not only to act in DNA replication and repair, but also to constitute octamers with histones H3 and H4. They are also involved in the packaging of DNA in nucleosomes.[17] Interestingly, a study by Ventura et al.[18] showed that histone isoforms can be considered a strong prognostic biomarker in patients with head and neck cancer. This shows how an analysis of the AEP can benefit the patient by providing previous diagnosis and adequate treatment.

AEP proteins have long been known for their protective effect in the homeostasis of the oral cavity.[11] It is interesting to highlight that the focus on these proteins a few years ago was placed on those that were secreted in saliva.[11] However, other important sources of AEP proteins are exfoliated oral mucosa cells, and gingival cells that deliver their content to saliva. In this context, many proteins with an affinity for hydroxyapatite might be immobilized in the AEP, and play an important protective role against acid challenges. This is the case of histones, as well as other proteins recently identified in the AEP as acid-resistant, such as hemoglobin (HB).[7,19-22] Nevertheless, HB was evaluated in the present study because it was found to be higher in the AEP[7] and saliva[22] of GERD patients without ETW, compared with GERD patients with ETW. Moreover, bear in mind that HB has a strong affinity for hydroxyapatite, and that hydroxyapatite columns are used to purify this protein.[23] Interestingly, the adsorption rate of HB to hydroxyapatite increases as pH decreases.[24] GERD patients have an oral pH typically lower than that of healthy people.[10] This might increase the chance of HB adsorption onto dental surfaces.

Furthermore, a high number of phosphorylated and calcium-binding proteins were found among the proteins identified exclusively in the GNE group. Nearly 50% of a total of 40 distinct proteins of the GNE group (the vast majority being intracellular proteins) are phosphorylated or Ca-binding proteins, thus suggesting that they might interact intensively with hydroxyapatite. This finding was also observed in our previous publication[7], and might also be implicated in acid resistant mechanisms.

Another important finding was that several isoforms of the spectrin beta-chain were found only in the AEP collected from the GNE group. These proteins interact with actin. Curiously, there were two actin isoforms that increased more than twofold in the GNE group, in comparison with the GE group. The spectrin beta-chain can also form protein complexes. Because it binds to actin, it could be involved in the formation of supramolecular aggregates in the second stage of AEP formation.[25]

As mentioned above, several intracellular proteins delivered to saliva after exfoliation of oral mucosa cells have the potential to bind to hydroxyapatite, or participate in supramolecular aggregates that bind to the precursor proteins in the AEP. This has also been observed in other recent studies,[26-31] and could have a central role in protecting the tooth surface against acid dissolution. Hence, it is worthwhile noting that not only do secreted salivary proteins participate in AEP formation, but oral mucosa proteins, gingival crevicular fluid and even bacteria also act strongly on the enamel pellicle. This indicates that the AEP protein could play a protective role against dental erosion caused by intrinsic acids. This topic should be investigated further in future studies.

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