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# Proteins from whole mouth saliva mediate greater protection against severe erosive tooth wear than proteins from parotid saliva using an in vitro model.

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## Abstract

**Objectives:** to investigate the protective properties of the acquired enamel pellicle (AEP) formed from whole mouth saliva (WMS) or parotid saliva (PS) against Erosive tooth wear (ETW) in an in-vitro model.

**Methods**: 60 enamel specimens were prepared from extracted human teeth and were randomly assigned to 4 experimental groups: WMS (n=20), PS (n=20), artificial saliva (AS, n=10) and deionised water (DW, n=10). AEP samples were eluted from WMS (n=5) and PS (n=5) groups after five cycles (acid, wash and saliva) using filter papers soaked in sodium dodecyl sulfate by mechanically rubbing before mean step height was evaluated using a non-contacting profilometer for all groups (n=10 each). Total protein in AEP was quantified using BCA assay, individual protein components of AEP were separated and analysed using SDS-PAGE and western blot for [mucin5b, albumin, carbonic anhydrase VI (CA VI), statherin].

Specific antibody binding was quantified using purified protein standards of known concentration. Samples of AEP were also analysed by LC/MS/MS sequencing.

**Results:** WMS group had significantly ($p<0.0001$) less acid-induced erosion (step height [4.16 (0.9) μm]) than PS group [6.41 (0.3) μm]. The amount of total protein, mucin5b and albumin were more dominant in WMS pellicles than PS ($p<0.0001$) whereas CA VI and statherin were dominant in PS pellicles ($p<0.0001$).

**Conclusion:** The composition of the AEP influences the degree of protection from acid attack, possibly by altering the mechanism of protection. AEP from WMS was more protective against ETW than that of PS and this protection may be attributed to mucin5b and albumin rather than to statherin and CA VI. This protection appears to be through preventing the protons diffusion to the crystal surface rather than neutralising acid or calcium homeostasis.

**Significance**: The salivary composition has an important effect on protection against in-vitro ETW and that in-vitro salivary proteins models of ETW differ from *in-vivo* studies. Therefore, it can be recommended that in-vitro laboratory models of ETW need to be assessed carefully to represent the clinical environment more closely.

**Key words**: Saliva, human enamel, pellicle, erosion, profilometer, step height, proteins, ions, SDS-PAGE

# 1. Introduction

Erosive tooth wear (ETW) is a term used recently to describe the wear of teeth primarily as a result of erosion but in combination with attrition and abrasion (Lussi and Carvalho, 2014). The structures of dental enamel can be insulted by ETW which is a dynamic process and is affected by a number of chemical, biological and behavioural factors (Lussi and Jaeggi, 2008, Bartlett, 2005). It is well documented that saliva and acquired enamel pellicle (AEP) are important biological factors that influence ETW (Amaechi et al., 1999, Zero and Lussi, 2005, Lussi and Jaeggi, 2008, Wetton et al., 2006, Wetton et al., 2007, Hellwig et al., 2013,

Vukosavljevic et al., 2014). AEP starts forming moments after brushing and some studies have shown an improvement of AEP protection at longer pre-treatment period between 24 h to several days (Hannig and Balz, 1999, Hannig et al., 2004, Amerongen et al., 1987). AEP serves many functions against ETW such as acting as a diffusion barrier that reduces direct contact between acids and the tooth surface (Carlen et al., 1998, Vukosavljevic et al., 2014), a neutraliser of protons (Lussi and Jaeggi, 2008, Sreebny, 2000, Buzalaf et al., 2012) and as a reservoir zone that is rich in minerals that help remineralise the demineralised tooth tissues (Proctor et al., 2005). Certain protein components of AEP such as statherin and proline-rich proteins adhere quickly and strongly with the enamel crystals (Hay, 1973, Zimmerman et al., 2013) due to their phosphate groups, which attract calcium and phosphate ions to the enamel surface (Kosoric et al., 2007). Other proteins such as mucins, amylase, albumin and CA VI build up later by the constant flow of saliva over tooth surfaces (Amerongen et al., 1987, Hannig et al., 2008) and serve many functions such as lubrication (mucins (Amerongen et al., 1987), diffusion barrier (albumin (Hemingway et al., 2008) and acid neutralisation (CA VI) (Proctor et al., 2005, Leinonen et al., 1999).

When studying AEP using in-vitro models, whole mouth saliva is collected by spitting or drooling whereas saliva from a single salivary gland such as parotid gland is collected using a Lashley suction cup. Mucin 7 and mucin 5b are salivary mucins secreted by submandibular and sublingual glands but are absent from parotid glands. PS is a serous fluid which consists predominantly of proteins such as proline-rich proteins (PRPs), histatins and statherin (Humphrey and Williamson, 2001).

Owing to the structural and compositional complexity of WMS as well as the instantaneous interaction between salivary proteins and minerals, it is difficult to determine the exact components of AEP that have a role in the protection of the enamel surface against in-vitro ETW. Artificial saliva (AS) can also be used in in-vitro ETW studies to mimic the role of inorganic components of natural saliva and currently found in a number of formulations (Gibson and Beeley, 1994, Amaechi et al., 1999).

Previous in-vivo studies have shown that statherin (Mutahar et al., 2017c) alongside serum albumin (Taira et al., 2018) are possibly responsible for most of the protection conferred by the AEP. There is not yet an accurate in-vitro ETW model that closely represents the clinical studies due to many differences between in-vitro and in-vivo AEP. These include the unique features of the oral environment such as the dynamics of salivary flow, enzymatic activities, thickness of the AEP, mineral surface properties and health and age of patients (Yao et al., 2001, Hannig and Hannig, 2009). In addition, in-vitro studies use ground and polished enamel surfaces which differ in susceptibility to acid challenge and enamel mineral content compared to the outer natural enamel layer (Ganss et al., 2000, Carvalho and Lussi, 2015). However, a reliable, accurate, clinically relevant in-vitro ETW model to study the role of AEP against ETW would be of great benefit; reducing the reliance for expensive, time consuming in-situ models.

This study aimed to investigate how the composition of AEP affected in-vitro ETW model by measuring the total protein and a targeted approach to measure four key salivary proteins: mucin5b, albumin, CA VI and statherin. Our hypothesis was that the amount of total protein and targeted salivary proteins in AEP formed from WMS would differ, thus its protection against ETW than that formed from PS only.

# 2. Material and methods

## *2.1 Sample and solutions preparations*

Sixty enamel specimens were prepared from thirty previously extracted, caries free, permanent human molar teeth and stored in sodium hypochlorite (0.05%) at 4 °C. Teeth were collected from patients attending clinics at King's College London Dental Institute, Guy's hospital London who were informed about the possibility of using their teeth and written consent was obtained. The experiment was carried out in accordance with the approved guidelines and regulations of the National Research Ethics Committee, London (REC ref 12/LO/1836). The power calculation for comparing the mean step height (the erosive loss of tissue) was carried out using Gpower version 3.1.5. based on ANOVA and paired t-test as

well as on previous studies (Martins et al., 2013, O'Toole et al., 2015) For protein analysis, a power calculation for comparing the mean protein levels between WMS group and PS group was carried out based on paired t test as well as on previous studies (Martin et al., 2013; Carpenter et al., 2014; Mutahar et al., 2017). The buccal and lingual surfaces of extracted teeth were cut with a 4-inch diamond blade (Isomet 1000 with an Extex diamond waffering blade; Buehler, Coventry, UK) at 300 rpm and a load of 200 N using a cutting machine (Buehler GmbH, Düsseldorf, Germany). The cut specimens were then placed into a custom-made silicone mould (specimen size 8 × 21.5 × 24 mm) and embedded in cold cure acrylic resin (Oracryl; Bracon, East Sussex, UK) and the natural surface polished (Metaserv 3000 variable speed grinder-polisher; Buehler, Coventry, UK) using the Federation of European Producers of Abrasives (FEPA) standard silicon carbide sandpaper, starting at 80 grit, followed by the 180, 600, 1200, 2400 and 4000 grit. This resulted in a smooth, polished surface and large enough for analysis, approximately 2 X 3 mm wide with a reference area 1 mm wide on either side to create two intact reference areas (Figure 1). Specimens were randomised and immersed in 80 ml of DW and ultrasonicated (Nusonics GP-70, T310) at 60 Hz for 15 min, after which they were rinsed and allowed to dry. Specimens were then taped with PVC adhesive tape to create a window of exposed enamel approximately 2 mm by 3 mm wide with a reference area on either side.

Four solutions were used in this study: WMS, PS, AS and DW. Paraffin-stimulated WMS samples were collected from healthy volunteers. Twenty participants (5 female and 15 male ranging in age from 24 to 60 years) from students and staff at King's College London Dental Institute, Guy's hospital London took part in the WMS and PS collection for this study. For WMS, participants were asked to expectorate saliva immediately after starting to chew the paraffin wax and continue for 5 minutes. 2% solution citric-acid stimulated PS samples were also collected from the same participants using a Lashley cup which was placed on the opening of Stenson's duct. A 10-minute collection period was applied where 2 drops of the stimulant were applied every 30 seconds to the posterior lateral surface of the tongue. The

protocol for collecting PS was adapted from Sreebny and Vissink (2010) (Sreebny and Vissink, 2010). The mean flow rate (4.2 ml/min), mean pH (6.7) and buffering capacity of the collected saliva were immediately measured to ensure that they fell within the normal range. The protocol for WMS and PS collection from healthy volunteers was approved by the ethical review committee (Northampton REC, 14/EM/0183) and written informed consent was obtained from each participant taking part in the study. The collected WMS and PS were ice-chilled and pooled immediately after collection at - 80 °C for long-term storage. Prior to use, the frozen saliva samples were defrosted for the same length of time (3 h) at 22 ± 1°C. Thawed saliva was mixed vigorously with a vortex mixer to re-suspend precipitation of proteins on thawing to avoid the loss of a specific proteins of less than 14 kDa such as statherin and/or histatins (Francis et al., 2000). The AS was prepared according to the protocol used by Eisenburger et al. (2001) (Eisenburger et al., 2001). It contained the following ingredients in DW: $CaCl_2.2H_2O$ 0.7 mmol/L; $MgCl_2$ 0.2 mmol/L; $KH_2PO4$ 4.0 mmol/L; HEPES buffer (acid form) 20.0 mmol/L; KCl 30.0 mmol/L. The pH of the prepared solution was adjusted to 7.0 by adding sodium hydroxide (NaOH) and using a pH meter (Oakton pH 510 bench top meter, Eutech Instruments Pte Ltd, Singapore) and was always used within 24 hours of its preparation.

## *2.2 Immersion in solutions and Cycling procedure*

The prepared 60 specimens were randomly allocated by an independent investigator using SPSS random sample generator to 4 experimental groups according to the solutions used: WMS (n=20), PS (n=20), AS (n=10) and DW (n=10). Enamel specimens were immersed and stored un-agitated overnight at 22 ° C ± 1 in the corresponding solution for 24 h. There were two cycling procedures: at control and after five cycles of erosion as can be seen in Figure 2.

***Control Cycle:***

After enamel specimens were immersed in the corresponding solution for 24 h, 10 specimens were taken out from WMS (n=5) and PS (n=5) groups then were rinsed in DW for 2 minutes

before AEP was eluted as will be described below ready for protein analysis (Total protein, Electrophoresis, proteomics) . This group of specimens served as the control group where AEP was eluted prior to acid erosion.

***Five cycles of erosion:***

The remaining enamel specimens [WMS (n=15), PS (n=15), AS (n=10) and DW (n=10)] were then exposed to five cycles of erosion (Figure 2). Each cycle consisted of a further 30 min immersion in the corresponding solution (either WMS, PS, AS or DW) prior to exposure to a 10-minute citric acid followed by 2-minute water rinse. The 10-min acid erosion consisted of 80 ml 0.3% citric acid (Sigma Aldrich), 0.02 M, pH=3.2, at 22°C±1, agitated with an orbital shaker (Bibby Scientific, Staffordshire, UK) at 60 rpm. After the completion of the five cycle erosion, AEP samples were then eluted from 10 specimens [WMS (n=5) and PS (n=5)] ready for protein analysis (Total protein, Electrophoresis, proteomics). The remaining specimens [WMS (n=10), PS (n=10), AS (n=10) and DW (n=10)] were then air-dried for 24 h after which the tape was carefully removed ready for profilometric measurement.

## 2.3 AEP collection and recovery

The AEP was eluted using previously published protocols (Svendsen et al., 2008, Carpenter et al., 2014). AEP samples were collected by mechanically rubbing the soaked filter paper against the enamel surface for 15 seconds using sterile tweezers to hold a filter paper soaked in sodium dodecyl sulfate (SDS) (0.5 % w/v). Filter papers carrying the AEP were microcentrifuged and the adsorbed proteins were recovered by adding 15 µl SDS (Sigma-Aldrich, Steinheim, Germany) (0.5 %) and 5 µl LDS buffer (1:4) (Novex, Thermo Fisher Scientific Inc, UK). The AEP eluents were microcentrifuged for 8 min at 8000 rpm and dithiothreitol (DTT) (1.8 µl, 0.5 mM) reducing agent (1:10) (Sigma-Aldrich, Poole, Dorset, UK) was added to the eluent. Samples were vortexed for 1 min with a vortex mixer and then heat denatured at 100 º C for 5 min.

## 2.4 Testing

### Step height

The erosive loss of tissue (Step height) was measured after five cycles of erosion for the four groups: WMS, PS, AS, and DW (n=10 specimens each) using a surface non-contacting profilometer (SNCP) (Taicaan XYRIS 2000, Taicaan™ Technologies Ltd., Southampton, UK). SNCP assessment used a white laser light with a 7 µm spot size scanned the enamel specimens, over a 6 mm X 3 mm, in a raster pattern 10 µm apart. Ten randomly selected step height measurements were selected from each specimen and mean step height in µm recorded (Boddies 2D v1.4 TaiCaan Technologies Ltd., Southampton, UK) (Rodriguez and Bartlett, 2010) Mutahar et al, 2017).

### Total protein testing

The AEP samples (control and after five cycles of erosion) were prepared into microtiter plates (96-wells, Fisher Scientific, Leicestershire) and the total protein was measured using the bicinchoninic acid assay (BCA) with bovine serum albumin (BSA) protein as a standard protein (2mg/mL) (Pierce Chemical, Rockford, Ill., USA). A spectrophotometer (BioRad laboratories Ltd, Hemel Hempstead, UK) at wavelength of 562 nm was used to measure the absorbance of all AEP samples.

### Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was used for separation of protein fractions in their denatured state from the recovered AEP samples. Each AEP prepared sample (15 µL) was loaded onto each lane on two 4–12% Bis-Tris gels (control and after erosion AEP samples). Electrophoresis was carried out in MES-SDS running buffer according to manufacturer's instructions. In each gel, 10 lanes were occupied by the AEP samples (WMS: n=5 and PS: n=5) and the other 4 lanes were occupied by a mixture of four purified proteins of standards of known concentration (Figure 3). The purified standards used in the mixture were mucin5b (156 µg/ml) (kind gift of Faculty of Odontology, Malmö University, Malmö, Sweden), albumin (1 µg/ml) (Alpha Diagnostic Intl. Inc, San Antonio, Texas 78244 USA), CA VI (140 µg /ml) (Jena Bioscience, D-07749 Jena Germany) and statherin (382

µg/ml). Statherin was prepared by the author as described previously by Proctor et al., 2005 and Harvey et al., (2011) (Proctor et al., 2005, Harvey et al., 2011). Briefly, fresh PS from a single donor at King's College London was collected and aliquoted into 10 petri-dishes, 1 ml each and exposed to air to form a statherin-rich film at the air/saliva interface after 1 h. The residual saliva underneath the film was carefully pipetted out and washed three times with 100 µl of DW. A 100 µL wash of 10 mM EDTA was then added to solubilise the statherin layer which was then separated and tested for identity and purity using SDS-PAGE gel stained with Coomassie Brilliant Blue and antibody detection (Harvey et al., 2011, Proctor et al., 2005). The statherin content in the purified film protein was 382 µg/ml.

The volume of purified proteins used in the mixture were mucin5b (10 µL), albumin (10 µL), CA VI (5 µL) and statherin (5 µL) to make a 30 µL mixture of purified standards optimised to give a linear standard curve with the antibodies used. This volume was tripled to produce a 90 µl mixture to generate sufficient amounts for the two SDS-PAGE gels. In each SDS-PAGE gel, four different volumes of the purified standard mixtures were loaded into the gels alongside the AEP samples. These volumes were 15 µL/lane 1, 7.5 µL/lane 2, 3.8 µL/lane 3 and 1.5 µL/lane 4 as shown in Figure 3. The loaded protein samples in the precast gels were then transferred onto a nitrocellulose membrane using western blot technique.

### Immunoblotting

Western blotting was completed according to the manufacturer's instructions and used to transfer proteins onto a nitrocellulose membrane. Using a sterile razor, each nitrocellulose membrane was cut transversely into four sections corresponding to the four specific proteins of interest. At room temperature, the nitrocellulose membranes were then blocked in Tris Tween buffer solution with 1% Tween (TTBS) pH 7.6 for 1 h before membranes were probed with primary antibodies: mucin5b (1:1000) (GENTAUR Ltd. 1910 Kampenhout, Belgium), albumin (1:1000) (Sigma-Aldrich, Saint Louis, MO 63103, USA), carbonic anhydrase VI (CA VI) (1:5000) (R&D Systems UK Abingdon, OX14 3NB, UK) and statherin (1:1000) (Abcam, Cambridge, UK). The nitrocellulose membranes were then washed in TTBS for 15 min (5 min

X 3 times) and then followed by incubation with the required secondary antibody. A final 15-min wash in TTBS was completed before the membranes were imaged.

### Imaging analysis of the blotted membrane

ChemiDoc MP imaging analysis (Bio-Rad) was used to quantify the light intensity of the chemiluminescent reaction and exposure times optimised to prevent pixel saturation. The amounts of proteins on the blotted nitrocellulose membranes were quantified using tools of ImageLab software version 4.1 (Bio-Rad Laboratories Ltd., Hertfordshire, UK) to select and determine the background-subtracted density of the bands in all the gels (n=2) using purified protein standards of known concentration. The standard curves of purified proteins were generated from the mean volume intensities (n=2) against the absolute quantities of the corresponding purified standard. This was used to generate a calibration curve (Appendix 1) using a linear formula. This formula was used to calculate the amount of each protein in the AEP samples. The bands of standard proteins on different SDS-PAGE gels (n=2) were used to assess quantity and reproducibility.

## Proteomic analysis

Two equal AEP samples from WMS and PS (control groups) were prepared into two tubes. 40 µL sample buffer was added to each tube and heated at 96 ºC for 10 minutes prior to centrifugation at 14,000 rpm. Boiled protein samples were loaded onto an SDS 4-20% stacking gel. Protein bands were prepared and excised before they were digested with trypsin and analysed using LC-MS/MS which was carried out at the Centre of Excellence for Mass Spectrometry, King's College London, Institute of Psychiatry, Psychology and Neuroscience.

## 2.5 Statistical analysis:

Descriptive statistics were used to summarise the step height and the protein data using IBM SPSS Statistics version 23.0 (IBM Corporation, Armonk, New York). Linear regression models were used to test the significant difference between the solutions with respect to step height. If any differences between solutions was significant, then further post hoc analyses were carried out to find out which one was statistically significant. The total and four specific protein

data did not follow normal distribution and hence they were transformed to achieve normality. Therefore, data were described using mean and standard deviation as well as median and interquartile range. Total protein, mucin5b, albumin were log transformed to attain normality and log transformed values were used for the analysis. CA VI and statherin were square root transformed and the transformed values were used for the analysis. Linear regression models were used to find out the effect of saliva type (WMS and PS) as well as the erosion condition (before erosion and after five cycles). If the interaction was statistically significant, the post hoc Boneferroni analysis testing the linear combinations of groups and saliva was used to find out which group and saliva were statistically significant. All such p values were to be adjusted for multiple testing. If the interaction effect was not statistically significant, then the final model included only the main effects of groups and saliva.

# 3. Results

## 3.1 Step height

Figure 4 shows the mean (standard deviation, SD) step height after five erosion cycles;.

WMS group [4.14 (0.9) µm] and PS group [6.42 (0.3) µm] had significantly less step height change than AS group [7.47 (1.0) µm] and DW group [10.89 (1.3) µm]. WMS group showed significantly less step height than PS group ($p < 0.0001$).

## 3.2 Total protein

Table 1 shows the total protein concentration in the in-vitro AEP samples derived from WMS and PS before erosion [after 24 h in the corresponding saliva (control)] and after five cycles of erosion. The mean (+/- SD) concentration of total proteins in AEP from WMS before erosion was [1.65 (0.16) µg/µL] and after five erosion cycles was [0.38 (0.10) µg/µL]. The mean (SD) concentration of total protein in AEP from PS at control was [0.67 (0.12) µg/µL] and after five erosion cycles was [0.15 (0.05) µg/µL]. AEP from PS had significantly lower concentration of total protein than AEP from WMS in all groups [before and after erosion ($p<001$)]. For AEP from WMS, the concentration of total protein after five erosion cycles [0.38 (0.10) µg/µL] was significantly lower than that before erosion [1.65 (0.16) µg/µL] ($p<0.000$). For AEP from PS,

the total protein concentration in AEP after five erosion cycles [0.15 (0.05) µg/µL] showed significantly lower concentration than that before erosion [0.67 (0.12) µg/µL] ($p<0.001$).

## 3.3 Specific proteins

Table 2 shows the mean (SD) and median (interquartile range, IQR) amount of the four specific proteins in in-vitro AEP from WMS and PS after 24 h immersion in the corresponding solution [before erosion (control)] and after five erosion cycles (EV).

In all groups and conditions, the amount of muc 5b and albumin were significantly more dominant in AEP from WMS than that of PS ($p<0.0001$), whereas the amount of CA VI and statherin were significantly dominant in PS ($p < 0.0001$).

The amount of mucin5b in AEP from WMS before erosion [57.5 (33.3) ng] significantly increased [121.5 (19.9) ng $P< 0.0001$] after five erosion cycles. The amount of albumin in AEP from WMS before erosion [1.4 (0.74) ng] increased after five cycles erosion [1.9 (0.8) ng] but this was not significantly different ($p>0.05$).

The amount of CA VI in AEP from WMS before erosion [6.3 (2.3) ng] significantly decreased after five erosion cycles [(0.14 (0.09) ng $p<0.0001$]. The amount of CA VI in AEP from PS before erosion [60.7 (22.6) ng] increased significantly after five cycles erosion [92.3 (19.15) ng $p<0.0001$].

The amount of statherin in AEP from WMS before erosion [19.4 (6.3) ng] significantly decreased after five erosion cycles [0.2 (0.04) ng $P<0.0001$].

## 3.4 Proteomics

LC/MS/MS sequencing successfully identified many proteins within each of the two AEP samples (WMS and PS) after 24 h immersion in the corresponding saliva. A much larger number of unique proteins were detected in the AEP from WMS (53 proteins) when compared to the AEP from PS. In total, 133 proteins were detected in the AEP from WMS. 88 proteins were detected in the AEP from PS with only 8 unique proteins to this sample when compared to the AEP from WMS.

Figure 5 shows the fold change of a wide range of proteins derived from WMS and PS including mucin5b, albumin and CA VI. They were identified from the two gel bands of the AEP from WMS versus the AEP from PS following database searching against the human portion of the uniprot database. As can be seen from Figure 5, mucin5b, albumin, amylase, lactotransferrin, lysozyme, serotransferrin, IgM, cystatin SN, D, C and SA were dominant in AEP from WMS whereas CA VI and PRPs were dominant in the AEP from PS.

# 4. Discussion

Our previous paper demonstrated that proteins within the AEP increased protection from acidic attack compared to the ions alone (Mutahar et al., 2017a). In the present study we demonstrate that the composition of the proteins can also affect the degree of protection using an *in vitro* model. Forming pellicles from WMS or parotid saliva created different composition of absorbed proteins. The mass spec analysis revealed a greater variety of proteins adhering from WMS compared to parotid saliva and total protein concentration was nearly double that from parotid saliva despite the starting concentration being similar for the two types of saliva. Further analysis of specific proteins suggested mucin5b and albumin within the WMS AEP but statherin/ carbonic anhydrase-rich, mucin/ albumin deficient parotid AEP. Based on recent in-vivo studies (Carpenter et al., 2014, Mutahar et al., 2017c, Taira et al., 2018) the statherin-rich pellicle might have been expected to be more protective. However the opposite was apparent, based on surface loss measurements. We therefore discuss possible reasons why this might be.

One possible factor could be the degree of acid attack. Current in-vitro ETW models use erosive conditions sufficient to cause measureable differences in surface height which may not always be the case in the in-vivo situation where shorter exposure to acid causes softening rather than surface loss (Jager et al., 2011). It is well-documented that the salivary protein layer formed on hard substrates is of globular nature and its response to acidic

challenge depends on the severity of acidic challenge (Hannig et al., 2004, Hara et al., 2006, Ash et al., 2014). A recent in-vivo study has identified serum albumin alongside statherin as key resistant proteins in AEP to removal by HCl acid in both short and long-term formed AEP (Taira et al., 2018). Albumin was also found to be abundant in AEP and amongst the first proteins to adsorb to enamel (Siqueira et al., 2012). Both studies (Siqueira et al., 2012, Taira et al., 2018) agree with the results in this in-vitro study that albumin has high affinity to enamel surfaces and that neither the severity of acid nor the maturity of AEP altered its adsorption.

Two in-vitro studies have compared the protection level of WMS and PS against ETW (Amerongen et al., 1987, Martins et al., 2013). These studies used different measurement techniques than used in the present study. Martins et al., (2013) measured the amount of calcium and phosphate released from enamel specimens after the demineralisation period and found that WMS and PS, and in particular small molecular proteins, provided an effective protection against 12 days enamel demineralisation (Martins et al., 2013). The combination of ions and proteins in saliva, independently of the type of saliva sample (WMS or PS) was found to further improve the reduction of enamel demineralisation as compared to proteins without ions (dialyzed samples) (Martins et al., 2013). However, a recent study disagreed with the results of Martins et al., (2013) by demonstrating that proteins alone (WMS depleted from all ions) can provide better protection against ETW than WMS (proteins and ions) or AS (ions only) (Baumann et al., 2016).

The greater resistance of AEP from WMS to ETW compared to AEP from PS in this in-vitro study may be related to the mucin5b-rich AEP. A more viscous and diffuse AEP from WMS was observed as opposed to the more elastic and compact AEP from PS (Ash et al., 2014). Vissink et al., (1985) added mucin of high concentrations (30 g/L) to saliva substitutes, demonstrating that mucin had a rehardening role against demineralised enamel as compared to saliva substitutes without mucin (Vissink et al., 1985). In the same way, the addition of gastric human mucin (2.7 g/L) to a remineralising solution led to mineral gains as

compared to mucin-free mineralising solutions due to perhaps calcium deposition into the lesion area (Meyer-Lueckel et al., 2004). Another observation in our results was that CA VI and statherin were not abundant in the AEP from WMS. This can be explained by the high proteolytic effect of WMS enzymes as opposed to the PS which has a weak proteolytic activity that makes its salivary proteins less susceptible to proteolysis as opposed to WMS (Martins et al., 2013, Zimmerman et al., 2013). The results of the present study support the dominance of lactotransferrin, amylase, lysozyme and serotransferrin in the AEP from WMS compared to AEP from PS. Previous studies have also demonstrated that proteolytic salivary enzymes such as the ones we found (lysozyme, serotransferrin, lactotransferrin) were dominant in WMS which can degrade some salivary proteins in WMS such as histatins, statherin and PRPs undermining their functionality (Helmerhorst et al., 2006, Siqueira et al., 2010). Other proteins are known to be abundant in saliva such as PRPs which accounts for up to 70 % of PS (Beeley et al., 1991) while others have recently shown to have a great potential to protect the teeth against ETW such as cystatin (Santiago et al., 2017).

A five cycle ETW model was adopted in this in-vitro study to measure four targeted proteins in AEP for several reasons. First, the four proteins in this study were selected based on their different protection mechanisms against ETW as in the case of our published in-vivo work (Mutahar et al., 2017c) for useful comparison. Second, multiple erosion cycles would generate measurable erosive loss of tissue (in line with the specification of the white light used in the SNCP) to confirm the occurrence of in-vitro ETW before measuring the amount of adhered proteins on the eroded enamel surfaces. This model has also been used previously to assess in-vitro ETW (O'Toole et al., 2015, Mutahar et al., 2017b) and has added information about the role of in-vitro AEP and its role in protection against ETW, but also casts some doubt about the application of in-vitro data in the literature to application to in-vivo and clinical application. Future in-vitro ETW studies may be recommended to make the current in-vitro ETW model more clinically relevant by using fresh saliva, mimic the action of the tongue and cheek more accurately or even studying the fluid dynamics that might occur in the oral cavity and applying

them to an in-vitro model by developing specialised stirrers. Further tests are also required to understand the relevant acidic concentration that best mimics in-vivo ETW.

# 5. Conclusions

The data presented in this study help to further define the mechanisms leading to the protection against in-vitro ETW model and demonstrate that salivary composition has an important effect on such protection. Mucin5b-rich AEP from WMS gave better protection against in-vitro ETW than statherin-rich AEP from PS. Therefore, the likely mechanisms contributing to protection against ETW in laboratory models seem to be as a physical barrier rather than stabilising the crystal structure

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## Figures

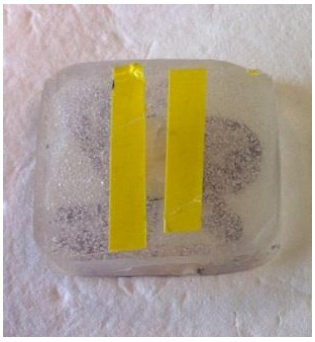

**Figure 1:** A photograph of polished and taped enamel specimen embedded in cold cure acrylic resin using the custom-made silicone mould.

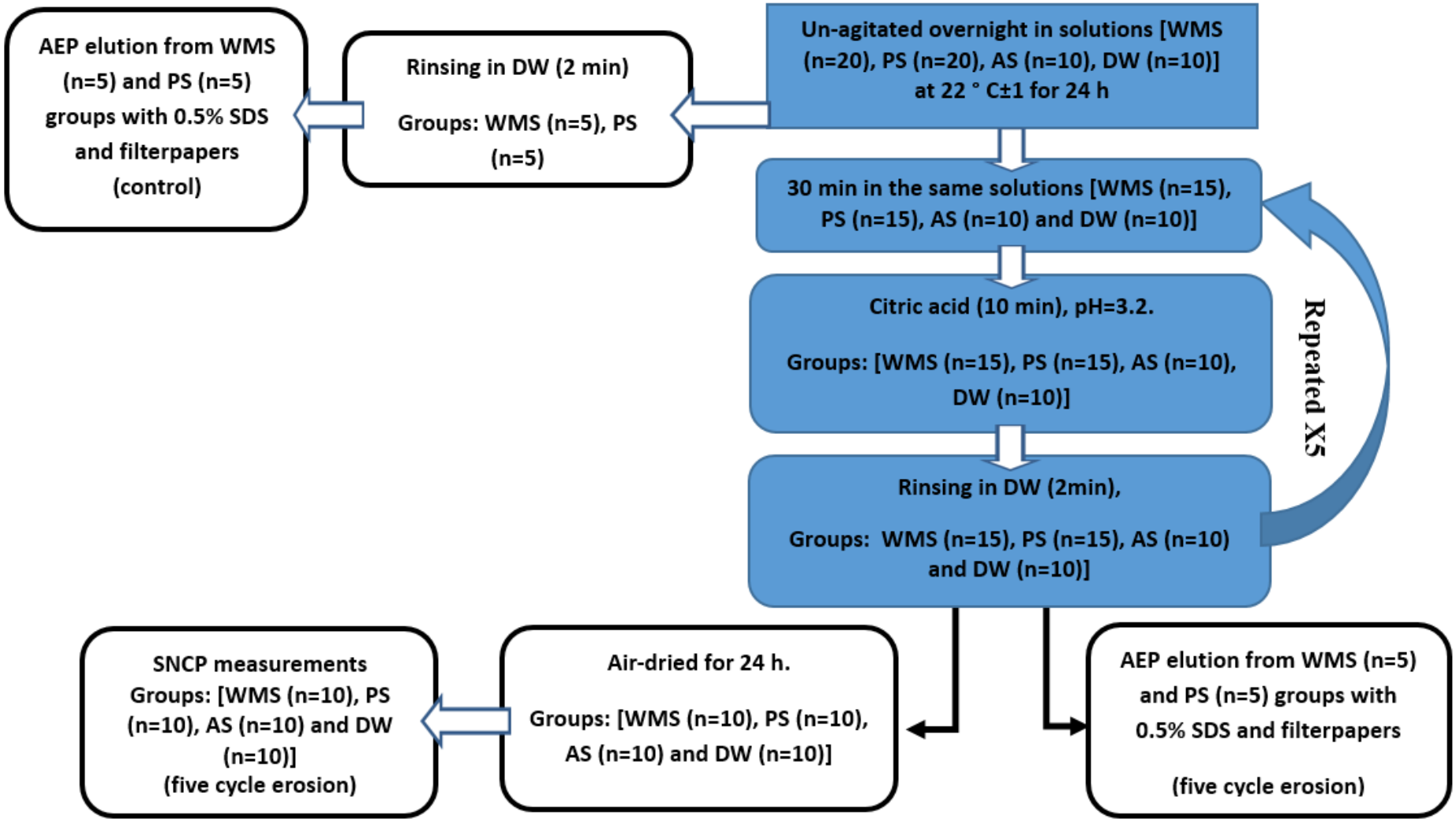


**figure 2**: A flowchart representation of specimens immersed in solutions: (WMS:n=20, PS:n=20, AS:n=10, DW:n=10) showing in-vitro AEP formation after 24 hours before erosion cycle [(control group) WMS(n=5), PS (n=5)] and after five cycle erosion cycles [WMS(n=5), PS (n=5)] including SNCP measurements [WMS (n=10), PS (n=10), AS (n=10) and DW (n=10)].

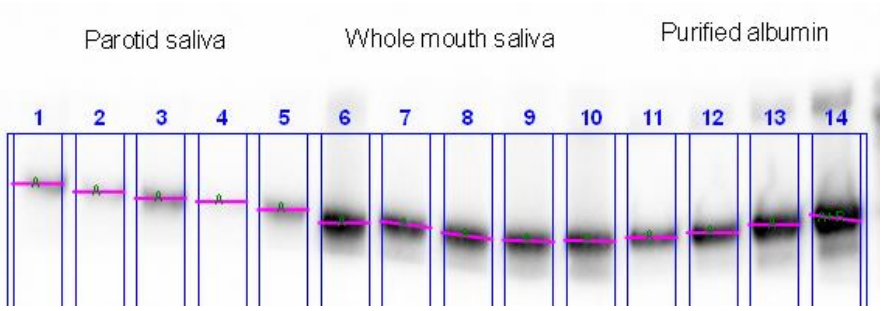


**Figure 3:** A randomly selected example of SDS-PAGE and western blotting of AEP samples (WMS: n=5 and PS: n=5) and purified protein standards (n=4) probed with albumin antibody. AEP samples from PS (Lanes 1-5), AEP from WMS (Lanes 6-10) and standard purified albumin of different volumes (Lanes 11-14).

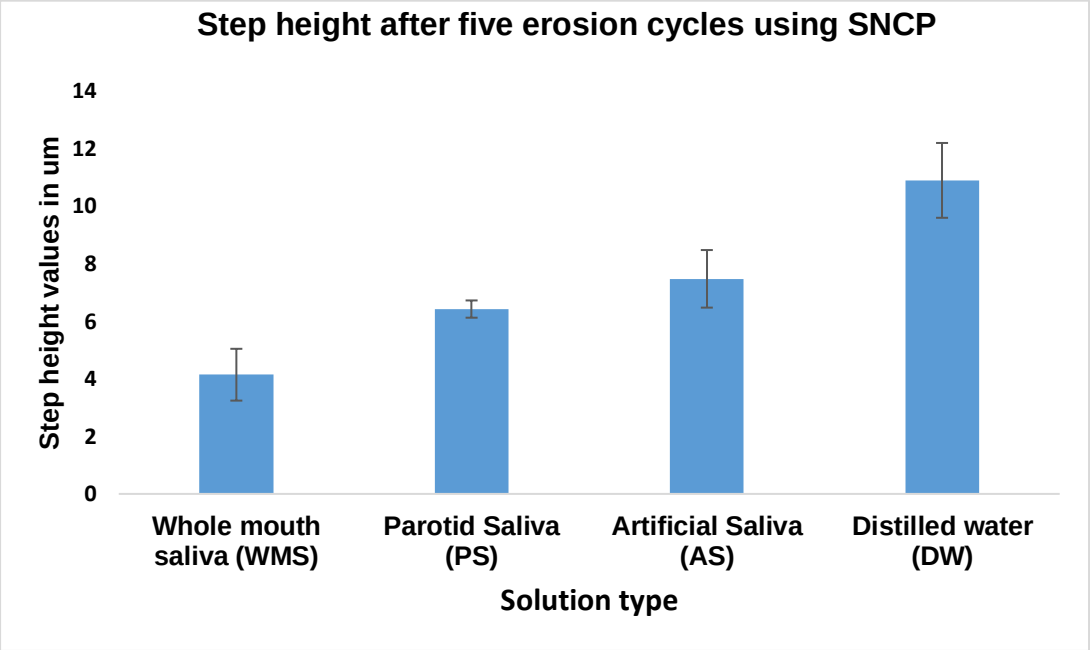


**Figure 4:** Mean (SD) step height (µm) for enamel surfaces for four groups ( 10 specimens each) after the five cycles of erosion using white optical light profilometer. Significant differences between all groups ($p<0.0001$).

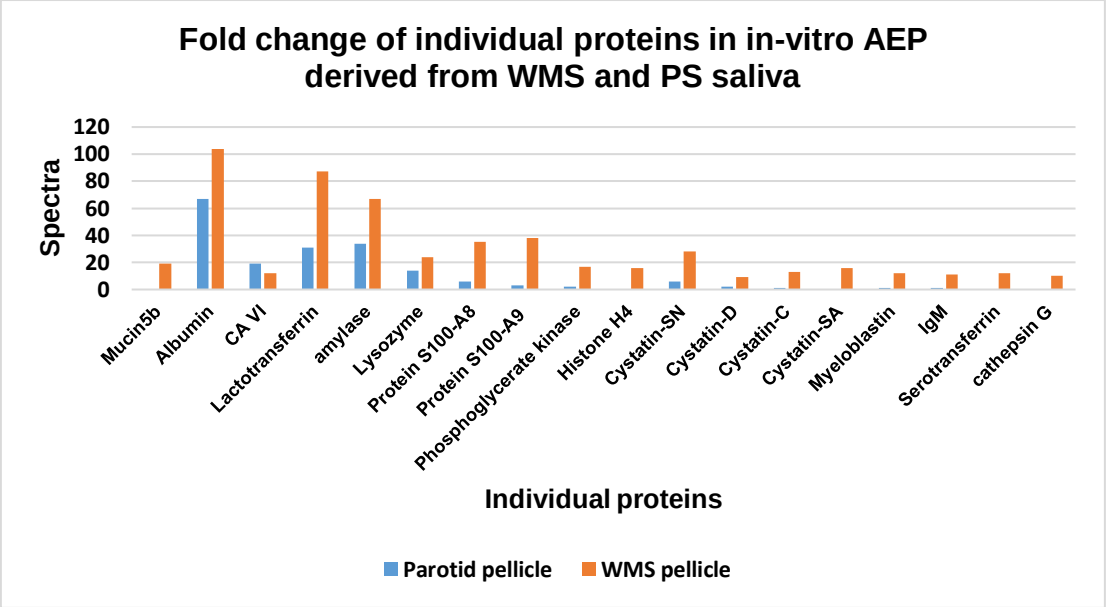


**Figure 5:** Proteomic profile of individual proteins identified in the in-vitro formed AEP. AEP was eluted from enamel surfaces after 24 h immersion in either WMS (n=1) or PS (n=1) and was analysed by LC-MS/MS searched using the uniprot database selecting Human Taxonomy (HT).

## Tables:

| Saliva type and erosion condition | Concentration of total protein (µg/µL) | |
|---|---|---|
| | **Mean (SD)** | **Median (IQR)** |
| Whole mouth saliva at control (WMSC) | 1.65 (0.16)µπ | 1.66 (0.19) |
| Whole mouth saliva after five cycle erosion (WMSEV) | 0.38 (0.10)µΔ | 0.37 (0.18) |
| Parotid saliva control (PSC) | 0.67 (0.12)#π | 0.65 (0.07) |
| Parotid saliva after five cycle erosion (PSEV) | 0.15 (0.05)#Δ | 0.12 (0.11) |

**Table 1**: Mean (SD) and median (interquartile range) concentration of total protein (µg/µL) in-vitro salivary AEP formed on enamel samples immersed in either WMS (n=10) or PS (n=10) for 24 h. AEP were then eluted before [WMS: n=5 and PS: n=5 (control)] or after five cycles erosion [WMS: n=5 and PS: n=5] using 0.5% SDS and quantified using BCA assay. Same symbols in the table indicate significant differences ($p<0.0001$). (WMSC: whole mouth saliva at control; WMSEV: whole mouth saliva after 5 erosion cycles; PSC: parotid saliva at control; PSEV: parotid saliva after 5 erosion cycles)

| Saliva type and erosion condition | Mucin5b Amount of protein (ng) | | Albumin Amount of protein (ng) | | CA VI Amount of protein (ng) | | Statherin Amount of protein(ng) | |
|---|---|---|---|---|---|---|---|---|
| | **Mean (SD)** | **Median (IQR)** | **Mean (SD** | **Median (IQR)** | **Mean (SD** | **Median (IQR)** | **Mean (SD)** | **Median (IQR)** |
| **Whole mouth saliva Control (no acid exposure) (WMSC)** | 57.5 (33.3)β | 38.0 (46.0) | 1.4 (0.8) α | 1.2 (0.1) | 6.3 (2.3)€ | 6.6 (3.7) | 19.4 (6.3)¥ | 21.0 (9.0) |
| **Whole mouth saliva Five cycle erosion (WMSEV)** | 121.5 (19.9)β | 119.0 (21.0) | 1.9 (0.8)∆ | 1.3 (1.5) | 0.14 (0.1)€ | 0.10 (0.01) | 0.20 (0.04)¥∑ | 0.10 (0.01) |
| **Parotid saliva Control (no acid exposure) (PSC)** | | | 0.3 (0.2) α | 0.3 (0.3) | 60.7 (22.5)€ | 61.2 (27.0) | 210.4 (25.8)¥ | 205.2 (7.6) |
| **Parotid saliva Five cycle erosion (PSEV)** | | | 0.3 (0.1)∆ | 0.3 (0.1) | 92.3 (17.1)€ | 94.9 (5.2) | 180.6 (23.4)∑ | 170.0 (23.1) |

**Table 2**: Mean (SD) and median (interquartile range) amount of proteins (nanogram) in-vitro AEP formed on enamel specimens immersed in WMS (n=10) or PS (n=10) for 24 h. The AEP was then eluted before (control) or after five cycles erosion using 0.5% SDS and quantified using ImageLab software. Same symbols in the table indicate significant differences ($p<0.0001$). (WMSC= whole mouth saliva at control; WMSEV: whole mouth saliva after 5 erosion cycles; PSC: parotid saliva at control; PSEV: parotid saliva after 5 erosion cycles).