UNIVERSIDADE DE SÃO PAULO
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Changes in the proteomic profile of acquired enamel pellicles formed in vivo for different times, after exposure to hydrochloric acid

Alterações no perfil proteômico da película adquirida formada in vivo e em diferentes tempos, após exposição ao ácido Clorídrico

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Dissertation presented to the Bauru School of Dentistry of the University of São Paulo to obtain the degree of Master in Science in the Applied Dental Science Program, Stomatology and Oral Biology concentration area.

Supervisor: Prof. Drª Marília Afonso Rabelo Buzalaf

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Orientadora: Prof. Drª Marília Afonso Rabelo Buzalaf

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"A felicidade não está na estrada que leva a algum lugar. A felicidade é a própria estrada."

Bob Dylan

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"Você tem que ser o espelho da mudança que está propondo. Se eu quero mudar o mundo, tenho que começar por mim."

Mahatma Gandhi

ABSTRACT

Changes in the proteomic profile of acquired enamel pellicles formed in vivo for different times, after exposure to hydrochloric acid

Saliva it is an important factor against enamel and dentin damages. When the saliva enter in contact with the dental surface, results in a selective adsorption of salivary proteins, glycoproteins and lipids. This adsorption formed an organic free-bacterial film, which when formed in the enamel, is denominated acquired enamel pellicle. The presence of this proteins covering the enamel tissues, has the function of lubrication, buffering and remineralization capabilities, making it an important factor against dental erosion. The objective of this study was detected changes in the protein profile of acquired enamel pellicles (AEP) formed *in vivo* for different times, after application of hydrochloric acid (HCl). The experimental was realized in 12 consecutive days. On each day, nine subjects, (aged 18 to 35 years, non-smokers, with good general and oral health) were submitted to dental prophylaxis with pumice. After 3 or 120 min, time of formation of the acquired pellicle, the teeth were isolated with cotton rolls and, submitted for a 3 different procedures, one procedure of each day, 50 µL of 0.1 M HCl (pH = 1.0), 0.01 M HCl (pH = 2.0) or deionized water were applied on the buccal surface of the teeth for 10 s. The application of HCl was in all teethes from the superior and lower arch, in vestibular surface. In sequence the AEP was collected using an electrode filter paper pre-soaked in 3% citric acid. This procedures was repeted for one more day. After protein extraction, the samples were submitted to reverse phase liquid chromatography coupled to mass spectrometry (nLC-ESI-MS/MS). Label-free quantification was performed (Protein Lynx Global Service software). A total of 180 proteins were successfully identified in the AEP samples. The number of identified proteins increased with the time of pellicle formation. Only 4 proteins were present in all the groups (isoforms of IgA, *Serum albumin* and *Statherin*). The greatest number of proteins identified uniquely in one of the groups was obtained for the groups treated with HCl after 2 h of pellicle formation (~ 50 proteins). Conclusion: Proteins resistant to removal by HCl, such as *Serum Albumin* and *Statherin*, were identified even in the

short-term AEP. In addition, 120-min pellicle present many proteins that are resistant to removal by HCl. This suggests an increase in the protection against intrinsic acids along the time of pellicle formation, which should be evaluated in future studies.

Keywords: Acquired Pellicle; enamel; dental erosion; gastroesophageal reflux; proteomics.

RESUMO

A saliva é um importante meio de proteção contra danos ao esmalte e dentina, e é quando ela entra em contato com a superfície dentária, que ocorre uma adsorção seletiva de proteínas salivares, glicoproteínas e lipídeos. Esta adsorção forma um filme orgânico, que é isenta de bactérias, que quando formada sobre esmalte dentário é denominada de película adquirida do esmalte (PAE). A presença destas proteínas recobrindo os tecidos dentários auxilia na lubrificação, tem capacidades de tamponamento e de remineralização, tornando-se um importante fator de proteção contra erosão dentária. O objetivo deste trabalho foi detectar as alterações no perfil protéico na película adquirida do esmalte (PAE) formada *in vivo,* após a exposição ao ácido clorídrico. Os experimentos foram realizados em 12 dias consecutivos. Em cada dia, os voluntários (n=9), com idade entre 18 a 35 anos, não fumantes, e com um bom estado de saúde geral e bucal, eram submetidos a uma profilaxia dentária com pedra pomes. Depois de 3 min ou 120 min e após a formação PAE, os dentes eram isolados com rolos de algodão e submetidos a 3 procedimentos distintos, sendo um deles realizado a cada dia: aplicação de 50μL de ácido clorídrico (0,1 M, pH 1), ácido clorídrico (0,01 M, pH 2) ou água deionizada por 10 segundos. A aplicação foi feita, em todos os dentes dos arcos superiores e inferiores na face vestibular. Na sequência, a película foi removida com um papel de filtro umedecido em ácido cítrico a 3%. Este procedimento foi repetido por mais uma vez e foi feito um “pool’ com os papeis de filtro obtidos dos 9 voluntários, para cada procedimento e tempo de formação (Água-3min, Água-2h, pH2-3min, pH2-2h, pH1-3min e pH1-2h). Após extração das proteínas, as mesmas foram submetidas à cromatografia líquida de fase reversa interligada a um espectrômetro de massas (nLC-ESI-MS/MS). Quantificação proteômica livre de marcadores foi feita utilizando o *software* (Protein Lynx Global Service software). Um total de 180 proteínas foram encontradas nas amostras de PAE. E o número de proteínas identificadas crescia conforme aumentou-se o seu tempo de formação da película. Somente 4 proteínas foram presentes em todos os grupos sendo estas isoforms de IgA, *Serum albumin* e *Statherin*. Um grande número proteínas foram identificadas como sendo únicas dos grupos tratados com HCl, depois de 2h de formação de película (~ 50 proteínas). Em conclusão as proteínas são

resistentes a remoção por HCl, e tanto que Serum Albumin e Statherin, foram identificadas em películas formadas em tempos precoces. Para películas formadas no tempo de 120-min foram encontradas muitas proteínas que são resistentes a remoção por HCl. Este fato sugere um aumento da proteção contra ácidos intrínsecos conforme o tempo de formação de película, o que deverá ser avaliada em estudos futuros.

Palavras-chave:. Película Adquirida. Esmalte Dentário. Erosão Dental. Refluxo Gastroesofágico. Proteômica.

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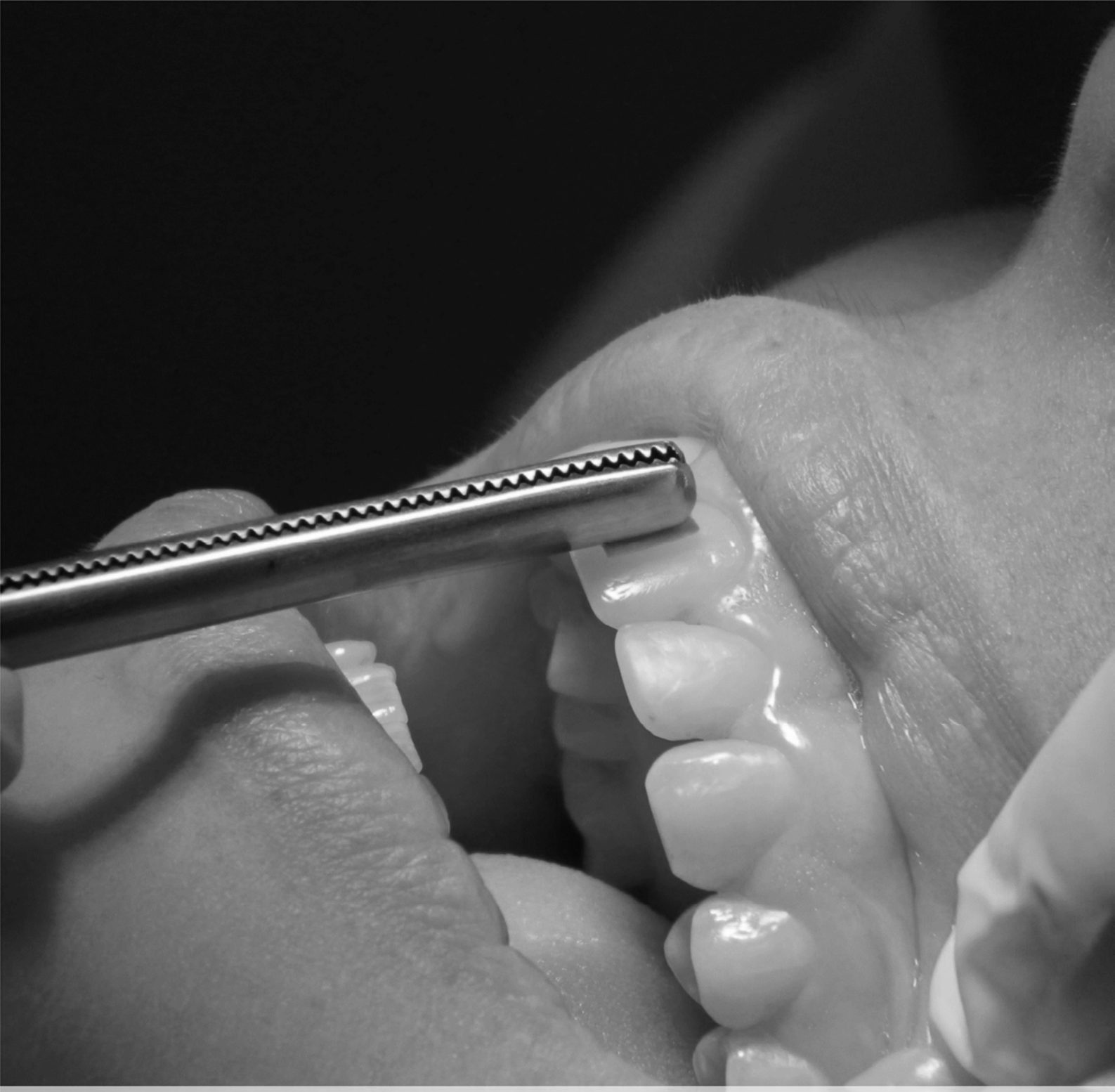


1-INTRODUCTION

1 INTRODUCTION

In the last decades, it has been observed that the dental losses, are being studied extensively, especially those caused by non-bacterial acids. Dental erosion is one of the most documented lesion with more studies in the last few years. This lesion it is a multifactorial condition caused by a complex interaction the patient and the nutritional factors, and also influenced by other factors such as education, behavior, occupation, level of knowledge, and health. In the first instance, there is a softening of the tooth surface and this process is followed by a dissolution of the enamel crystals layer-by-layer, leading to permanent volume tooth loss with a remaining softened surface (LUSSI et al., 2011).

The dental erosion could be classified, taking into account their etiology, could be extrinsic or intrinsic (MAGALHAES et al., 2009). The intrinsic erosion is the consequence of the action of endogenous acids, originating from gastric reflux, chronic regurgitation, alcoholism, pregnancy, or disorders of the nervous system, such as anorexia and / or bulimia. Intrinsic tooth erosion is due to the chronic performance of gastric acid over the dental surface for a long period of time (MOAZZEZ; BARTLETT, 2014). The extrinsic erosion, is caused by exogenous acids, for example from dietary (BARBOUR; LUSSI, 2014) and drug formulations (HELLWIG; LUSSI, 2014). The main extrinsic etiological factor of erosion is would be derived from acids from the diet. Most of the low pH food and beverages (below 4.5) would have the potential to cause tooth erosion, since at this pH there is a sub saturation of the oral fluids in relation to hydroxyapatite and fluorapatite (SHELLIS; FEATHERSTONE; LUSSI, 2014). This disorder occurs in patients with, regurgitation, bulimia, rumination and gastroesophageal reflux disease (GERD) (MOAZZEZ; ANGGIANSAH; BARTLETT, 2005). It has been reported that around 10-20% of the population suffer from GERD (DENT et al., 2005). Considering that the pH and titratability of gastric acids is greater than those of dietary acids, the destruction of the tooth structure is usually more severe (MOAZZEZ et al., 2005).

All solid surfaces exposed in the oral cavity are covered by a proteinaceous layer called the acquired pellicle (HANNIG, C.; HANNIG; ATTIN, 2005; HANNIG, M.; BALZ, 1999; HANNIG, M.; JOINER, 2006; LENDENMANN; GROGAN; OPPENHEIM,

2000). It is an organic film, bacterial-free, covering soft and hard tissues. Glycoproteins and proteins, including several enzymes (HANNIG, C.; HAMKENS; et al., 2005; SIQUEIRA; CUSTODIO; MCDONALD, 2012), compose the Acquired Pellicle.

The presence of these organic components, confer important functions to this protein-rich film, such as lubrication, buffering and remineralizing, also acts as a diffusion barrier that reduces dissolution of the tooth.(BUZALAF; HANNAS; KATO, 2012; VUKOSAVLJEVIC et al., 2014). It is remark that part of these proteins remains on the pellicle composition, even after exposure to erosive challenges which makes the acquired pellicle an extreme important factor in the etiology of dental erosion (HANNIG, C. et al., 2009; HANNIG, M.; BALZ, 1999;2001).

There are many studies that focus on the protector impact of the acquired pellicle in the enamel surface (HANNIG, M.; BALZ, 1999;2001; HANNIG, M. et al., 2004; HANNIG, M. et al., 2003; VUKOSAVLJEVIC et al., 2014). Additionally, they also investigated changes in the power of protection along its time of formation of the acquired pellicle, and even with several published studies, there still is many controversies regarding the protective potential of the acquired pellicle as a function of its time of formation. Some previously published studies, has been concluded that the resistance of the acquired pellicle are dependent of time of formation, one study demonstrated pellicles with 2 hours formation, had a fast dissolutions when compares with pellicles formed in 6, 12 and 24 hours (HANNIG, M. et al., 2003). It has been suggested, in some studies, that acquired pellicle must reach an ideal thickness, and for a significant protection against the acids challenges, the dental surface must be in contact with saliva for at least 1 hour, and also was verified that protection factor does not significantly increased, if the pellicle through process of maturation for 24h. In addition, were not found significantly differences in erosives challenges, when the pellicles was formed in 24h or 7 days. For Acquired Pellicles formed in short periods of exposure of the dental surface with saliva (less than 30 minutes), apparently the pellicles does not seem to be able to confer good protection against erosion (HANNIG, M.; BALZ, 1999; HANNIG, M. et al., 2003; WETTON et al., 2006). However, some studies are not consistent with previously studies mentioned. An example, studies made in pellicles formed in situ, for 30 min, 1 or 2 hours (HANNIG, M. et al., 2004) or for 2, 6, 12 and 24h (HANNIG, M. et al., 2003), respectively, did not obtain results that significantly differed in the hability of reduce enamel desmineralization. Another study (HANNIG, M. et al., 2004), concluded that is not possible find differences on the effect

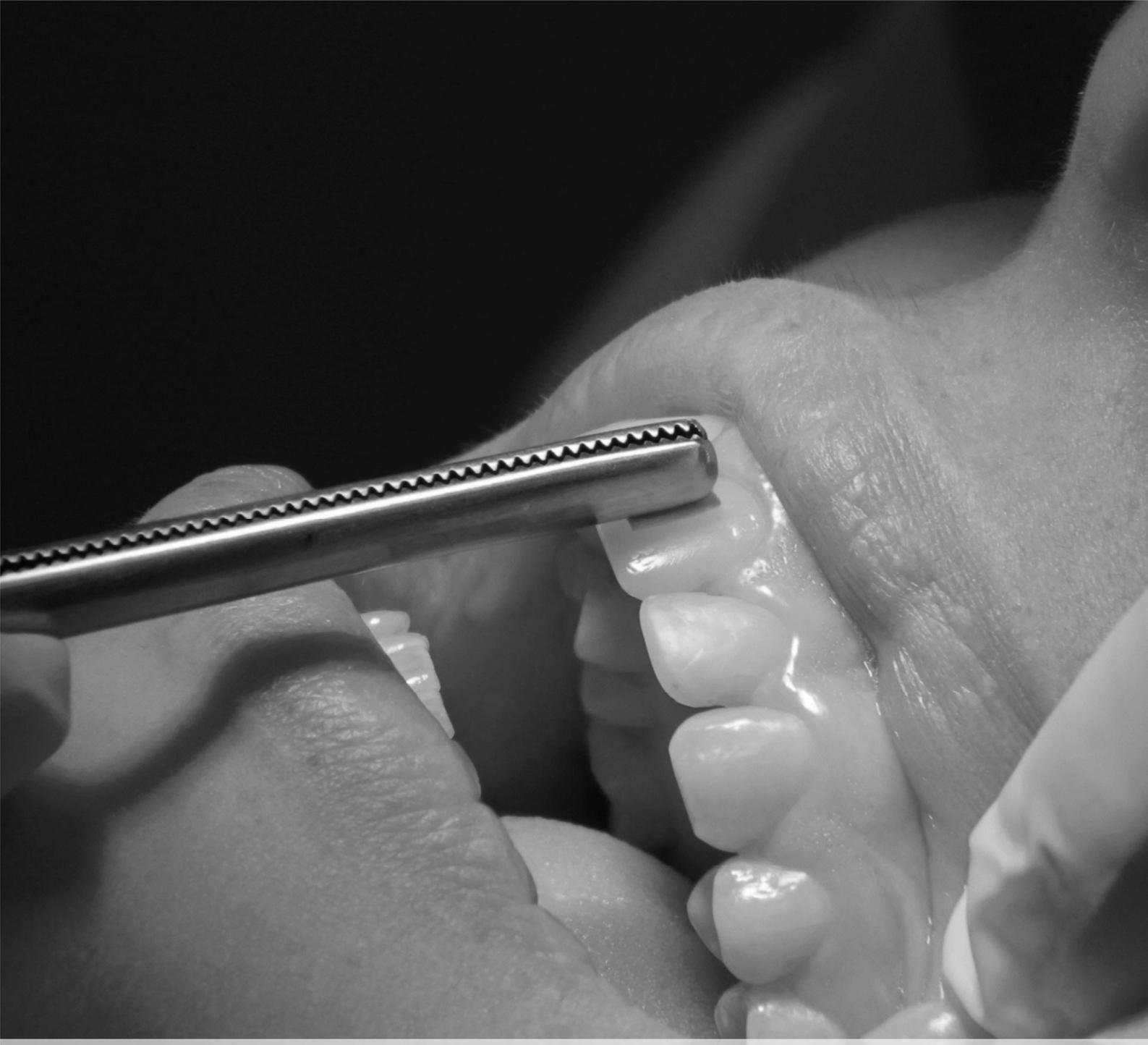
protector of Pellicle formed after 3 minutes when this compares with the pellicle formed in 2 hours. These results can be assigned to the fact that the formation of the Acquired Pellicle, especially in the protein adsorption, that happens seconds after the dental surface enter in contact with salivary proteins (HANNIG, M. et al., 2004), resulting in a basal pellicle electrondense, even after 1 minute of contact (ERICSON et al., 1982). Once that subsequent layers of pellicle are much less electrondense, compares with basal pellicle that seems to offer lack additional protection against acids attack (HANNIG, M. et al., 2004). This is consistent with the observations whereof, after consumed acids beverages, the external globular layers are removed in different extension, according to the localization of the samples in the buccal cavity, while the basal pellicle not affected. In that way, would be interesting knows which proteins compound the basal layer from the acquired Pellicle, that who could be able to protect against acids attack, this justify the pellicle collect formed in short periods.

Since different acids vary in their ability to demineralize bovine enamel (HANNIG, C.; HAMKENS; et al., 2005) the protection from the pellicle formed in situ in the enamel and dentine erosion, caused by hydrochloric, citric and phosphoric acids were analyzed (WIEGAND et al., 2008). Samples of enamel and dentine bovine were exposed by 120 minutes in the oral cavity from 10 healthy volunteers. In sequence, the samples (enamel and dentine) covered by acquired pellicle, were steeped, out of the oral cavity, in 1mL of hydrochloric, citric or phosphoric acid (pH 2,6 60 seconds, N=30 for each type of acid). Samples without covered with pellicle (N=10) were the control group. The calcium release was analysed by atomic absorption spectroscopy. The samples that were covered by acquired pellicle, had calcium loss significantly reduce, when compares to the group control, for all types of acid. Similar findings were obtained in other studies. Were observed a reduction in the calcium loss between 60 and 78% for enamel samples, eroded by citric acid (1% for 60 seconds) (HANNIG, M. et al., 2004; HANNIG, M. et al., 2003). To sum up another study in vitro also showed that the acquired pellicle offers significantly protection better for enamel (44%) than dentine (14%) (WETTON et al., 2006).

Due to the protector function from the acquired pellicle against acid challenges, fact that are widely reported in the literature (BUZALAF et al., 2012; VUKOSAVLJEVIC et al., 2014) and the observation that even in severs acids challenges (exposed by 5 minutes in citric acid 1%), the acquired Pellicle is not total removed from the enamel surface (HANNIG, M.; BALZ, 2001; HANNIG, M.; JOINER, 2006). It becomes

important to investigate the possible components of the pellicle that are most directly associated with the protective potential against tooth erosion, in other words, which proteins remain on the enamel after erosive challenges.

Recently studies by our group has evaluated, using proteomic strategies, which proteins remain adhered to the acquired pellicle formed over the dentin in situ (DELECRODE et al., 2015) and over the enamel in vivo, after citric acid challenge (1%), simulating extrinsic erosion. In conclusion, it would be interesting to determine the quantitative proteomic profile from acquired pellicles formed in enamel after hydrochloric acids challenges, simulating intrinsic erosion. This becomes of great importance because, recently, it was reported that patients with eating disorders had more risk of erosion (Or = 12.4), which is increased when vomiting is self-induced (OR = 19.6). 4 In addition, around 10-20% of the population suffer from GERD 5 (DENT et al., 2005). In that way, knowledge about which proteins from the acquired pellicle are removal resistant by Hydrochloric acid could be new preventives strategies against intrinsic erosion. In futures studies we could employ procedures that “Acquired Pellicle engineer”, leading to a formation of acquired pellicle with that resistant proteins, and making possible better enamel protection against intrinsic erosion, in susceptible patients. These procedures may involve application of mouthwash solutions, toothpaste or gels rich with these acid resistant proteins.



2-Article

2 ARTICLE

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Changes in the proteomic profile of acquired enamel pellicle as a function of its time of formation and hydrochloric acid exposure.

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Running title: Proteomics of acquired enamel pellicle changes after exposure to HCl

Keywords: Acquired Pellicle; Enamel; dental erosion; gastroesophageal reflux; proteomics.

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Abstract

Objective: Changes in the protein profile of acquired enamel pellicles (AEP) formed *in vivo* for different times, after application of hydrochloric acid (HCl) were evaluated. Methods: Nine subjects were submitted to dental prophylaxis with pumice. After 3 or 120 min, the teeth were isolated with cotton rolls and 50 µL of 0.1 M HCl (pH = 1.0), 0.01 M HCl (pH = 2.0) or deionized water were applied on the buccal surface of the teeth for 10 s. The AEP was then collected using an electrode filter paper pre-soaked in 3% citric acid. After protein extraction, the samples were submitted to reverse phase liquid chromatography coupled to mass spectrometry (nLC-ESI-MS/MS). Label-free quantification was performed (Protein Lynx Global Service software). Results: A total of 180 proteins were successfully identified in the AEP samples. The number of identified proteins increased with the time of pellicle formation. Only 4 proteins were present in all the groups (isoforms of IgA, *Serum albumin* and *Statherin*). The greatest number of proteins identified uniquely in one of the groups was obtained for the groups treated with HCl after 2 h of pellicle formation (~ 50 proteins). Conclusion: Proteins resistant to removal by HCl, such as *Serum Albumin* and *Statherin*, were identified even in the short-term AEP. In addition, 120-min pellicle present many proteins that are resistant to removal by HCl. This suggests an increase in the protection against intrinsic acids along the time of pellicle formation, which should be evaluated in future studies.

Introduction

Dental erosive wear develops ultimately due to the action of non-bacterial acids that initially provoke softening of the dental surface. If the acidic challenge persists, layer-by-layer dissolution of the enamel crystals takes place, with the remaining surface becoming softened [1]. Despite the lesion itself is caused by acids, erosive wear is in fact a multifactorial condition developed due to a complex interaction of factors related to the patient combined with nutritional factors. The etiology is also influenced by other factors such as education, behavior, occupation, knowledge, employment and general health [2]. Among the factors related to the patient are some conditions that lead to the return of gastric acids, also known as intrinsic acids, to the oral cavity. This occurs in patients with bulimia, regurgitation, rumination and gastroesophageal reflux disease (GERD) [3]. Recently, it was reported that patients with eating disorders had more risk of erosion (Or = 12.4), which is increased when vomiting is self-induced (OR = 19.6). [4] In addition, around 10-20% of the population suffer from GERD [5]. Considering that the pH and titratability of gastric acids is greater than those of dietary acids, the destruction of the tooth structure is usually more severe [3], which means that preventive and therapeutic measures are necessary.

One of the most important preventive factors against erosive wear is saliva, since it is supersaturated with respect to hydroxyapatite, has the potential to clean the oral cavity, washing out the acids, and contributes to the formation of the acquired enamel pellicle (AEP) [6]. The AEP is a bacteria-free organic film that covers the hard and soft tissues in the oral cavity. It is composed mainly by proteins and glycoproteins [7], but it also contains lipids [8]. These organic components confer important functions to the AEP, such as lubrication and protection of the underlying tooth surface. The AEP also acts as a diffusion barrier that reduces the direct contact between the acids and the tooth surface, thus reducing the degree of dissolution of the tooth [6, 9]. Most of the protective ability of the AEP against acidic dissolution of the teeth derives from its protein composition. It is noteworthy that part of the pellicle remains on the tooth surface, even after severe erosive challenges [10, 11]. Recently, proteomic tools were employed to identify proteins within the AEP that are resistant to removal by citric acid [12]. These acid-resistant proteins seem to have good potential to prevent tooth dissolution under acidic conditions. However, proteins resistant to removal by hydrochloric acid (HCl), the main constituent of the gastric juice, have never been identified so far, which was the main aim of the present study. Additionally, we also investigated changes in the protein profile of the AEP

along its time of formation, both under normal conditions and after challenges with two concentrations of HCl. The null hypotheses tested were that the protein composition of the AEP does not change as a function of its time of formation neither after exposure to different concentrations of HCl.

Materials and Methods

Ethical Aspects and Subjects

Nine young (18-35 years old) adult subjects (2 male, 7 female) participated in this crossover study, approved by the local Institutional Ethics Committee (No. 44737115.4.0000.5417). Subjects signed an informed consent document prior to the beginning of the study. Sample size was based on *in vivo* studies conducted with similar research protocol [12, 13]. The inclusion criteria were: non-smokers, good oral (without caries, gingivitis, periodontitis and other oral conditions that could affect the composition of oral fluid), good general health and no restorative treatment on the buccal surface of the upper and lower teeth. Participants who had risk factors for dental erosion, such as excessive consumption of carbonated drinks, fruit juices or acidic fruits, swimmers, or that had gastric disorders such as bulimia and gastroesophageal reflux were excluded. In addition, pregnant women, patients with systemic diseases and using chronic medication were not eligible to participate. Volunteers stimulated salivary flow was higher than 1 mL/min and the unstimulated salivary flow was higher than 0.25 mL/min. Following a crossover protocol, each volunteer took part in all the groups under study. The factors under study were the time of formation of the AEP in two levels (3 min or 2 h, in order to analyse the proteins present in the basal layer and in the subsequent layers, respectively[14]) and the type of treatment solution applied over the AEP in three levels: deionized water, 0.01 M HCl (pH 2.0) or 0.1 M HCl (pH 1.0), totalling 6 groups (Water-3min, Water-2h, pH2-3min, pH2-2h, pH1-3min and pH1-2h). The HCl concentrations of 0.1 and 0.01 M were chosen because they correspond to pH 1 and 2, respectively and the pH of the gastric juice typically ranges between 1 and 3 [15]. For each volunteer, AEP was collected on 12 different days (2 days for each of the six groups/treatments), as detailed below.

AEP Formation and Collection

In order to avoid circadian effects on the composition of the pellicle, the experiment began in the morning each day [16]. The subjects underwent a dental prophylaxis employing coarse pumice containing no additives. The volunteers waited 3 or 120 min deprived of food and beverage consumption, to allow the formation of acquired pellicle on enamel [17]. After the periods allowed for the formation of the AEP (3 or 120 min), each quadrant of the mouth was rinsed with deionized water and dried with compressed air twice and isolated with cotton rolls. Then 50 µL of deionized water, 0.1 M HCl (pH 1) or 0.01 M HCl (pH 2), depending on the phase under study, was gently applied with a pipette on the buccal surface of the upper and lower teeth (from second molar to second molar in each arch) for 10 sec. The solutions were carefully removed with deionized water using the dental syringe and the teeth were air-dried [12]. The remaining AEP was then collected with the aid of 5X10 mm electrode filter paper (Bio-Rad, Hercules, CA) pre-dipped in 3% citric acid (pH 2.5; Sigma-Aldrich, USA). The filter paper was rubbed (without pressure) on the coronal two-thirds (to avoid contamination of the gingival margin) of the buccal surfaces of the teeth with tweezers [17]. The wick filters were placed in 2 mL cryotubes and stored at -80°C until prepared for proteomic analysis. The filters collected from all the participants on two different days, corresponding to the same treatment and time of pellicle formation were pooled in order to have enough material to be analysed. Six pools were obtained, corresponding to the 6 groups.

Preparation of the AEP samples

The AEP samples were prepared as previously reported [18].The Eppendorf tubes containing the filter papers were removed from the -80° C freezer. After defrost, the papers were cut into small pieces, using sterile scissors and tweezers. These pieces were kept together in an Eppendorf tube, constituting a pool for each group. Approximately 400 µL (until the papers were covered) of a solution containing 6 M urea, 2 M thiourea in NH_4HCO_3 50 mM pH 7.8 was added to each tube, vortexed for 10 minutes at 4°C, sonicated for 5 minutes and centrifuged for 10 minutes at 14,000 g at 4°C. The supernatant was collected and transferred to a new tube. This procedure was repeated once more. The papers were then placed in tube filters (Corning Costar®Spin-X® Plastic Centrifuge Tube Filters, Sigma-Aldrich, New York, USA) and centrifuged at 14,000 g for 10 minutes at 4°C. The supernatant was recovered, added to

that collected previously and centrifuged once more at 14,000 g at 4°C. The supernatant was transferred to a 15-ml falcon tube. In sequence, 50 mM NH_4HCO_3 (1.5 X the sample volume) was added to dilute the urea and thiourea. The samples were transferred to Falcon Amicon tubes (Amicon Ultra - 15 Centrifugal Filter Units - Merck Millipore, Tallagreen, Ireland), centrifuged at 5,000 g at 4°C and concentrated to approximately 150 µL. Reduction was then performed by adding 5 mM dithiothreitol (DTT) followed by incubation for 40 min at 37ºC. Samples were then alkylated by adding 10 mM iodoacetamide (IAA) and incubated in the dark for 30 min. In sequence, 100 µL of 50 mM NH_4HCO_3 was added and samples were digested for 14 h at 37ºC by adding 2% (p/p) trypsin (Promega, Madison, USA). Then 10 µL of 5% formic acid was added to stop the action of trypsin. Samples were then desalted and purified using C18 Spin columns (Thermo Scientific, USA). Then an aliquot of 1 µL of each sample was removed and protein quantification was performed using the Bradford method (Bio-Rad Bradford Assays, USA). The total amount of protein recovered ranged between 63 and 115 µg for the different groups. The samples were resuspended in a solution containing 3% acetonitrile and 0.1% formic acid to be submitted to nano LC-ESI-MS / MS.

Shotgun Label-free Quantitative Proteomic Analysis

Peptides identification was performed on a nanoACQUITY UPLC-Xevo QTof MS system (Waters, Manchester, UK). The nanoACQUITY UPLC was equipped with nanoACQUITY HSS T3, analytical reverse phase column (75 µm X 150 mm, 1.8 µm particle size, Waters). The column was equilibrated with mobile phase A (0.1 % formic acid in water). The peptides were separated with a linear gradient of 7-85% mobile phase B (0.1 % formic acid in ACN) for 70 min at a flow rate of 0.35 µL/min. The column temperature was kept at 55°C. The Xevo G2 Q-TOF mass spectrometer was operated in positive nanoelectrospray ion mode and data were collected using the MSE method in elevated energy (19-45 V), which allows data acquisition of both precursor and fragment ions, in one injection. Source conditions used included capillary voltage, 2.5 kV; sample cone, 30 V; extraction cone, 5.0 V and source temperature, 80°C. Data acquisition occurred over 70 min and the scan range was 50–2000 Da. The lock spray, used to ensure accuracy and reproducibility, was run with a [Glu1] fibrinopeptide solution (1 pmol/µL) at a flow rate of 1 µL/min, as a reference ion in positive mode at m/z 785.8427. ProteinLynx Global Server (PLGS) version 3.0 was used to process and

search the continuum 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 May 2016 from UniProtKB (http://www.uniprot.org/). The identified proteins were classified and assigned by biological function [16, 18, 19], origin and molecular interaction (http://www.uniprot.org/).

For label-free quantitative proteome, three MS raw files from each pooled group were analysed using the Protein Lynx Global Service (PLGS, v 2.2.5, Waters Co., Manchester, UK) software. All the proteins identified with a score 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. Difference in expression among the groups was expressed as $p<0.05$ for down-regulated proteins and $1-p>0.95$ for up-regulated proteins. The following relevant comparisons were made: Water-2h X Water-3min, pH2-2h X pH2-3min, pH1-2h X pH1-3min, Water-3min X pH2-3min, pH2-2h X Water-2h, pH1-3min X Water-3min, pH1-2h X Water-2h, pH1-3min X pH2-3min, pH1-2h X pH2-2h.

Results

The identified proteins when classified according to their function, molecular interaction and origin are displayed in Tables 1 and 2 and Supplementary table (S1). In total, 180 proteins were identified (Table S1). Figure 1 shows the number of proteins common to the distinct groups, as well as the numbers of proteins found in only one of the groups. Four proteins were identified in all the groups (*Ig alpha-1 chain C region*, *Ig alpha-2 chain C region*, *Serum albumin* and *Statherin*; Figure 1, Table S1). The number of identified proteins increased with the time of pellicle formation (Table S1). Fifteen proteins were found only in groups with 2 h of formation, such as *Lysozyme C*, *Myeloperoxidase*, *Protein S100-A8, Lactotransferrin* and isoforms of keratin. Many proteins (140) were found exclusively in one of the groups: 13 in Water-3min, 28 in Water-2h, 1 in pH2-3min, 47 in pH2-2h and 52 in pH 1-2h. No unique protein was identified in the group pH1-3min (Table 1).

Regarding quantitative analysis, nine comparisons were made among the six groups (Table 2). For the group treated with water (comparison Water-2h X Water 3-min), along time there was an increase in *Actin cytoplasmic 2*, *Neutrophil defensin 3*, *Actin cytoplasmic 1, Neutrophil defensin 1* and a decrease in *Cystatin-SN*. When the AEP was challenged with HCl pH 2, along time (comparison pH2-2h X pH2-3 min) there was an increase in *Serum albumin*

and decrease in *Statherin*, *Ig alpha-2 chain C region* and *Ig alpha-1 chain C region*. *Serum albumin* also increased along time when the AEP was challenged with HCl pH 1(comparison pH1-2h X pH1-3min). Besides *Serum albumin*, *Statherin*, *Ig alpha-1 chain C region* an *Ig alpha-2 chain C region* also increased in this comparison.

When the groups treated with HCl at different pH were compared, for the short-term pellicle under stronger challenge (comparison pH1-3min X pH2-3min) there was an increase in *Serum albumin* only that was also increased in the long-term pellicle (comparison pH1-2h X pH2-2h). In this comparison, there was also an increase in 9 other proteins, including *Cystatin-SN, Lysozyme C*, *Statherin* and isoforms of IgA and keratin. On the other hand, for this comparison there was a decrease in 11 proteins, including *Protein S100-A8, Neutrophil defensin 3* and isoforms of actin and POTE Ankyrin (Table 2).

Discussion

The main aim of this study was to identify proteins in the AEP that are resistant to removal by HCl, an intrinsic acid that is found in the oral cavity after episodes of gastroesophageal reflux, vomiting and bulimia. The identification of these acid-resistant proteins is of utmost importance, since they have a great potential to protect the teeth after acidic challenges. A secondary aim was to evaluate changes in the protein profile of the AEP along its time of formation, both under normal conditions as well as after challenges with HCl. It was observed an increase in the number of proteins identified in the AEP along time, which is expected and has been reported in a previous study [13]. Some proteins were identified only in the groups with 2 hours of AEP formation, such as *Lysozyme C*, *Myeloperoxidase*, *Protein S100-A8, Lactotransferrin* and isoforms of keratin, which suggests that these proteins probably do not bind to hydroxyapatite but bind to the precursor proteins. An interesting finding of the present study was that only quite few proteins were identified in the 3-min pellicle after challenge with HCl (mainly Immunoglobulins, *Serum Albumin* and *Statherin*). Since it has been reported no difference in the protective effect against acids of a pellicle formed after three minutes compared to one formed after two hours [14], it is possible that these proteins are responsible for most of the protection conferred by the short-term pellicle. It should be

highlighted, however, that this study [14] was conducted in situ and that the acidic challenge was performed with orange juice, while the present study had an in vivo design and the acidic challenge was done with HCl.

We employed an in vivo design that had been used before to evaluate proteins in the AEP that are resistant to removal by acids that mimic extrinsic erosion (citric acid) and caries (lactic acid) [12]. Thus, the results obtained here are more realistic than those described in studies involving in vitro [20-22] or in situ [23] protocols. The preparation of the AEP for MS was done according to a recently developed methodology that increases the identification of proteins in the AEP samples [18]. This allowed the identification of 180 proteins. From these, four deserve special attention because they were present in all the groups, regardless of the time of pellicle formation, and even after exposure to HCl: *Statherin*, *Serum Albumin* and isoforms of Ig alpha. *Statherin* is a 43-residue phosphorylated salivary protein with primary sequence similarities to osteopontin and casein that binds calcium and hydroxyapatite. Its negative charge density and helical conformation at the N-terminus are important for the interaction with hydroxyapatite [24]. This interaction seems to be strong, since not even challenge with 0.1 M HCl was able to detach this protein from the enamel surface. In fact, solid-state nuclear magnetic resonance (NMR) studies confirmed that the N-terminus of *Statherin* strongly binds to hydroxyapatite, while the middle and C-terminal regions are mobile and dynamic [25]. In order to protect against demineralization, it is suggested that statherin-like peptides containing at least 15 N-terminal residues or more, are required [26]. It was recently reported that the calcium concentration in the AEP of patients with dental erosion is 50% reduced, while the concentration of *Statherin* is 35% reduced [27]. This emphasizes the potential of this protein to protect against dental erosion. Another protein that was identified in all the conditions was *Serum Albumin,* which comes from plasma and it is able to bind ions as calcium [28]. This can also be important for acid protection, since ovalbumin was shown to reduce the dissolution of hydroxyapatite by acid solutions in vitro [29, 30]. These results indicate that *Albumin* and *Statherin* are resistant to removal by HCl. These proteins are then potential candidates to be included in anti-erosion dental products. The presence of Ig alpha among these proteins deserves further investigation, since at the moment this protein has not been related to resistance of enamel against erosive demineralization.

Interestingly, the greatest number of proteins identified uniquely in one of the groups was found for the groups treated with HCl after 2 h of pellicle formation (around 50 proteins for each of these groups). This means that many proteins remain in the AEP after exposure to HCl, even at low pH. The fact the groups treated with HCl had a higher number of proteins than

the group treated with deionized water might be related to the fact that HCl might remove at least part of the most abundant proteins, thus allowing that the present in much lower amounts can be detected by mass spectrometry. Furthermore, it should be highlighted that potentially protective proteins, such as *Serum albumin* (both for HCl pH 1 and HCl pH 2) and *Statherin* (for HCl pH 1) increased along time of formation after application of HCl. As mentioned above, an in situ study employing orange juice to perform the erosive challenge did not find any difference in the protective effect of AEPs formed after three minutes or two hours [14]. It must be taken into account that the pH of HCl is lower than that of orange juice. Thus, it is possible that for the AEP challenged with intrinsic acid (HCl), a long time of maturation is important for the protection against demineralization. In line with this hypothesis, an in situ study showed that exposure to saliva for 30 min for AEP formation promoted less enamel resistance to HCl erosive attack than exposure to saliva for 2 hours [31]. This suggests that more mature pellicles might provide higher protection against intrinsic erosion than short-term pellicle.

In conclusion, the present study identified potentially protective proteins that are resistant to removal by HCl even in the short-term AEP, such as *Serum Albumin* and *Statherin*. In addition, it was observed that the mature pellicles present many proteins that are resistant to removal by HCl, even at pH 1. This suggests that there is an increase in the protection against intrinsic acids along the time of pellicle formation, which should be evaluated in future studies.

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Figure legend

Figure 1. Organogram showing the number of proteins identified in the acquired enamel pellicle collected after different times of formation (3 minutes or 2 hours) and treatments (Water, HCl pH 2 or HCl pH 1).

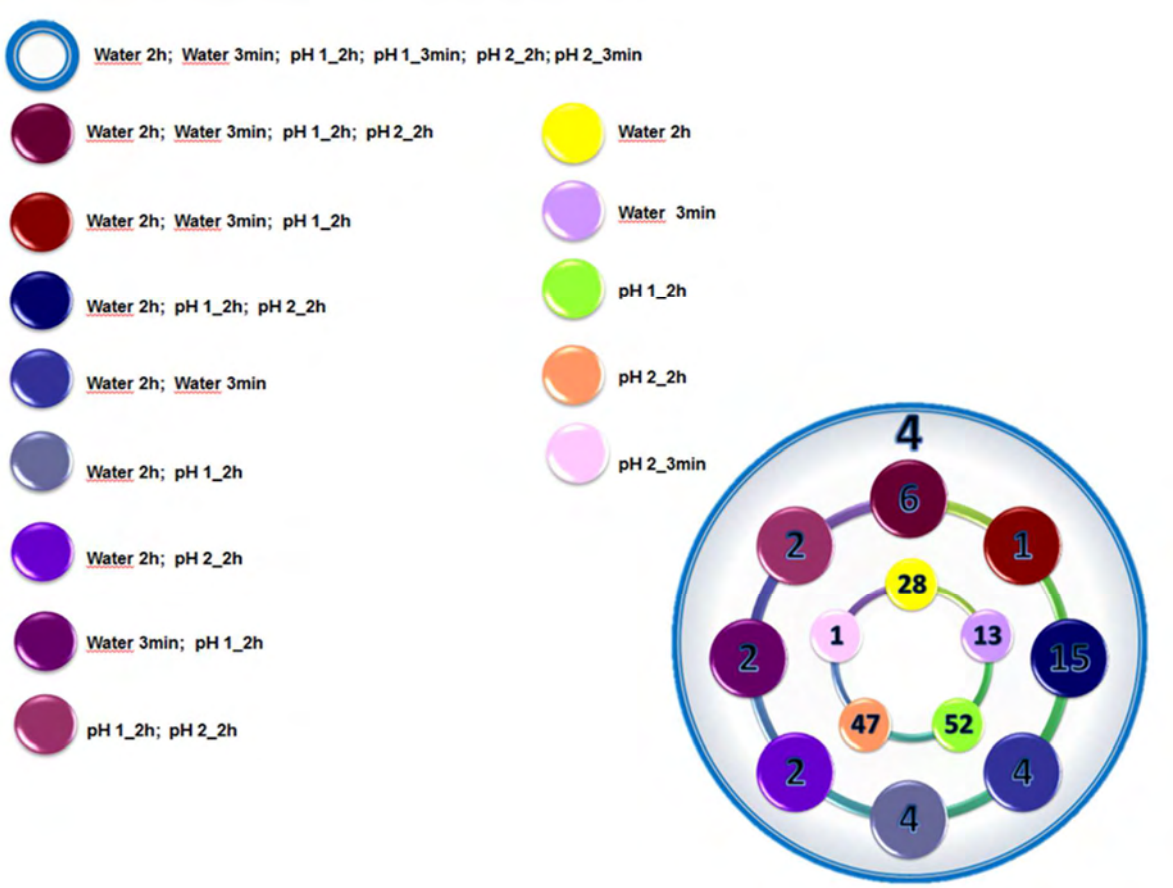


Table 1. Classification of the proteins identified in only one of the groups.

Water 3 minutes	Accession name	Protein name and Classification	PLGS Score
	O75419	Cell division control protein 45 homolog [a. b. g. n. p. u. w]	62.00
	O43187	Interleukin-1 receptor-associated kinase-like 2 [b. g. j. n. p. s. u]	54.33
	Q13485	Mothers against decapentaplegic homolog 4 [f. g. h. j. n. p. u. w]	110.93
	D6RGX4	Protein FAM90A26 [b. m. t. u]	34.91
	Q9P1P4	Putative trace amine-associated receptor 3 [b. g. m. s. x]	41.63
	F8WDV7	RING finger protein 121 [b. c. m. s. u. w]	104.86
	Q13126	S-methyl-5'-thioadenosine phosphorylase [a. b. g. n. p. u. w]	127.04
	A6NLX3	Speedy protein E4 [b. g. t. w]	144.9
	Q96NU1	Sterile alpha motif domain-containing protein 11[e. m. p. u]	46.14
	Q7Z7G0	Target of Nesh-SH3 [b. d. m. o. u. w]	67.43
	Q9NNW7	Thioredoxin reductase 2. mitochondrial [a. b. g. n. u. w]	42.25
	F2Z2F3	Uncharacterized protein [b. m. n. w]	127.04
	P25311	Zinc-alpha-2-glycoprotein [a. b. g. o. u. w]	62.92

Water 2 hours	Accession name	Protein name and Classification	PLGS Score
	P22303	Acetylcholinesterase [a. e. g. o. s. u. w]	56.19
	P03973	Antileukoproteinase [a. g. j. o. u]	127.13
	Q6PIW4	Fidgetin-like protein 1 [a. b. g. h. p. u. w]	77.58
	H7BXF5	Histone deacetylase complex subunit SAP130 [a. b. g. p. u]	97.64
	P31276	Homeobox protein Hox-C13 [b. e. g. p. u]	92.86
	Q5TA45	Integrator complex subunit 11 [b. e. g. n. p. u]	139.67
	Q5JVA3	Interleukin-15 receptor subunit alpha (Fragment) [b. e. h. j. p. u]	125.69
	F8W0C6	Keratin. type II cytoskeletal 5 (Fragment) [d. h. o. p. u. w]	119.78
	Q9NSK0	Kinesin light chain 4 [a. c. g. n. u]	145.84
	Q8WV93	Lactation elevated protein 1 [a. g. n. u]	96.68
	O43148	mRNA cap guanine-N7 methyltransferase [a. e. g. p. u]	130.73
	E9PK18	Rho GTPase-activating protein 27 [a. b. e. g. n. s. u]	267.7
	Q15633	RISC-loading complex subunit TARBP2 [f. g. n. p. u. w]	191.49
	Q9C0I3	Serine-rich coiled-coil domain-containing protein 1 [b. m. t. u]	179.96
	I3L0M1	Sex hormone-binding globulin [b. m. o. u. w]	107.92
	O76082	Solute carrier family 22 member 5 [f. g. s. u. w]	115.98
	O00338	Sulfotransferase 1C2 [a. b. g. n. u. w]	129.21

	B8ZZF7	Sulfotransferase [b. m. t. x]	129.21
	P60508	Syncytin-2 [b. m. s. u. w]	79.25
	Q12799	T-complex protein 10A homolog [b. m. n. u]	91.62
	X6REB3	Transcription factor E2-alpha [f. g. p. u. w]	76.38
	P07437	Tubulin beta chain [b. d. m. n. q. u. w]	100.25
	Q13885	Tubulin beta-2A chain [b. d. m. n. q. u. w]	100.25
	Q9BVA1	Tubulin beta-2B chain [b. d. m. n. q. u. w]	100.25
	P04350	Tubulin beta-4A chain [b. d. m. n. q. u. w]	100.25
	P68371	Tubulin beta-4B chain [b. d. m. n. q. u. w]	100.25
	Q96KH6	Uncharacterized protein C18orf12 [b. m. t. w]	109.41
	F8W0F8	Voltage-dependent L-type calcium channel subunit beta-3 [f. g. n. s. v. u]	157.48
pH 2 - 3 minutes	**Accession name**	**Protein name and Classification**	
	Q9UBC2	Epidermal growth factor receptor substrate 15-like 1 [b. c. g. p. s. u. v]	
pH 2_2 hours	**Accession name**	**Protein name and Classification**	
	Q8TE56	A disintegrin and metalloproteinase with thrombospondin motifs 17 [b. m. o. u]	
	Q5T085	Alpha-amylase (Fragment) [a. m. t. u]	
	P04745	Alpha-amylase 1 [a. g. o. u]	
	P19961	Alpha-amylase 2B [a. g. o. u]	

Q6NXT1	Ankyrin repeat domain-containing protein 54 (a. b. c. e. g. n. p. u)
G5E9V7	Armadillo repeat containing 8. isoform CRA_d (m. t. u)
G5E9V6	Armadillo repeat containing 8. isoform CRA_e (b. m. t. u)
Q8IUR7	Armadillo repeat-containing protein 8 (f. m. t. u)
Q9H4G0	Band 4.1-like protein 1 (a. b. g. p. u)
Q9NXV6	CDKN2A-interacting protein (a. b. g. m. p. u. w)
P18847	Cyclic AMP-dependent transcription factor ATF-3 (a. b. e. g. h. p. u)
E9PDJ4	Dedicator of cytokinesis protein 8 (f. m. n. s. u. u. w)
Q8IYM9	E3 ubiquitin-protein ligase TRIM22 (a. t. g. i. n. p. u. w)
F6RJU0	Enoyl-CoA hydratase domain-containing protein 2. mitochondrial (a. b. m. t. x)
Q9NQT5	Exosome complex component RRP40 (b. g. n. p. u. w)
Q6P050	F-box and leucine-rich protein 22 (a. m. n. u)
F8WF38	Gamma-aminobutyric acid type B receptor subunit 1 (a. b. g. h. s. u. w)
Q9H4A5	Golgi phosphoprotein 3-like (a. b. c. g. n. u)
P16520	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3 (a. b. g. n. o. s. u)
U3KQK0	Histone H2B ((b. m. p. u. w)
P33778	Histone H2B type 1-B (b. m. p. u. w)
P62807	Histone H2B type 1-C/E/F/G/I (b. i. j. p. u. w)

P58876	Histone H2B type 1-D [b. m. p. u. w]
Q93079	Histone H2B type 1-H [b. m. p. u. w]
P06899	Histone H2B type 1-J [b. l. j. p. u. w]
O60814	Histone H2B type 1-K [b. l. j. p. u. w]
Q99880	Histone H2B type 1-L [b. m. p. u. w]
Q99879	Histone H2B type 1-M [b. m. p. u. w]
Q99877	Histone H2B type 1-N [b. m. p. u. w]
P23527	Histone H2B type 1-O [b. m. p. u. w]
Q16778	Histone H2B type 2-E [b. i. j. p. u. w]
Q5QNW6	Histone H2B type 2-F [b. m. p. u. w]
Q8N257	Histone H2B type 3-B [b. m. p. u. w]
P57053	Histone H2B type F-S [b. l. j. p. u. w]
P01859	Ig gamma-2 chain C region [b. e. j. o. u. w]
P01860	Ig gamma-3 chain C region [b. e. j. o. u. w]
P01861	Ig gamma-4 chain C region [b. e. j. o. u. w]
Q9NZM3	Intersectin-2 [a. b. g. l. n. u. w]
K7ERE3	Keratin. type I cytoskeletal 13 [d. m. o. p. q. u]
Q92615	La-related protein 4B [b. g. n. u]

	C9JIR0	Mitotic spindle assembly checkpoint protein MAD1 (Fragment) [a.b. g. n. p. u. x]
	I3L0M3	MYC-associated zinc finger protein (Purine-binding transcription factor). isoform CRA_e [b. m. t. u]
	P80188	Neutrophil gelatinase-associated lipocalin [c. j. o. u]
	O15031	Plexin-B2 [b. g. o. s. u]
	Q8NHV4	Protein NEDD1 [b. g. n. u]
	K7EPI1	Queuine tRNA-ribosyltransferase (Fragment) [a. b. e. g. n. s. u]
	O43361	Zinc finger protein 749 [b. m. p. u]

pH 1_2 hours	**Accession name**	**Protein name and Classification**
	P54619	5'-AMP-activated protein kinase subunit gamma-1 [a. b. g. n. o. p. s. u]
	H0YDN9	ADP-ribosylation factor GTPase-activating protein 2 (Fragment) [m. t. u]
	Q9NVJ2	ADP-ribosylation factor-like protein 8B [a. b. c. g. n. m. s. w]
	C9JV77	Alpha-2-HS-glycoprotein [a. b. g. m. o. u]
	Q8WXJ9	Ankyrin repeat and SOCS box protein 17 [a. b. m. r. u]
	F8W696	Apolipoprotein A-I [a. c. g. o. u]
	F8WCU9	AT-rich interactive domain-containing protein 2 [b. m. p. s. u]
	Q8NAA4	Autophagy-related protein 16-2 [m. t. u]
	A6NG92	CCDC144A protein [b. m. t. x]
	Q00610	Clathrin heavy chain 1 [f. g. n. s. u. v. w]

A0A087WVQ6	Clathrin heavy chain [b. g. s. u]
A2RUR9	Coiled-coil domain-containing protein 144A [b. m. t. x]
Q3MJ40	Coiled-coil domain-containing protein 144B [b. m. t. u]
E9PLP0	Cysteine--tRNA ligase. cytoplasmic [b. c. g. n. u. w]
O60269	G protein-regulated inducer of neurite outgrowth 2 [b. g. t. u]
P19526	Galactoside 2-alpha-L-fucosyltransferase 1 [a. b. g. r. u]
E5RJS3	Gamma-aminobutyric acid receptor subunit alpha-1 (Fragment) [a. c. g. s. u]
P07098	Gastric triacylglycerol lipase [a. b. g. n. r. u]
Q14789	Golgin subfamily B member 1 [d. m. s. u]
Q8IZP7	Heparan-sulfate 6-O-sulfotransferase 3 [a. b. g. s. u]
P01777	Ig heavy chain V-III region TEI [b. e. j. o. x]
A0A0G2JMZ3	Leukocyte immunoglobulin-like receptor subfamily B member 3 [f. l. j. s. u. w]
C9JPF8	MAP6 domain containing 1. isoform CRA_b [d. m. n. x]
Q9H9H5	MAP6 domain-containing protein 1 [b. d. g. n. u]
I3L170	Microtubule-associated protein [m. n. x]
P10636	Microtubule-associated protein tau [f.g.n.u.w]
Q9Y618	Nuclear receptor corepressor 2 [a.g.p.u]
Q6ZVD8	PH domain leucine-rich repeat-containing protein phosphatase 2 [b. m. n. p. u]

Q9BUL5	PHD finger protein 23 [b. g. n. p. u. x]
P00558	Phosphoglycerate kinase 1 [a. b. g. h. n. o. s. u]
Q9HAU0	Pleckstrin homology domain-containing family A member 5 [b. g. n. s. u. x]
A0JP02	PLEKHA5 protein [b. g. n. p. s. u]
O00180	Potassium channel subfamily K member 1 [a. b. c. g. n. s. u. x]
H3BTH7	Protein ADGRG1 (Fragment) [f. m. s. u. x]
A0A087WW89	Protein IGHV3-72 [m. t .x]
Q5T7Y6	Protein S100 [b. l. t. u. v]
P23297	Protein S100-A1 [a. b. g. n. u. v. w]
Q9UPX0	Protein turtle homolog B [b. g. s. u]
Q8IYA2	Putative coiled-coil domain-containing protein 144C [b. m. t. u]
H0Y9A8	Rho-related BTB domain-containing protein 3 (Fragment) [a. b. g. n. u]
O96013	Serine/threonine-protein kinase PAK 4 [f. h. n. u. w]
Q16650	T-box brain protein 1 [b. m. p. u. w]
Q5T1B5	Type I inositol 1.4.5-trisphosphate 5-phosphatase [b. g. m. o. s. u. w]
O94966	Ubiquitin carboxyl-terminal hydrolase 19 [b. g. n. u. w]
Q14157	Ubiquitin-associated protein 2-like [b. m. t. u. w]
P49459	Ubiquitin-conjugating enzyme E2 A [a. b. m. n. p. w]

Q8N7F7	Ubiquitin-like protein 4B [b. m. n. w]
A0A087WSY3	Uncharacterized protein [b. m. n. w]
Q9UM54	Unconventional myosin-VI [a. b. d. g. n. p. s. u]
P08670	Vimentin [b. j. n. u. w]
Q14584	Zinc finger protein 266 [b.m.p.u]
Q969W8	Zinc finger protein 566 [b. m. p. u]

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.

Table 2. Classification and relative quantification of proteins identified in the acquired enamel pellicle collected after different times of formation (3 minutes or 2 hours) and treatments (Water, HCl pH 2 or HCl pH 1).

Accession number	Protein name	Ratio Water-2h / Water-3min	*P*
P63261	Actin. cytoplasmic 2 [a, d, g, j, n, q, u, w]	2.70	1.00
P59666	Neutrophil defensin 3 [b, i, j, o, u]	2.70	1.00
P60709	Actin. cytoplasmic 1 [b, m, n, q, u, w]	2.63	1.00
P59665	Neutrophil defensin 1 [b, i, j, o, u]	1.85	0.99
P01037	Cystatin-SN [a, b, g, o, u]	0.77	0.03
Accession number	**Protein name**	**Ratio pH2-2h/ pH2-3min**	***P***
P02768	Serum albumin [a, b, g, h, o, u, w]	1.58	1.00
P02808	Statherin [b, I, o, u, v]	0.79	0.00
P01877	Ig alpha-2 chain C region [b, e, I, j, o, u]	0.45	0.00
P01876	Ig alpha-1 chain C region [b, e, I, j, o, u]	0.44	0.00
Accession number	**Protein name**	**Ratio pH1-2h/ pH1-3min**	***P***
P02768	Serum albumin [a, b, g, h, o, u, w]	2.44	1.00
P02808	Statherin [b, I, o, u, v]	2.32	1.00
P01876	Ig alpha-1 chain C region [b, e, I, j, o, u]	1.18	1.00
P01877	Ig alpha-2 chain C region [b, e, I, j, o, u]	1.16	0.99

Accession number	**Protein name**	**Ratio pH2-3min/ Water-3min**	***P***
P02768	Serum albumin [a, b, g, h, o, u, w]	0.56	0.00
P02808	Statherin [b, l, o, u, v]	0.48	0.00
Accession number	**Protein name**	**Ratio pH2-2h/Water-2h**	***P***
P05109	Protein S100-A8 [b, i, j, n, o, p, q, u, w]	1.20	0.98
P63261	Actin. cytoplasmic 2 [a, d, g, j, n, q, u, w]	1.09	0.96
P05164	Myeloperoxidase [a, b, g, j, , r, u]	0.75	0.00
P02788	Lactotransferrin [b, c, i, j, n, o, p, u, w]	0.74	0.00
P15515	Histatin-1 [b, l, l, o, u]	0.65	0.00
P59666	Neutrophil defensin 3 [b, i, j, o, u]	0.58	0.00
P01037	Cystatin-SN [a, b, g, o, u]	0.54	0.02
P59665	Neutrophil defensin 1 [b, i, j, o, u]	0.54	0.00
P01877	Ig alpha-2 chain C region [b, e, l, j, o, u]	0.51	0.00
P01876	Ig alpha-1 chain C region [b, e, l, j, o, u]	0.48	0.00
P02808	Statherin [b, l, o, u, v]	0.38	0.00
P61626	Lysozyme C [a, b, i, o, u]	0.30	0.00
Accession number	**Protein name**	**Ratio pH1-3min/Water-3min**	***P***
P02808	Statherin [b, l, o, u, v]	0.44	0.00

P02768	Serum albumin [a, b, g, h, o, u, w]	0.36	0.00

Accession number	**Protein name**	**Ratio pH1-2h/Water-2h**	***P***
P02768	Serum albumin [a, b, g, h, o, u, w]	2.1	1.0
C9JKR2	Albumin. isoform CRA_k [c, m, o, u]	2.0	1.0
P02787	Serotransferrin [e, d, h, i, m, o, u, w]	2.0	1.0
P02538	Keratin. type II cytoskeletal 6A [d, h, o, p, s, u]	1.8	1.0
P48668	Keratin. type II cytoskeletal 6C [d, m, o, u]	1.8	1.0
P04259	Keratin. type II cytoskeletal 6B [d, m, o, u]	1.7	1.0
P01876	Ig alpha-1 chain C region [b, e, I, j, o, u]	1.3	1.0
P01877	Ig alpha-2 chain C region [b, e, I, j, o, u]	1.3	1.0
P62736	Actin aortic smooth muscle [b, m, n, q, u, w]	0.88	0.04
P63261	Actin. cytoplasmic 2 [a, d, g, j, n, q, u, w]	0.86	0.00
P68032	Actin. alpha cardiac muscle 1 [b, m, n, q, u, w]	0.85	0.02
P60709	Actin. cytoplasmic 1 [b, m, n, q, u, w]	0.85	0.00
P0CG38	POTE ankyrin domain family member I [b, m, o, u]	0.84	0.04
P63267	Actin. gamma-enteric smooth muscle [b, m, n, q, u, w]	0.84	0.02
P68133	Actin. alpha skeletal muscle [b, m, n, q, u, w]	0.84	0.02
Q6S8J3	POTE ankyrin domain family member E [b, m, o, u]	0.84	0.01

A5A3E0	POTE ankyrin domain family member F [b, m, o, u]	0.84	0.01
P02788	Lactotransferrin [b, c, i, j, n, o, p, u, w]	0.64	0.00
P59665	Neutrophil defensin 1 [b, i, j, o, u]	0.51	0.00
P59666	Neutrophil defensin 3 [b, i, j, o, u]	0.49	0.00
P61626	Lysozyme C [a, b, i, o, u]	0.42	0.00
Accession number	**Protein name**	**Ratio pH1-3min /pH2-3min**	***P***
P02768	Serum albumin [a, b, g, h, o, u, w]	1.43	0.97
Accession number	**Protein name**	**Ratio pH1-2h/ pH2-2h**	***P***
P01876	Ig alpha-1 chain C region [b, e, I, j, o, u]	2.78	1.00
P02808	Statherin [b, I, o, u, v]	2.78	1.00
P01877	Ig alpha-2 chain C region [b, e, I, j, o, u]	2.56	1.00
P02768	Serum albumin [a, b, g, h, o, u, w]	2.17	1.00
C9JKR2	Albumin. isoform CRA_k [c, m, o, u	2.08	1.00
P01037	Cystatin-SN [a, b, g, o, u]	2.08	1.00
P04259	Keratin. type II cytoskeletal 6B [d, m, o, u]	1.75	1.00
P48668	Keratin. type II cytoskeletal 6C [d, m, o, u]	1.75	1.00
P02538	Keratin. type II cytoskeletal 6A [d, h, o, p, s, u]	1.72	1.00
P61626	Lysozyme C [a, b, i, o u]	1.43	1.00

P59666	Neutrophil defensin 3 [b, i, j, o, u]	0.85	0.01
P63267	Actin. gamma-enteric smooth muscle [b, m, n, q, u, w]	0.83	0.02
A5A3E0	POTE ankyrin domain family member F [b, m, o, u]	0.83	0.00
P62736	Actin. aortic smooth muscle [b, m, n, q, u, w]	0.82	0.00
P68133	Actin. alpha skeletal muscle [b, m, n, q, u, w]	0.82	0.00
Q6S8J3	POTE ankyrin domain family member E [b, m, o, u]	0.82	0.00
P68032	Actin. alpha cardiac muscle 1 [b, m, n, q, u, w]	0.81	0.02
P0CG38	POTE ankyrin domain family member I [b, m, o, u]	0.80	0.01
P60709	Actin. cytoplasmic 1 [b, m, n, q, u, w]	0.79	0.00
P63261	Actin. cytoplasmic 2 [a, d, g, j, n, q, u, w]	0.79	0.00
P05109	Protein S100-A8 [b, i, j, n, o, p, q, u, w]	0.68	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.

Table S1. Classification of the identified proteins in the acquired enamel pellicle collected after different times of formation and treatments 3 minutes ou 2 hours, water or hydrochloric acid pH 1 or hydrochloric acid pH 2.

Accession	Description	Water 3 min	Water 2 Hours	pH 2 / 3min	pH 2 / 2 Hours	pH 1 / 2 Hours	pH 1/ 3min
P54619	5'-AMP-activated protein kinase subunit gamma-1 [a,b,g,n,o,p,s,u]					Yes	
Q8TE56	A disintegrin and metalloproteinase with thrombospondin motifs 17 [b,m,o,u]				Yes		
P22303	Acetylcholinesterase [a,e,g,o,s,u,n]		Yes				
P68032	Actin, alpha cardiac muscle 1 [b,m,n,q,u,w]		Yes		Yes	Yes	
P68133	Actin, alpha skeletal muscle [b,m,n,q,u,w]		Yes		Yes	Yes	
P62736	Actin, aortic smooth muscle [b,m,n,q,u,w]		Yes		Yes	Yes	
P60709	Actin, cytoplasmic 1 [b,m,n,q,u,w]	Yes	Yes		Yes	Yes	
P63261	Actin, cytoplasmic 2 [a,d,g,j,n,q,u,w]	Yes	Yes		Yes	Yes	
P63267	Actin, gamma-enteric smooth muscle [b,m,n,q,u,w]		Yes		Yes	Yes	
H0YDN9	ADP-ribosylation factor GTPase-activating protein 2 (Fragment) [m,t,x]					Yes	
Q9NVJ2	ADP-ribosylation factor-like protein 8B [a,b,c,g,n,m,s,w]					Yes	
C9JKR2	Albumin, isoform CRA_k [c,m,o,u]	Yes	Yes		Yes	Yes	
C9JV77	Alpha-2-HS-glycoprotein [a,b,g,m,o,u]					Yes	
Q5T085	Alpha-amylase (Fragment) [a,g,t,u]				Yes		
P04745	Alpha-amylase 1[a,g,o,u]				Yes		

P19961	Alpha-amylase 2B [a,g,o,u]			Yes	
Q8WXJ9	Ankyrin repeat and SOCS box protein 17 [a,b,m,r,u]				Yes
Q6NXT1	Ankyrin repeat domain-containing protein 54 [a,b,c,e,g,n,p,u]			Yes	
P04083	Annexin A1 [a,b,g,j,n,o,p,s,u,w]			Yes	Yes
P03973	Antileukoproteinase [a,g,i,j,o,u]		Yes		
F8W696	Apolipoprotein A-I [a,c,g,o,u]				Yes
G5E9V7	Armadillo repeat containing 8, isoform CRA_d [m,t,u]			Yes	
G5E9V6	Armadillo repeat containing 8, isoform CRA_e [b,m,t,u]			Yes	
Q8IUR7	Armadillo repeat-containing protein 8 [f,m,t,u]			Yes	
F8WCU9	AT-rich interactive domain-containing protein 2 [b,m,p,s,u]				Yes
Q8NAA4	Autophagy-related protein 16-2 [m,t,u]				Yes
Q9H4G0	Band 4.1-like protein 1 [a,b,g,p,u]			Yes	
Q562R1	Beta-actin-like protein 2 [b,m,n,u,w]		Yes		Yes
A6NG92	CCDC144A protein [b,m,t,x]				Yes
Q9NXV6	CDKN2A-interacting protein [a,b,g,m,p,u,w]			Yes	
O75419	Cell division control protein 45 homolog [a,b,g,n,p,u,w]	Yes			
A0A087WVQ6	Clathrin heavy chain [b, m, n, q, u, w]				Yes

Q00610	Clathrin heavy chain 1 [f,g,n,s,u,v,w]					Yes
A2RUR9	Coiled-coil domain-containing protein 144A [b,m,t,x]					Yes
Q3MJ40	Coiled-coil domain-containing protein 144B [b,m,t,u]					Yes
P18847	Cyclic AMP-dependent transcription factor ATF-3 [a,b,e,g,h,p,u]				Yes	
P01037	Cystatin-SN [a,b,g,o,u]	Yes	Yes		Yes	Yes
E9PLP0	Cysteine--tRNA ligase, cytoplasmic [b,c,g,n,u,w]					Yes
E9PDJ4	Dedicator of cytokinesis protein 8 [f,m,s,u,w]				Yes	
Q8IYM9	E3 ubiquitin-protein ligase TRIM22 [a,f,g,i,n,p,u,w]				Yes	
F6RJU0	Enoyl-CoA hydratase domain-containing protein 2, mitochondrial [a,b,m,t,x]				Yes	
Q9UBC2	Epidermal growth factor receptor substrate 15-like 1 [b,c,g,p,s,u,v]			Yes		
Q9NQT5	Exosome complex component RRP40 [b,g,n,p,u,w]				Yes	
Q6P050	F-box and leucine-rich protein 22 [a,m,n,u]				Yes	
Q6PIW4	Fidgetin-like protein 1 [a,b,g,h,p,u,w]		Yes			
P15408	Fos-related antigen 2 [b,g,p,u,x]		Yes			Yes
O60269	G protein-regulated inducer of neurite outgrowth 2 [b,g,t,u]					Yes
P19526	Galactoside 2-alpha-L-fucosyltransferase 1 [a,b,g,r,u]					Yes
E5RJS3	Gamma-aminobutyric acid receptor subunit alpha-1 (Fragment) [a,c,g,s,u]					Yes
F8WF38	Gamma-aminobutyric acid type B receptor subunit 1 [a,b,g,h,s,u,w]				Yes	

P07098	Gastric triacylglycerol lipase [a,b,g,n,r,u]				Yes
Q9H4A5	Golgi phosphoprotein 3-like [a,b,c,g,n,u]			Yes	
Q14789	Golgin subfamily B member 1 [d,m,s,u]				Yes
P16520	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3 [a,b,g,n,o,s,u]			Yes	
P00738	Haptoglobin [a,b,g,i,j,o,u,w]	Yes			Yes
P04792	Heat shock protein beta-1 [b,d,m,n,p,q,u,w]	Yes	Yes		
Q8IZP7	Heparan-sulfate 6-O-sulfotransferase 3 [a,b,g,p,u]				Yes
P15515	Histatin-1 [b,i,l,o,u]		Yes	Yes	
H7BXF5	Histone deacetylase complex subunit SAP130 [a,b,g,p,u]		Yes		
U3KQK0	Histone H2B [b,m,p,u,w]			Yes	
P33778	Histone H2B type 1-B [b,m,p,u,w]			Yes	
P62807	Histone H2B type 1-C/E/F/G/I [b,i,j,p,u,w]			Yes	
P58876	Histone H2B type 1-D [b,m,p,u,w]			Yes	
Q93079	Histone H2B type 1-H [b,i,j,p,u,w]			Yes	
P06899	Histone H2B type 1-J [b,i,j,p,u,w]			Yes	
O60814	Histone H2B type 1-K [b,I,j,p,u,w]			Yes	
Q99880	Histone H2B type 1-L [b,m,p,u,w]			Yes	
Q99879	Histone H2B type 1-M [b,m,p,u]			Yes	

Q99877	Histone H2B type 1-N [b,I,j,p,u,w]				Yes		
P23527	Histone H2B type 1-O [b,I,j,p,u,w]				Yes		
Q16778	Histone H2B type 2-E [b,i,j,p,u,w]				Yes		
Q5QNW6	Histone H2B type 2-F [b,m,p,u,w]				Yes		
Q8N257	Histone H2B type 3-B [b,m,p,u,w]				Yes		
P57053	Histone H2B type F-S [b,I,j,p,u,w]				Yes		
P31276	Homeobox protein Hox-C13 [b,e,g,p,u]		Yes				
P01876	Ig alpha-1 chain C region [b,e,I,j,o,u]	Yes	Yes	Yes	Yes	Yes	Yes
P01877	Ig alpha-2 chain C region [b,e,I,j,o,u]	Yes	Yes	Yes	Yes	Yes	Yes
P01859	Ig gamma-2 chain C region [b,e,j,o,u,w]				Yes		
P01860	Ig gamma-3 chain C region [b,e,j,o,u,w]				Yes		
P01861	Ig gamma-4 chain C region [b,e,j,o,u,w]				Yes		
P01766	Ig heavy chain V-III region BRO [b,j,o,x]		Yes			Yes	
P01777	Ig heavy chain V-III region TEI [b,e,j,o,x]					Yes	
Q5TA45	Integrator complex subunit 11 [b,e,g,n,p,u]		Yes				
O43187	Interleukin-1 receptor-associated kinase-like 2 [b,g,j,n,p,s,u]	Yes					
Q5JVA3	Interleukin-15 receptor subunit alpha (Fragment) [b,e,h,j,p,u]		Yes				
Q9NZM3	Intersectin-2 [a,b,g,l,n,u,w]				Yes		

P13646	Keratin, type I cytoskeletal 13[d,m,o,p,q,u]			Yes	
P13647	Keratin, type II cytoskeletal 5 [d,h,o,p,s,w]	Yes	Yes		
F8W0C6	Keratin, type II cytoskeletal 5 (Fragment) [d,h,o,p,s,w]		Yes		
P02538	Keratin, type II cytoskeletal 6A [ad,h,o,p,s,u]		Yes	Yes	Yes
P04259	Keratin, type II cytoskeletal 6B [d,m,o,u]		Yes	Yes	Yes
P48668	Keratin, type II cytoskeletal 6C [d,m,o,u]		Yes	Yes	Yes
Q9NSK0	Kinesin light chain 4 [a,c,g,n,u]		Yes		
Q8WV93	Lactation elevated protein 1 [a,g,n,u]		Yes		
P02788	Lactotransferrin [b,c,i,j,n,o,p,u,w]		Yes	Yes	Yes
Q92615	La-related protein 4B [b,g,n,u]			Yes	
A0A0G2JMZ3	Leukocyte immunoglobulin-like receptor subfamily B member 3 [f,i,j,s,u,w]				Yes
P61626	Lysozyme C [a,b,i,o,u]		Yes	Yes	Yes
C9JPF8	MAP6 domain containing 1, isoform CRA_b [d,m,n,x]				Yes
Q9H9H5	MAP6 domain-containing protein 1 [b,d,g,n,u]				Yes
P08493	Matrix Gla protein [b,m,o,u]	Yes	Yes		
I3L170	Microtubule-associated protein [m,n,x]				Yes
P10636	Microtubule-associated protein tau [f,g,n,u,w]				Yes

C9JIR0	Mitotic spindle assembly checkpoint protein MAD1 (Fragment) [a,b,g,n,p,u,x]			Yes	
Q13485	Mothers against decapentaplegic homolog 4 [f,g,h,j,n,p,u,w]	Yes			
O43148	mRNA cap guanine-N7 methyltransferase [a,e,g,p,u]		Yes		
I3L0M3	MYC-associated zinc finger protein (Purine-binding transcription factor), isoform CRA_e [b,m,t,u]			Yes	
P05164	Myeloperoxidase [a,b,g,j,r,u]		Yes	Yes	Yes
P59665	Neutrophil defensin 1 [b,i,j,o,u]	Yes	Yes	Yes	Yes
P59666	Neutrophil defensin 3 [b,i,j,o,u]	Yes	Yes	Yes	Yes
P80188	Neutrophil gelatinase-associated lipocalin [c,j,o,u]			Yes	
Q9Y618	Nuclear receptor corepressor 2 [a,g,p,u]				Yes
C9J5S7	Peptidyl-prolyl cis-trans isomerase [a,b,e,j,n,o]		Yes	Yes	
P62937	Peptidyl-prolyl cis-trans isomerase A [b,d,m,n,o,u,w]		Yes	Yes	
Q6ZVD8	PH domain leucine-rich repeat-containing protein phosphatase 2 [b,m,n,p,u]				Yes
Q9BUL5	PHD finger protein 23 [b,g,n,p,u,x]				Yes
P00558	Phosphoglycerate kinase 1 [a,b,g,h,n,o,s,u]				Yes
Q9HAU0	Pleckstrin homology domain-containing family A member 5 [b,g,n,s,u,x]				Yes
A0JP02	PLEKHA5 protein [b,g,o,s,u]				Yes
O15031	Plexin-B2 [b,g,o,s,u]			Yes	

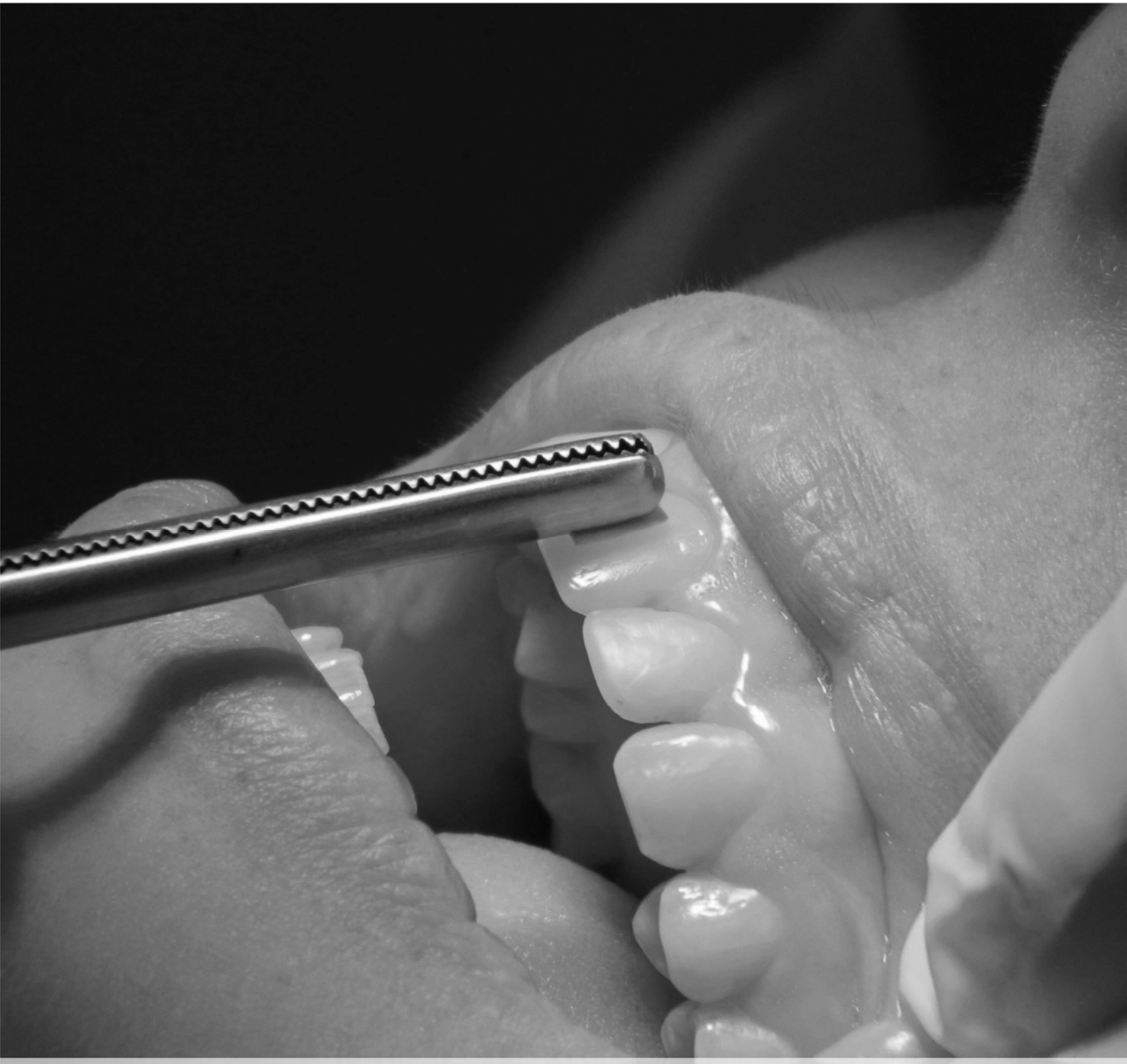
O00180	Potassium channel subfamily K member 1 [a,b,c,g,n,s,u,x,]				Yes
Q6S8J3	POTE ankyrin domain family member E [b,m,o,u]		Yes	Yes	Yes
A5A3E0	POTE ankyrin domain family member F [b,m,o,u]		Yes	Yes	Yes
P0CG38	POTE ankyrin domain family member I [b,m,o,u]		Yes	Yes	Yes
H3BTH7	Protein ADGRG1 (Fragment) [f,m,s,u,x]				Yes
D6RGX4	Protein FAM90A26 [b,m,t,u]	Yes			
A0A087WW89	Protein IGHV3-72 [m,t,x]				Yes
S4R460	Protein IGHV3OR16-9 [b,j,o,w]		Yes		Yes
Q8NHV4	Protein NEDD1 [b,g,n,u]			Yes	
Q5T7Y6	Protein S100 [b,l,t,u,v]				Yes
P23297	Protein S100-A1 [a,b,g,n,u,v,w]				Yes
P05109	Protein S100-A8 [b,i,j,n,o,p,q,u,w]		Yes	Yes	Yes
Q9UPX0	Protein turtle homolog B [b,g,s,u]				Yes
Q9BYX7	Putative beta-actin-like protein 3 [b,g,s,u]		Yes	Yes	Yes
Q8IYA2	Putative coiled-coil domain-containing protein 144C [b,m,t,u]				Yes
Q9P1P4	Putative trace amine-associated receptor 3 [b,g,m,s,x]	Yes			
K7EPI1	Queuine tRNA-ribosyltransferase (Fragment) [a,g,n,p,u]			Yes	

E9PK18	Rho GTPase-activating protein 27 [a,b,e,g,n,s,u]		Yes				
H0Y9A8	Rho-related BTB domain-containing protein 3 (Fragment) [a,b,e,g,n,u]					Yes	
F8WDV7	RING finger protein 121 [b,c,m,s,u,w]	Yes					
Q15633	RISC-loading complex subunit TARBP2 [f,g,n,p,u,w]		Yes				
O96013	Serine/threonine-protein kinase PAK 4 [f,h,n,u,w]					Yes	
Q9C0I3	Serine-rich coiled-coil domain-containing protein 1 [b,m,t,u]		Yes				
P02787	Serotransferrin [e,d,h,i,m,o,u,w]	Yes	Yes			Yes	
P02768	Serum albumin [a,b,g,h,o,u,w]	Yes	Yes	Yes	Yes	Yes	Yes
I3L0M1	Sex hormone-binding globulin [b,m,o,u,w]		Yes				
Q13126	S-methyl-5'-thioadenosine phosphorylase [a,b,g,n,p,u,w]	Yes					
O76082	Solute carrier family 22 member 5 [f,g,s,u,w]		Yes				
A6NLX3	Speedy protein E4 [b,g,t,w]	Yes					
P02808	Statherin [b,i,o,u,v]	Yes	Yes	Yes	Yes	Yes	Yes
Q96NU1	Sterile alpha motif domain-containing protein 11 [e,m,p,u]	Yes					
P02814	Submaxillary gland androgen-regulated protein 3B [m,o,w]	Yes	Yes				
B8ZZF7	Sulfotransferase [b, m, t, x]		Yes				
O00338	Sulfotransferase 1C2 [a,b,g,n,u,w]		Yes				
P60508	Syncytin-2 [b,m,s,u,w]		Yes				

Q7Z7G0	Target of Nesh-SH3 (b,d,m,o,u,w)	Yes		
Q16650	T-box brain protein 1 (b,m,p,u,w)			Yes
Q12799	T-complex protein 10A homolog (b,m,n,u)		Yes	
Q9NNW7	Thioredoxin reductase 2, mitochondrial (a,b,g,n,u,w)	Yes		
X6REB3	Transcription factor E2-alpha (f,g,p,u,w)		Yes	
P07437	Tubulin beta chain (b,d,m,n,g,u,w)		Yes	
Q13885	Tubulin beta-2A chain (b,d,m,n,q,u,w)		Yes	
Q9BVA1	Tubulin beta-2B chain (b,d,m,n,q,u,w)		Yes	
P04350	Tubulin beta-4A chain (b,d,m,n,q,u,w)		Yes	
P68371	Tubulin beta-4B chain (b,d,m,n,q,u,w)		Yes	
Q5T1B5	Type I inositol 1,4,5-trisphosphate 5-phosphatase (b,g,m,o,s,u,w)			Yes
O94966	Ubiquitin carboxyl-terminal hydrolase 19 (b,g,n,u,w)			Yes
Q14157	Ubiquitin-associated protein 2-like (b,m,t,u,w)			Yes
P49459	Ubiquitin-conjugating enzyme E2 A (a,b,m,t,u,w)			Yes
Q8N7F7	Ubiquitin-like protein 4B (b,m,n,w)			Yes
F2Z2F3	Uncharacterized protein (b,m,n,w)	Yes		
A0A087WSY3	Uncharacterized protein (b,m,t,x)			Yes

Q96KH6	Uncharacterized protein C18orf12 [b,m,t,w]		Yes		
Q9UM54	Unconventional myosin-VI [a,b,d,g,n,p,s,u]				Yes
P08670	Vimentin [b,j,n,u,w]				Yes
F8W0F8	Voltage-dependent L-type calcium channel subunit beta-3 [f,g,n,s,v,u]		Yes		
Q14584	Zinc finger protein 266 [b,m,p,u]				Yes
Q969W8	Zinc finger protein 566 [b,m,p,u]				Yes
O43361	Zinc finger protein 749 [b,m,p,u]			Yes	
P25311	Zinc-alpha-2-glycoprotein [a,b,g,o,u,w]	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.



3-Discussion

3 DISCUSSION

We employed a different protocol according to a recently developed methodology to extract and prepare the acquired enamel pellicle proteins for analyses, and this protocol was choose, since that increased the number of identified proteins, and this fact was verify in another study (VENTURA et al., 2017).

The main objective of this study was, collect Acquired pellicles and identify proteins that are not removal after challenges with HCl. Hydrochloric acid was choose due to is an intrinsic acid, with a low pH, the HCl reaches the oral cavity through regurgitation, vomiting, bulimia, anorexia nervosa and gatroesophageal reflux (MOAZZEZ; BARTLETT, 2014). Therefore, the lesions of dental erosion have been commonly found in patients with these diseases (MEURMAN et al., 1994). With all this knowledge, the identification of these acids-resistant proteins is very important, since they have a great potential to protect the teeth against intrinsic erosion.

Since it has been reported, no significantly differences in the protector potential against demineralization by acids, in pellicles formed in three minutes or 120 minutes formation (HANNIG, M. et al., 2004) the second aim of this present study was evaluate changes in the protein profile of the AEP along its time of formation, under normal conditions and after acids challenges with HCl.

In the identify, most of the proteins were found in the groups with 2 hours of formation, so we can conclude that the number of proteins increase along the time of formation, which is expected and has been reported in a previous study (LEE et al., 2013). Analyzing the results of this study, an interesting finding was that only quite few number of proteins were find in the groups with 3 minutes of formation after exposure to HCl, which is *Serum albumin, Statherin and immunoglobulins*, and it is possible that these proteins are responsible for most of the protection conferred by the short-term pellicle.

There is some proteins that are only identify in the groups of 2 hours of formation, like *Myeloperoxidase, Lysozyme C, Protein S-100-A8, Lactotransferrin* and isoforms of keratin, this finding instigate that these proteins probably do not bind to hydroxyapatite, but bind to another proteins, like the precursor proteins.

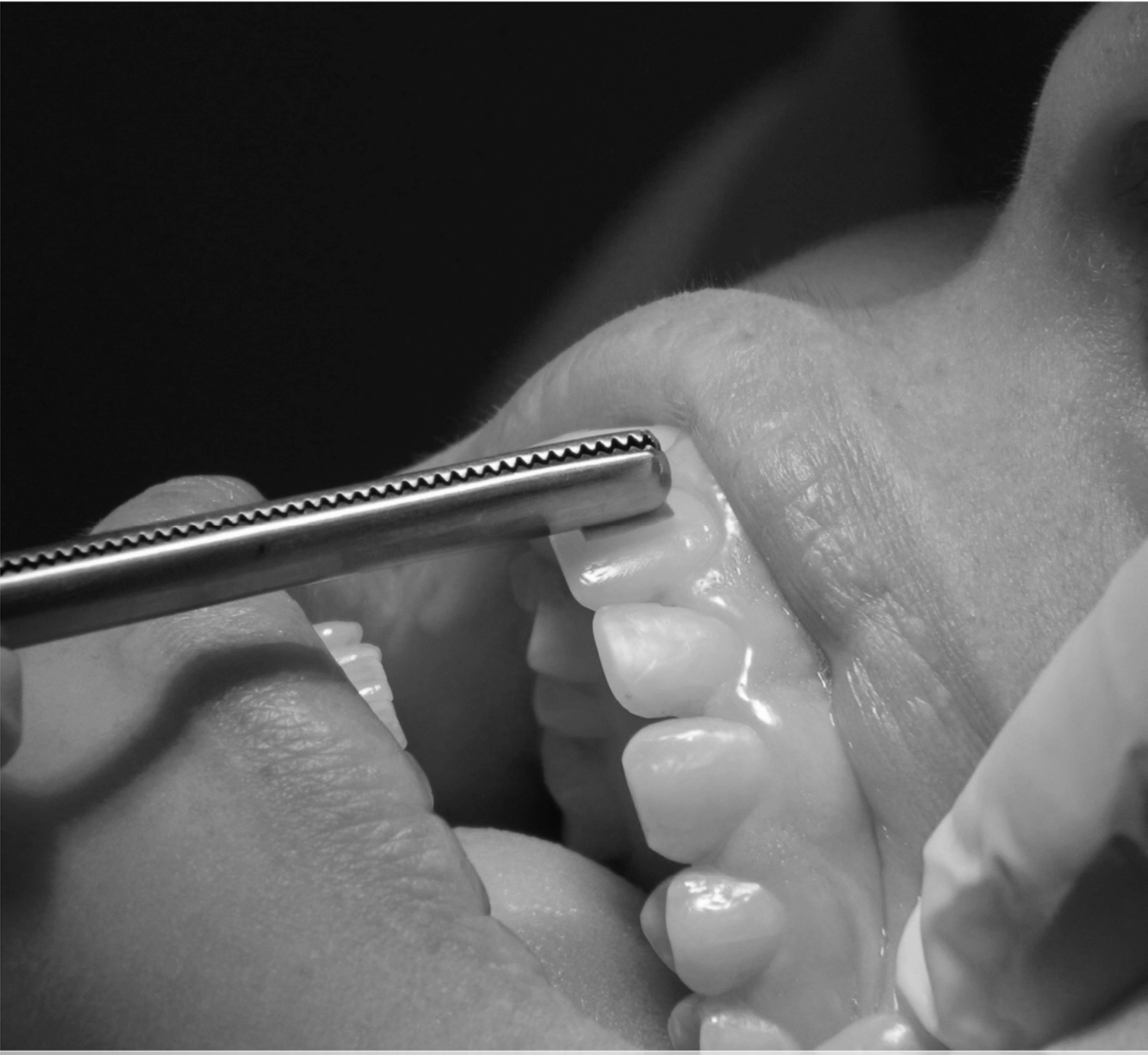
This protocol allowed the identification of 180 proteins. From these four proteins are important and should be detach, because this four were in all the groups, even in the short time of formation, regardless of the treatment received. *Serum albumin* is one of this proteins they are present in all groups, this protein originary from plasma and its capable to bind ions as calcium (HEMINGWAY et al., 2008)(HEMINGWAY et al., 2008). This fact can be important for protection against acids, since that ovalbumin was verify in another study and was able to reduce the dissolution of hydroxyapatite by acids challenges in vitro (HEMINGWAY et al., 2008; KOSORIC; HECTOR; ANDERSON, 2010). Another protein is *Statherin*, a 43-residue phosphorylated protein typical found in saliva, with primary sequence likeness to osteopontin and casein that was able to bind hydroxyapatite and calcium. For the interaction with hydroxyapatite are important the helical conformation at the N-terminus and the negative charge (RAJ et al., 1992). This interaction apparent to be powerful, since not even exposed to HCl (0,1 M) was able to separate and detach this protein from the teeth surface. In fact solid-state nuclear magnetic resonance (NMR) studies confirmed that the N-terminus of *Statherin* strongly binds to hydroxyapatite, while the middle and C-terminal regions are dynamic and flexible (NAGANAGOWDA; GURURAJA; LEVINE). In order to protect against loss mineral teeth, it is prompting that statherin-like peptides containing at least 15 N-terminal residues or more, are needed (SHAH et al., 2011). It was recently found in studies that concentration of calcium from acquired pellicle collected in patients with dental erosion it is 50% reduced, while the concentration of *Statherin* is 35% reduced, showing that erosion and this protein is related (CARPENTER et al., 2014). These results suggested that these 2 proteins are resistant to removal by an intrinsic acid, and potential candidates to be more studied, and included in products to aim helps patients with dental erosion.

About the time formation of AEP, it should be emphasize that possible protective proteins, such as Serum albumin increased along time of formation, even after application of HCl (both for HCl pH1 and HCl pH 2) and this also happens to Statherin (for HCl pH 1). As mentioned above, one study did not find any difference in the protection of AEP formed in 3 minutes or 120 minutes. It must be taken into account this study was designed in situ, while the present study was conducted in vivo, and the results obtained here are more close to reality, than those described in studies in vitro (MASSON et al., 2013; SIQUEIRA et al., 2012) or in situ (DELECRODE et al., 2015) protocols. Another different point it is, an in situ study was employed orange juice as

erosive solution, mimicking intrinsic erosions. For this study the acid had the lower pH (HCl pH 1.0 or 2.0), compared to the study with orange juice (pH 2.5. It is possible that for the AEP exposure to the intrinsic acid, time of maturation is important for the protection against lost mineral by intrinsic desmineralization. In agreement of this assumption, an in situ study showed that contact to the dental surface to saliva for 30 minutes for AEP formation, provided less enamel resistance to HCl erosive attack than the contact to saliva for 2 hours (MENDONCA et al., 2017). Proposing that more mature pellicles might promoted better protection against erosion by intrinsic acid, than short-term pellicle.

Prepossessing, a great number of proteins was identify, like uniquely in one of the groups, for the groups exposed by HCl after 2 hours of formation. (approximately 50 proteins for each of these groups). Suggesting that many proteins are resistant to HCl, and remain, even at low pH.

Concluding, the present study identify *Serum albumin* and *Statherin* as potentially protective protein and these proteins are resistant and remain after HCl exposure, even in the pellicles formed in short time (3min). Moreover, was observed that are many proteins in the mature pellicles, and these proteins are more resistant to removal by an intrinsic acid, even at pH 1. This point to there is an increased in the power of protection against intrinsic acids along the time of pellicle formation, which is necessary to gain further knowledge and be evaluated in future studies.



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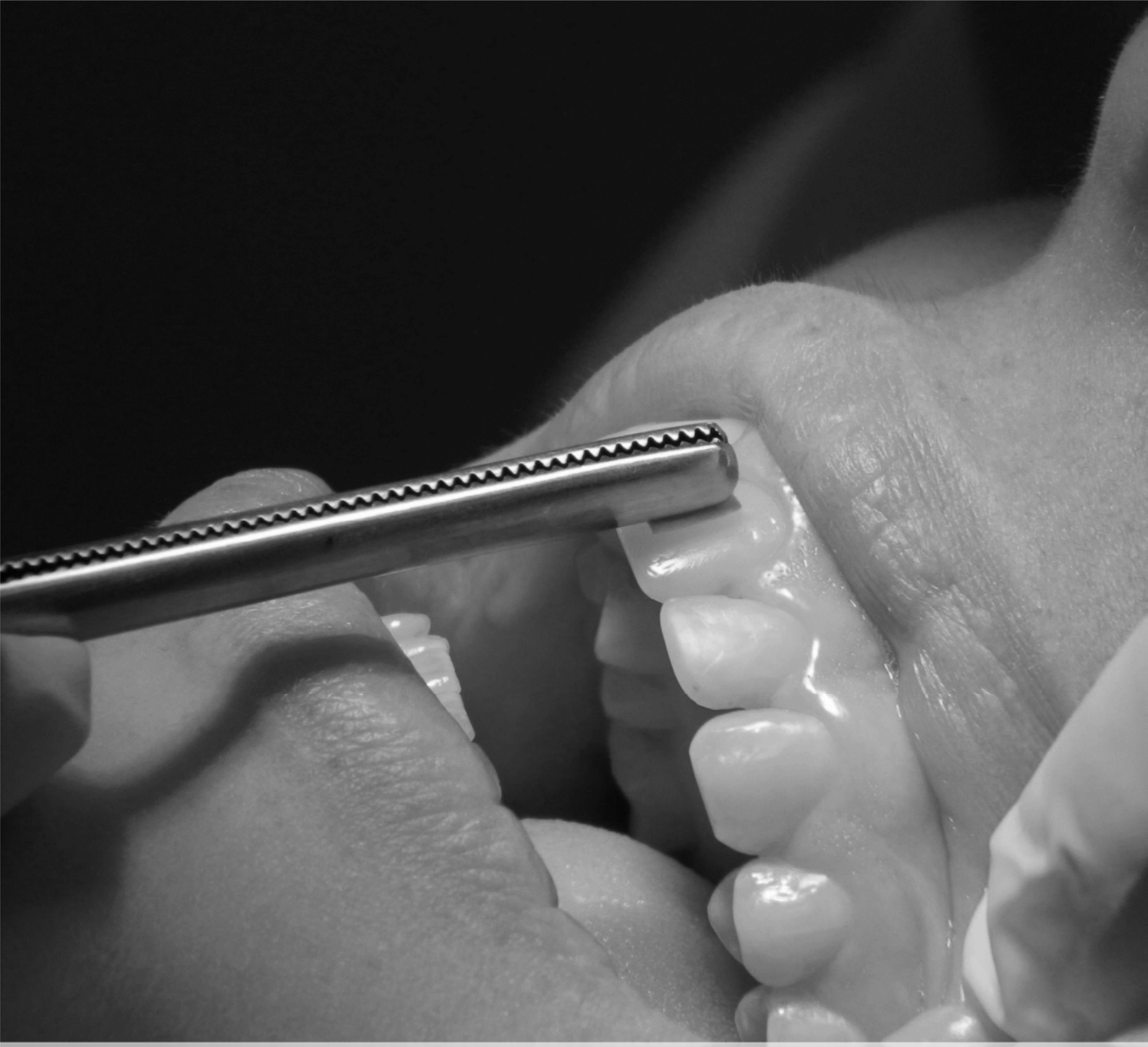

Annex

ANNEX 1

Universidade de São Paulo
Faculdade de Odontologia de Bauru
Departamento de Ciências Biológicas
Disciplina de Bioquímica

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Convido você estudante da pós graduação desta faculdade a participar como voluntário no trabalho experimental intitulado "Determinação de mudanças na composição da película adquirida do esmalte formada *in vivo* após exposição a ácido clorídrico: estudo proteômico." Você deverá comparecer ao laboratório de Bioquímica da Faculdade de Odontologia de Bauru – USP, no dia e horário que será estabelecido pelo pesquisador que entrará em contato com você no dia anterior de cada etapa da coleta de película adquirida, totalizando 12 dias que serão necessários a sua presença.

O objetivo deste trabalho será verificar se haverá alterações na película adquirida, após exposição ao ácido clorídrico, mimetizando pessoas que sofrem de doenças gastresofágicas como refluxos, bulimia etc. Será também verificado, quais das proteínas presentes na película adquirida, teve resistência a tal exposição ao ácido e se futuramente ela poderá exercer papel protetor aos dentes contra processos erosivos.

A película adquirida é uma fina camada transparente formada sobre os dentes assim que eles começam a aparecer na boca e entram em contato com a saliva. É sobre essa camada que se forma a placa dentária, ou seja, os restos de alimentos se grudam.

Assim, se o(a) Sr.(a) concordar a participar do projeto, para sua participação você terá que vir a Faculdade de Odontologia de Bauru – FOB para duas etapas, uma etapa será para a coleta de película após 3 minutos de formação e em outra etapa será para a coleta de película após 2 horas de formação, sendo que, para cada etapa, serão 6 dias, totalizando 12 dias consecutivos, tendo início sempre às 8 horas da manhã. Estas etapas serão realizadas em dias distintos. Você receberá uma meticulosa profilaxia dentária com pedra pomes, para que a película adquirida (camada de proteínas originárias da saliva que se ligam à superfície do dente) se forme naturalmente sobre o esmalte dentário. Depois de 3 minutos ou 2 horas após a formação da película adquirida, será aplicado 50μL de ácido Clorídrico (0,1 mol, pH 1), 50μL de ácido Clorídrico (0,01mol pH 2) ou água durante apenas 10 segundos (não causando nenhum dano ao esmalte do seu dente), dependendo da fase da pesquisa em que você estiver. A película será removida com um papel de filtro umedecido em ácido cítrico a 3%. Durante este período, você não poderá consumir alimentos ou bebidas. Todas as amostras coletadas serão depois de feita a análise, serão descartadas de forma adequada no laboratório de bioquímica da Faculdade de Odontologia - FOB.

Este projeto traz como benefícios á importância o conhecimento sobre quais proteínas da película adquirida do esmalte são resistentes à remoção por ácido clorídrico que irá nos permitir elaborar novas estratégias preventivas para proteção contra a erosão dentária intrínseca. Em estudos futuros poder-se-á empregar procedimentos de "engenharia" de película adquirida, levando à formação de uma película com composição tal, que possa proteger o tecido dentário contra desafios erosivos intrínsecos em pacientes susceptíveis.

Quanto aos benefícios oferecidos a você, no início do estudo será feito um exame clínico em relação às suas condições bucais e o resultado deste exame será prontamente informado a você. Caso seja detectado algum problema, faremos o encaminhamento à triagem da FOB, e você será atendido conforme agendamento. Além disto, no final do estudo, serão dadas instruções sobre higiene bucal, por escrito e verbalmente. A participação será voluntária e entende-se que você poderá fazer qualquer pergunta sobre os procedimentos, sendo que será livre para desistir de participar a qualquer momento, sem nenhum prejuízo de sua parte. Em adição, você terá, também, por parte dos pesquisadores, a garantia do sigilo que assegura a sua privacidade.

Destacamos ainda que não há risco nenhum à sua saúde com a participação nesta pesquisa, já que os tratamentos com o ácido serão de pequena duração assim sendo não ocorrerão prejuízo algum aos voluntários da pesquisa.

Os gastos que forem gerados por este trabalho ficarão a cargo da responsável pelo projeto. Importante ressaltar que não está sendo considerado nenhum pagamento ou recompensa material pela participação do sujeito neste estudo. Você terá garantido o direito à indenização compensatória caso fique comprovado que a sua participação acarretou algum problema a você.

Concordando em participar, você entende que este estudo será realizado em benefício das ciências médica e odontológica, e desta forma concorda com a divulgação dos dados obtidos por meio de publicações científicas. Esse termo de consentimento livre e esclarecido constará de duas vias, uma permanecerá com o pesquisador e outra será entregue a você.

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-901 – C.P. 73
e-mail: bioquimica@fob.usp.br– Fone/FAX (0xx14) **3235-8226/32234679**
http://www.fob.usp.br

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Caso queira apresentar reclamações em relação à sua participação na pesquisa, poderá entrar em contato com o Comitê de Ética em Pesquisa em Seres Humanos da FOB-USP, no endereço Al. Octávio Pinheiro Brisolla, 9-75 (sala no prédio da Biblioteca, FOB-USP) ou pelo telefone (14) 3235-8356. Para maiores esclarecimentos de duvidas soube a pesquisa você pode, a qualquer momento, contatar a pesquisadora (Even Akemi Taira) pelos telefones (14)3010-6317, (14) 98141-1707 ou pelo e-mail: even.taira@usp.br

Fica claro que o voluntário poderá, a qualquer momento, retirar seu CONSENTIMENTO LIVRE E ESCLARECIDO e deixar de participar do estudo alvo da pesquisa e ciente que todo trabalho realizado torna-se informação confidencial guardada por força do sigilo profissional (Art. 9º do Código de Ética Odontológica).

Pelo presente instrumento que atende às exigências legais, o Sr. (a) _____, portador da cédula de identidade _____ , após leitura minuciosa das informações constantes neste TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO, devidamente explicada pelos profissionais em seus mínimos detalhes, ciente dos serviços e procedimentos aos quais será submetido, não restando quaisquer dúvidas a respeito do lido e explicado, DECLARA e FIRMA seu CONSENTIMENTO LIVRE E ESCLARECIDO concordando em participar da pesquisa proposta. Fica claro que o sujeito da pesquisa, pode a qualquer momento retirar seu CONSENTIMENTO LIVRE E ESCLARECIDO e deixar de participar desta pesquisa e ciente de que todas as informações prestadas tornar-se-ão confidenciais e guardadas por força de sigilo profissional (Art. 9º do Código de Ética Odontológica).
Por fim, como pesquisador(a) responsável pela pesquisa, DECLARO o cumprimento do disposto na Resolução CNS nº 466 de 2012, contidos nos itens IV.3 e IV.4, este último se pertinente, item IV.5.a e na íntegra com a resolução CNS nº 466 de dezembro de 2012.

Por estarmos de acordo com o presente termo o firmamos em duas vias igualmente válidas (uma via para o sujeito da pesquisa e outra para o pesquisador) que serão rubricadas em todas as suas páginas e assinadas ao seu término, conforme o disposto pela Resolução CNS nº 466 de 2012, itens IV.3.f e IV.5.d.

Bauru, SP, _____ de _____ de _____.

_____ _____
Assinatura do Sujeito da Pesquisa Even Akemi Taira

O **Comitê de Ética em Pesquisa – CEP**, organizado e criado pela **FOB-USP**, em 29/06/98 (**Portaria GD/0698/FOB**), previsto no item VII da Resolução nº 466/12 do Conselho Nacional de Saúde do Ministério da Saúde (publicada no DOU de 13/06/2013), é um Colegiado interdisciplinar e independente, de relevância pública, de caráter consultivo, deliberativo e educativo, criado para defender os interesses dos participantes da pesquisa em sua integridade e dignidade e para contribuir no desenvolvimento da pesquisa dentro de padrões éticos.

Qualquer denúncia e/ou reclamação sobre sua participação na pesquisa poderá ser reportada a este CEP:
Horário e local de funcionamento:
Comitê de Ética em Pesquisa
Faculdade de Odontologia de Bauru-USP - Prédio da Pós-Graduação (bloco E - pavimento superior), de segunda à sexta-feira, no horário das **13h30 às 17 horas**, em dias úteis.
Alameda Dr. Octávio Pinheiro Brisolla, 9-75 Vila Universitária – Bauru – SP – CEP 17012-901
Telefone/FAX(14)3235-8356 e-mail: cep@fob.usp.br