An investigation of the relationship between erosive tooth wear and gastro-oesophageal reflux

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An Investigation of The Relationship Between Erosive Tooth Wear and Gastro-oesophageal Reflux

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Abstract

This thesis investigated predictive factors for erosive tooth wear in patients with Gastro-oesophageal Reflux disease symptoms. The studies included an in-vitro laboratory study, a case-control study and an in-vivo clinical study. The erosive effect of dietary and intrinsic acids on enamel samples and the protective effect of acquired enamel pellicle against an acidic challenge were investigated in-vitro. Human enamel samples (n=240) were exposed to citric acid (CA) (pH 3.2), hydrochloric acid (HCl) (pH 2.2) and artificial gastric juice (AGJ) (pH 1.1) with and without the presence of acquired enamel pellicle. The mean microhardness change increased with an increase in erosion time resulting in a softer enamel surface, from 30s to 300s for CA from 46.2 (2.8) KHN to 95.3 (2.8) KHN, CA with AEP from 55.1 (2.9) KHN to 116.0 (5.5) KHN, HCl from 52.5 (4.6) KHN to 109.5 (4.4) KHN, HCl with AEP from 66.02 (2.3) KHN to 123.7 (3.4) KHN. AGJ from 120s to 300s: 204.7 (37.5) KHN to 152 (22.1) KHN, AGJ with AEP from 206.5 (43.1) KHN to 245.4 (39.8) KHN. The mean step height increased with the increase in erosion time From 30s to 300 for CA 0.16 (0.11) um to 2.21 (0.94) um, CA with AEP 0.08 (0.04) um to 1.44 (0.46) um, HCl 0.54 (0.21) um to 4.58 (0.83) um, HCl with AEP 1.88 (0.98) um to 6.7 (0.58) um. AGJ from 120s to 300s: 16.6 (3.4) um to 27 (8.3) um and AGJ with AEP from 19.3 (4.5) to 36.3 (7.1) um. However, the presence of acquired enamel pellicle protected against dietary acids (citric acid) but not against intrinsic acids (HCl and artificial gastric juice).

A case-control study assessed the association of gastro-oesophageal reflux symptoms and erosive tooth wear on 261 participants. The predictors for ETW were age (+50) (OR 2.90, 95% CI: 1.83-6.00; p<0.0001), abnormal DeMeester score (OR 4.04, 95% CI: 0.95-17.15; p=0.05), inconclusive percentage of acid exposure time (OR 12.18, 95%

CI: 3.10-47.84; p<0.0001), abnormal percentage of acid exposure time (OR 8.5, 95% CI:1.81-39.89; p=0.007) and daily regurgitation reported by patients (OR 2.90, 95% CI:0.89-6.39; p=0.01). No association was observed between oesophageal hypomotility disorders and erosive tooth wear.

An in-vivo study assessed the total protein concentration of salivary film and acquired enamel pellicle from eroded and un-eroded tooth surfaces in 39 patients suffering from gastro-oesophageal reflux symptoms. No statistical difference was found when comparing eroded and un-eroded film/ acquired enamel pellicle from the same patient. However, when comparing those diagnosed with gastro-oesophageal reflux disease (GORD) and those without GORD, the acquired enamel pellicle total protein concentration was statistically lower in GORD patients compared to NO-GORD from both eroded (p=0.007) and un-eroded surfaces (p=0.008).

These studies summarise predictors of erosive tooth wear in patients with GOR symptoms and provide an insight on the protective role of acquired enamel pellicle in these patients.

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List of Abbreviations

(GORD) Gastro-oesophageal reflux disease

(ETW) Erosive tooth wear

(AEP) Acquired enamel pellicle

(BEWE) Basic erosive wear examination

(CA) Citric acid

(HCl) Hydrochloric acid

(AGJ) Artificial gastric juice

(DIW) deionised water

(SEM) Scanning electron microscopy

(ICP-MS) Inductively coupled plasma mass spectrometry

(NCSP) Non-contact surface profilometry

(SMH) Surface microhardness

(KHN) Knoop Hardness

(SMHb) Surface microhardness baseline

(SMHe) Surface microhardness erosion

(SMHc) Surface microhardness change

(HRM) High resolution manometry

(pH-MII) intraluminal pH-Impedance monitoring test

(RESQ) Reflux symptoms questionnaire

(GI) Gastrointestinal

(OGJ) Oesohpageo-gastric junction

(IOM) Ineffective oesophageal motility

(UOS) Upper oesophageal sphincter

(LOS) Lower oesophageal sphincter

(AET) Acid exposure time

(AE_UP) Acid exposure in upright position

(AE_SUP) Acid exposure in supine position

(SI) Symptoms index

(SAP) Symptoms association probability

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Preface

This thesis investigated possible risk factors for Erosive Tooth Wear (ETW) as a result of symptoms suggestive of Gastro-Oesophageal Reflux Disease (GORD). The studies included: A Laboratory/in-vitro study, a case control cross sectional study and an in-vivo study.

Chapter one- The literature review in chapter one provides an overview of the available evidence for predictive factors for the development of erosive tooth wear. Current diagnostic measurements and assessments of erosive tooth wear in vitro and in vivo are also discussed, in addition to discussing diagnostic tools for monitoring oesophageal motility and the movement of the refluxate in the gastrointestinal tract as well as oesophageal and extraoesophageal symptoms.

Chapter two- An in vitro study was done to understand the different effects of intrinsic and extrinsic acids on human polished enamel, mimicking real clinical situations by embedding the samples in human saliva, choosing the given time points and the stirring of acids to simulate a swishing behaviour.

Chapter three- A clinical study was conducted on patients attending the Oesophageal Department at Guy's hospital who were presenting with GORD symptoms. A symptoms diary was collected for a period of 7 days without medications prior to their appointment for the manometry and pH and impedance monitoring tests. These parameters were studied and analysed to assess the association between each parameter and the presence of ETW in order to assess predictive factors for ETW in this group of patients.

Chapter four- An in-vivo study was conducted to evaluate any differences in AEP between eroded and uneroded tooth surfaces in patients with GOR symptoms, with and without GORD diagnosis. This followed a similar study published by our group where differences were found in pellicle proteins on eroded vs uneroded surfaces in patients suffering from dietary ETW.

Chapter five- Provides a general discussion of the overall findings of this thesis and chapter six suggests clinical implications and possible future work.

1 Chapter 1: Literature Review

1.1 Tooth Wear

Tooth wear is a terminology described by the European Organization for Caries Research (ORCA) and the Cariology Research Group of the International Association for Dental Research (IADR) as the cumulative loss of dental tissue due to a chemical/physical process without bacterial involvement [Schlueter et al., 2020]. It's a multifactorial process and can occur as part of the normal physiological ageing process. Pathological tooth wear has been used to describe excessive wear, above physiological levels which would indicate a need for treatment [Bartlett and Dugmore, 2008]. Tooth wear includes abrasion, attrition and erosion [Davies et al., 2002].

Abrasion is described as wear of dental hard tissues due to a mechanical process under the impact of a foreign substance [Imfeld, 1996], it appears most commonly as a wedge-shaped lesion on the buccal surfaces of canine and premolars [Ganss and Lussi, 2014]. Tooth brushing is the most common cause of abrasive wear with less evidence for nail biting, use of toothpicks, abrasive dentifrices and other foreign objects [Addy and Hunter, 2003]. Attrition is the wear of dental hard tissues caused by tooth to tooth contact physiologically due to aging or pathologically caused by bruxism. Clinically it is seen as flattening of occlusal surfaces and loss of morphology [van 't Spijker et al., 2007], which may cause fractures in cusps or restorations. Erosion is wear of dental hard tissues due to a chemical process that does not include bacteria.

1.2 Erosive Tooth Wear (ETW)

In the oral cavity, erosion is generally accompanied by mechanical wear (attrition or abrasion) and hence the term “erosive tooth wear”, it is the most common type of tooth wear as clinically it is rare that wear is caused by a single aetiology.

The prevalence of ETW is steadily increasing worldwide, but it is often difficult to compare studies due to variability in scoring systems, diagnostic methods, and examination standards. It has been reported that ETW is “the third most common oral condition after dental caries and periodontal disease” [Bartlett et al., 2019]. The prevalence of ETW is thought to increase with age. A systematic review reported an increase in severe tooth wear from 3% at age 20 years to 17% at age 70 years [Van't Spijker et al., 2009] [Bartlett and O'Toole, 2019]. In the adult population, the reported prevalence ranges widely between 4-82% [Jaeggi and Lussi, 2014], with a reported mean of 20%-45% in permanent teeth globally [Schlueter and Luka, 2018]. One study assessed the prevalence of tooth wear in a sample of 3,200 adults in seven European countries using basic erosive tooth wear (BEWE) scoring system. The study reported that 29% of adults within the European countries show signs of ETW with UK scoring the highest levels of tooth wear [Bartlett et al., 2013]. The UK Adult Dental Health Survey (ADHS 2009) reported that among a sample of 5,654 adults 77% showed signs of anterior tooth wear [White et al., 2012].

The clinical presentation of ETW can vary depending on the severity, cause and site. In general, it is characterised by loss of natural morphology and reduction of the enamel thickness, cupping of the occlusal surfaces, loss of crown heights as well as chipping of incisal edges. During the early stages the surface can appear smooth and shiny. Further progression can result in loss of tooth substance affecting both enamel and dentine [Lussi

and Carvalho, 2014]. Removal of the smear layer and exposure if the dentinal tubules in some cases can result in dentine hypersensitivity [Wetselaar and Lobbezoo, 2016].

1.2.1 Aetiology Of ETW

ETW is caused by exposure of tooth tissue to acids in combination with attrition or abrasion. Acids originate from extrinsic sources, such as the diet or intrinsic sources, from the stomach [Schlueter et al., 2020].

1.2.1.1 *Extrinsic acids*

Extrinsic acids are most commonly present in foods and drinks. In some rare cases, the source of extrinsic acids is from the environment. Common acidic foods and drinks are fruit juices, carbonated drinks, citrus fruits and lemon juice as well as alcoholic beverages and sports drinks [Johansson, 2002; Shellis et al., 2013]. Citric acid is one of the most common acids present in frequently consumed acidic foods and drinks.

In addition to citric acid, other acids have been studied with regards to their erosive potential, such as phosphoric acid in carbonated drinks, malic acid in apples and lactic acid in food/ beverages subjected to fermentation [Hemingway et al., 2006; Hughes et al., 2000; Young and Tenuta, 2011].

Many factors influence the erosive potential of acids. These include the type of acid, pH, titratable acidity, buffering capacity, solution volume ratio to tooth surface, exposure time, chelating properties, and mineral concentration. These factors are dependant on each other, therefore it is difficult to define which of them is most important in regard to ETW.

The erosive potential of citric acid (0.3%) was compared to phosphoric acid (0.1%), the study showed higher enamel loss when polished human enamel samples were exposed to citric acid compared to phosphoric acid over a range of different pH values. It was

speculated that the result is due to the chelation property of citric acid that occurs at pH levels used in the study (3.9-6.0) [West et al., 2001]. Hughes et al.[2000] compared citric, malic and lactic acids at variable pH. They measured enamel loss using profilometry and concluded that citric and malic acids were comparable in their erosive potential, they both showed similar increase in enamel loss when decreasing the pH. Other studies have reported that varying the volume and type of acid added to the formula of foods and beverages can reduce the erosive potential, such as using malic acid rather than citric acid [Grenby, 1996].

The pH of dietary products is considered one of the predictors of the erosive potential. The pH at which hydroxyapetite dissolves has been reported to be 5.5 [Shellis et al., 2011]. When the solution reaches a pH lower than 5.5, it could result in demineralisation of the tooth surface, however this concept is based on dental caries which is different to ETW. The pH level varies in each product, the reported pH of citric acid in the literature ranges between 2.6 to 3.8 [Cheng et al., 2009c; Hjortsjo et al., 2010]. Many studies have demonstrated that as the level of pH decreases the rate of ETW increases regardless of the ETW measurement technique used [Grobler et al., 1990; Milosevic, 1997]. A study by Azadi-Schossig et al. [2016] investigated the erosive effect of citric acid on polished bovine enamel. They placed enamel samples in a chamber and flowed citric acid (0.3%) titrated to pH 7.0 through a peristaltic pump (flow rate of 2.47 mm/s) for 15 minutes. The results indicated that enamel loss caused by citric acid of pH 7.0 was insignificant compared to pH 2.17.

Buffering capacity and titratable acidity of beverages maintain the hydrogen ion concentration during their interaction with the tooth surface. However, it is essential to differentiate between the two factors: buffering capacity is dependent on the presence

of undissociated acid in a solution and measures the available hydrogen ions within a range of pH values. Whereas titratable acidity is defined for a specific pH value. The greater the buffering capacity of a solution the longer it takes for the acid to be neutralised by saliva [Johansson, 2002; Lussi et al., 2012a]. However, the effect of buffering properties could depend on other factors such as the ratio of solution volume to exposed tooth surface area and the length of exposure time. Hara and Zero [2008] reported that at low volumes of a solution, buffering properties are the most significant predictive factor for demineralisation, where at high volumes it was pH. Acid exposure time determines the length of contact of the acid with the tooth surface. Jensdottir et al. [2005b] investigated the erosive potential of soft drinks on human enamel samples in-vitro. Erosive potential was determined by measuring samples' weight and the calcium release in soft drink during immersion. They demonstrated that a decrease in the weight of samples was associated with a longer exposure of 24 hours and mainly associated with buffering properties. Whereas for a shorter exposure times (3 minutes) it was mainly the pH.

Chelating properties of acids is also an important factor. The chelating agent in a solution interacts with saliva and directly dissolves tooth minerals [Meurman and ten Cate, 1996; Shellis et al., 2014]. Citric acid attacks enamel crystal through a chelation process which occurs at pH ranges of 3.9-6.0 [West et al., 2001]. (the chelation process is described in detail in section 1.2.2).

Calcium, phosphate and fluoride concentration of acids could affect the erosive process; if the solution is oversaturated compared to the tooth surface, minerals are not lost. Whereas, if it is undersaturated it can result in initial tooth surface demineralisation. Jaeggi and Lussi [2014] reported that a high concentration of calcium and phosphate

within a solution at low pH resulted in minimal erosion and enamel softening. Moreover, promising results were reported when calcium and phosphate were added to erosive drinks [Lussi and Jaeggi, 2006]. Attin [2003] investigated the effect of addition of different concentrations of calcium, phosphate and fluoride to 1% citric acid (pH 2.2).The study reported that the addition of these minerals reduced the erosive effect. Furthermore, it was reported that enamel dissolution rate by citric acid could be best reduced by adding 0.5 mmol/1 calcium, 0.5 mmol/1 phosphate and 0.031 mmol/1 fluoride [Attin et al., 2005].

1.2.1.2 *Intrinsic acids*

A number of conditions result in gastric acid travelling up and reaching the oral cavity. The most common condition is gastro-oesophageal reflux disease (GORD), which will be explained in detail in (section 1.6). In addition, vomiting which might be involuntary, related to pregnancy sickness, migraines and stress or voluntary in the form of anorexia or bulimia nervosa could result in acids entering the oral cavity [Milosevic et al., 2008]. Another phenomenon called rumination can cause ETW, where the oesophageal sphincter is consciously relaxed to allow swallowed food to re-enter the oral cavity for re-mastication and swallowing again [Moazzez and Bartlett, 2014]. Gastric refluxate contains hydrochloric acid (HCl), which is the main acid in gastric juice, pepsin, a digestive enzyme that requires an acidic pH in order to become active, bile and rennin [Bartlett and Coward, 2001b].

Unlike extrinsic acids, pure gastric juice has a significantly lower pH ranging between 0.9 to 1.5 and is more destructive to dental tissues [Bartlett and Coward, 2001b]. Many studies in the literature modelling the effect of intrinsic acids for in-vitro studies, especially when mimicking the refluxate in patients with GORD, have used HCl [Austin et al., 2011]. The erosive effect of HCl on enamel at various pH levels and different time points has been assessed. Mann et al.[2014] investigated the effect of HCl on polished human enamel samples at different pH levels (pH 1.5 and pH 3.0), over periods of 30, 60 and 120 seconds. They found that surface roughness was statistically significantly higher when samples were eroded for 30, 60 and 120 seconds at both acid concentrations compared to baseline. A significant increase in surface roughness resulted when samples were immersed for 30 seconds between pH 1.5 and pH 3.0. Similarly, Derceli Jdos et al.[2016] investigated the effect of (0.1%) HCl (pH 2.0) on

polished bovine enamel samples over periods of 10, 20, 30 and 40 seconds. They reported that surface roughness changes occurred from 10 seconds of exposure to HCl.

Other studies have investigated the effect of intrinsic acid using human gastric juice on human enamel. Braga et al.[2011] used pooled gastric juice aspirated from patients during endoscopy to compare the erosive effect between gastric juice and orange juice on polished human enamel samples. The samples were placed in the solutions for 5 minutes, then rinsed and immersed in artificial saliva for 3 hours. They found that gastric juice resulted in statistically significantly higher calcium concentration loss compared to orange juice using atomic emission spectroscopy. However, due to the complexity and difficulty of collecting human gastric juice, laboratories have manufactured artificial gastric juice to investigate the effect of gastric reflux on human enamel. In general, it is important to mimic the clinical situation as closely as possible when modelling intrinsic acids in in-vitro studies [Young and Tenuta, 2011].

1.2.2 The Chemistry of ETW

Enamel is a complex structure composed mainly minerals, protein, lipid and water. The inorganic component is approximately 95-98%, composed mainly of minerals in the form of hydroxyapatite (HA) (Ca_{10} $(PO_4)_6$ $(OH)_2$), small particles of magnesium, sodium and calcium [Lussi et al., 2011]. The organic material is about 1%, composed of proteins presented as a thin layer over the enamel crystals and water occupying 1-4%. The structure of HA is a key-hole prism made of elongated crystallites of about 20-25 nm thick and 50-70 nm wide [Shelat, 2017]. Erosive tooth wear occurs when acid interacts with enamel crystals by diffusion of acid through the presented barriers, such as plaque, lipids/protein layer and acquired enamel pellicle.

The 'critical pH' concept is when the solution becomes undersaturated in minerals with respect to enamel surface. This leads to initial demineralisation of the tooth surface releasing minerals into the solution, resulting in an increase of the pH of the solution. The demineralisation process is terminated when the solution surface layer adjacent to the tooth becomes saturated with respect to the tooth surface [Dawes et al., 2015].

The chemical process of demineralisation of dental hard tissues differs with different acids (organic and inorganic acid). Organic acids contain carbon within their molecule, such as citric, malic, lactic and phosphoric acids found in foods and drinks. Citric acid is a weak organic acid that causes erosion through a chelation process. It dissociates in water releasing 3 hydrogen (H+) ions, citrate and undissociated molecules. The citrate ions act as a "chelator" attacking mineral crystals within the tooth surface releasing hydrogen ions. The hydrogen ions interact with either carbonate or phosphate forming a complex with calcium. This mechanism only occurs at basic pH of 7. At pH 2 the mechanism is

largely via the released hydrogen ions directly attacking minerals within the tooth tissue. Whereas between pH 2 and 5 both mechanisms apply.

Inorganic acids are mineral acids that do not contain carbon in their molecule. In a solution, they can partially or fully dissociate which is the reason behind their acidic strength. Hydrochloric acid is the most common inorganic acid in relation to erosion. It is found in gastric juice and hence the refluxate. At pH 1-2, hydrochloric acid fully dissociates in water providing hydrogen and chloride ions, the hydrogen ions rapidly dissolve mineral crystals.

1.3 Saliva and AEP in ETW

1.3.1 Saliva

The biological factors that influence and modify the extent of ETW, predominantly are saliva and acquired enamel pellicle (AEP) [Hara et al., 2006b].

Whole saliva is a complex biological matrix generated as a cumulative secretion from parotid, submandibular, sublingual glands; minor salivary glands and gingival crevicular fluid. Each gland produces different secretions depending on the sympathetic and parasympathetic stimulation, eating habits, medication intake, and health condition [Kaufman and Lamster, 2002]. Saliva is composed of water, electrolytes containing calcium, phosphate, magnesium, sodium and potassium, proteins such as mucin, immunoglobins and enzymes, lipids and other macromolecules [Buzalaf et al., 2012a; Humphrey and Williamson, 2001].

The volume of secreted saliva varies, ranging between 0.5-2.1 ml/min, and upon swallowing the residual saliva volume in the mouth ranges between 0.4-1.4 ml/min on hard tissues and mucosa. Natural saliva is present as a salivary film on hard tissue and mucosa [Dawes, 1987]. The film thickness has been measured using filter paper applied on different surfaces of the mouth, the tongue has a film thickness of 50-70 um, whereas on the buccal mucosa it is 40-50 um and on the anterior hard palate it is 10 um [Osailan et al., 2011]. However, enamel surface has been reported to have a film thickness of about 60-90 um [Watanabe and Dawes, 1990].

The film movement within the oral cavity and the rate of salivary secretion are of great importance as the rate of clearance of substances from the mouth is determined by swallowing [Proctor, 2016].

Saliva offers protection in various ways as listed below:

1. Mineral components in saliva, such as calcium, phosphate and fluoride, modulate the demineralisation/ remineralisation processes.
2. Proteins and glycoproteins, act as antibacterial, antifungal and antiviral agents. Proteins, like mucin, aggregate with microorganisms and inhibit the adherence and colonisation of these microorganisms. However, they have the ability to break down starch, lipids and proteins supplying the bacterial colonisation with nutrients [Dawes et al., 2015; Marsh et al., 2016; van 't Hof et al.].
3. Enzymes and immunoglobulin provide antibacterial and antimicrobial actions.
4. Normal salivary flow rate facilitates clearance of carbohydrates, acids and microorganisms from the oral cavity. A low flow rate may reduce clearance of acids from the oral cavity. However, studies are equivocal, with some showing that a reduced flow rate increases the risk of ETW [Gudmundsson et al., 1995; Lussi and Schaffner, 2000], whilst others, showing no correlation between the two conditions [Bartlett et al., 1998].
5. Salivary buffering system affects neutralisation of acids in the oral cavity. It is mainly composed of phosphate, carbonate and proteins buffers. When the pH is 5 or below the protein controls the buffering system, whereas if the pH reaches 6.3, carbonate controls the buffering system and phosphate when the pH is 7.2 [Bardow et al., 2000]. Salivary buffering system has been linked to the salivary flow rate. When salivary flow rate is reduced, salivary pH drops and the phosphate concentration increases [Dawes et al., 2015].

An impaired buffering capacity has been linked to increased rate of enamel dissolution in some studies [Gudmundsson et al., 1995], whereas other studies have shown no correlation between ETW and buffering capacity [Hara and Zero, 2014; Woltgens et al., 1985].

1.3.2 Acquired Enamel Pellicle (AEP)

In addition to the salivary film, there is an adherent layer forming the "acquired enamel pellicle" (AEP) on dental hard tissues [Cheaib and Lussi, 2011]. AEP is a bacteria-free organic layer formed in-vivo as a result of selective adsorption to the surface of the enamel. It is an accumulation of macromolecular components from saliva, blood, bacteria, gingival crevicular fluid, mucosa and diet [Hannig and Hannig, 2014].

AEP is commonly described as a sponge like structure consisting of two layers: the electron basal layer of 10 to 40 nm thickness and an outer globular and glandular layer [Hannig and Joiner, 2006b].

Numerous studies have supported the role of AEP as a protective interface against both chemical and bacterial acid-induced attack [Cheaib and Lussi, 2011; Hannig and Balz, 2001; Hara et al., 2006b]. The anti-erosive effect of AEP depends on many factors. These include composition, maturation time, location within the oral cavity [Hannig, 1999b], and individual variabilities [Finke et al., 2002; Sonju Clasen et al., 1997]. AEP plays a role as a protective physical interface forming a semi-permeable barrier between the tooth surface and the oral cavity [Mutahar et al., 2017a; Siqueira et al., 2010]. It modulates the demineralisation/remineralisation processes, decreasing demineralisation by controlling the calcium and phosphate diffusion from the tooth surface to the surrounding solution [Buzalaf et al., 2012a; Vukosavljevic et al., 2014]. During acid exposure, the outer globular layer is removed leaving the basal layer intact, protecting the surface from the acid.

The effect of In-vivo AEP maturation time against an erosive challenge varies in the literature. Hannig and Balz [1999] reported that 24 hours and 7 days formed in-vivo AEP had the same influence on protecting enamel surface. Moreover, it has been reported

that different levels of protection are provided at different locations in the oral cavity. Amaechi et al.[1999b] investigated the effect of 1 hour formed AEP in-situ on the distribution and severity of erosion. They observed that the severity of erosion was unevenly distributed in the oral cavity. Hannig and Balz [2001] investigated the protective effect of 24 hours in-situ formed AEP at different locations within the oral cavity (upper first molars buccal aspect and lower incisors lingual aspect), enamel samples with and without AEP were then exposed to 1% citric acid for 30 seconds and 5 minutes. They observed that AEP formed on enamel samples in the upper molars was less protective compared to AEP formed on lower incisors. However, they reported that even after 5 minutes of acid exposure, a layer of AEP was still present, concluding that the location-based protection is less important in the case of 24 hours formed AEP. In addition, the thickness of AEP may control the pattern of ETW distribution [Hannig and Hannig, 2014; Young and Khan, 2002]. The thinnest AEP was found on palatal surfaces of maxillary anterior teeth, which is a site commonly affected by ETW, whilst the thickest pellicle was found on lingual surfaces of mandibular anterior and posterior teeth where is usually not as prevalent [Lussi et al., 2004; Nekrashevych and Stosser, 2003a].

1.3.3 Proteins

Saliva contains thousands of proteins that have been extensively studied in proteomic research. Salivary proteins contribute to the biological functions, such as regulating soluble molecules, remineralisation of dental tissues, biofilm homeostasis, immune defence via antibodies, and nutrition [Schipper et al., 2007b].

Currently 3000 proteins have been identified in saliva, 363 of these proteins have been identified in AEP [Lee et al., 2013; Schweigel et al., 2016; Ventura et al., 2017]. Some salivary proteins are more abundant in AEP and contribute to the formation of AEP on enamel [Hannig and Joiner, 2006a; Siqueira et al., 2012], these include mucin, statherin, albumin and carbonic anhydrase (CA VI), histatin, cystatin and proline rich proteins [Algarni et al., 2015; Cheaib and Lussi, 2011, 2013]. The most abundant proteins found in the early stages of AEP formation have the ability to bind to calcium ions such as proline rich proteins (PRP), albumin and histatin. Whereas those abundant in the late stages of AEP formation, either have the ability to bind to other proteins, such as mucin forming complexes with histatin and statherin (MUC5B) or bind to phosphate such as Myosin-9 [Lee et al., 2013]. These proteins have an affinity to adhere to enamel and play a role in the protection of enamel against acidic attacks. Each individual protein plays a different mechanism of action in respect to ETW.

MUC5b and MUC7 play an important role in the formation of AEP, and selectively bind to hydroxyapatite [Siqueira et al., 2007d]. Mucins in AEP protect the enamel surface by forming a physical viscoelastic barrier [Hannig and Joiner, 2006b; Lamkin and Oppenheim, 1993; Siqueira et al., 2007b], acting as a lubricating membrane and providing antibacterial activity due to the carbohydrate portion of their molecule [Tabak, 1990].

Albumin is another key protein that contributes to the formation and protective mechanism of AEP. Hemingway et al.[2008] combined ovalbumin (a protein found in egg white) and casein proteins to investigate their acid resistance effect. They reported that these proteins inhibit ion diffusion, thus protecting enamel surface form dissolution. In addition, it was demonstrated that albumin binds to hydroxhyapitate and inhibits crystal growth [Robinson et al., 1992].

The function of statherin has been studied, in particular its ability to bind to calcium affecting demineralisation of enamel in-vitro [Kosoric et al., 2007] as well as maintaining a lubricating effect [Douglas et al., 1991; Hahn Berg et al., 2004; Harvey et al., 2011]. Furthermore, Calcium anhydrase (CA VI) in AEP functions as a catalyst between hydrogen ions in acids and bicarbonate ions in AEP, which increases enamel pH level and returns it to normal levels [Leinonen et al., 1999b].

1.4 Measurements techniques for Erosion and ETW

There are various quantitative and qualitative measurement techniques available for assessment of erosion and ETW in-vivo as well as in-vitro [Attin and Wegehaupt, 2014; Rios et al., 2008; Rodriguez et al., 2012; Schlueter et al., 2011a; Schlueter et al., 2014]. {Table 1-1} summarises the advantages and disadvantages of some of these methods. Characteristics of the techniques used in this thesis are detailed as follows:

In Vitro method	Advantages	Disadvantages
Surface Profilometry [Attin, 2006; Heurich et al., 2010]	• Measures both volume loss and vertical loss • Non-destructive • Considered gold standard for measuring tissue loss	• The contact type causes irreversible damage to the surface • Sensitive technique • Time consuming
Surface microhardness [Barbour and Rees, 2004]	• Simple and inexpensive • Detect early surface changes due to erosion	• Not accurate in natural unpolished enamel surfaces • Limited accuracy with natural samples • For accuracy it requires flat surface and precise alignment of the sample with the indentors

Scanning Electron Microscope (SEM) [Meurman and Frank, 1991]	• High in resolution measuring surface topography • For the use of wet samples	• Requires irreseversible preparation of the samples • Expensive
Tandem Scanning Confocal Microscopy (TSCM) [Mullan et al., 2018a]	• Rapid surface characterisation • Assess within species variations	The accuracy of images depends on the pin hole size
Atomic Force Microscopy (AFM): A. Confocal laser scanning microscopy (CLSM) [Barbour and Rees, 2004]	• Produce qualitative and quantitative data • High resolution images • Non-destructive	• Slow scanning rate • Expensive
B. Microradiography [Ganss et al., 2005]	• Direct technique • Sensitivity in thin sections	• Destructive
Optical Coherence Tomography (OCT) [Wilder-Smith et al., 2009]	• Non destructive • High 3-D resolution • Provides real-time structural imaging	• Limited penetration depth and scanning range • Difficult to find a ref point
In Vivo methods		
Quantitative laser fluorescence (QLF)	• Non-destructive	• Expensive

[Ablal et al., 2009]	• Available feedback to patients • Fast technique	• The presence of bacteria, electrolytic solutions, and blood considerably influence the intensity of fluorescence
Atomic absorption spectroscopy [Ganss et al., 2009]	• Non-destructive	• Expensive

Table 1-1: in-vitro and in-vivo tooth wear measurements

1.4.1 Indices

Clinical assessment of ETW is carried out by a combination of taking a history and assessing patients' habits and lifestyles as well as a clinical examination. This includes assessment of the shape, position and severity of any wear facets. The progress of ETW is generally slow and therefore objective clinical assessment is difficult. In addition, to date no reliable method exists, which can accurately measure changes on the tooth surface accurately, in-vivo. Many indices have been proposed for assessment of ETW. A systematic review by Wetselaar et al.[2016] reported that 114 different scoring systems were used to quantify tooth wear, the most frequently reported index is the Tooth wear index (TWI) followed by basic erosive tooth wear index (BEWE) and Lussi index. The first index was described by Eccles and Jenkins [1968], where erosion was classified as early, small and advanced. Smith and Knight [1984] introduced a more general concept developing the Tooth Wear Index (TWI) irrespective of the cause of wear, classifying tooth wear in scores from 0 to 4, 0 representing no loss of enamel or contour and 4, complete loss of enamel , pulp exposure, or defect more than 2 mm deep. Furthermore, Lussi in 1996 recommended to simplify the TWI index by classifying the wear by dentine

exposure level [Lussi, 1996], however, this index would not identify early enamel wear [Ganss and Lussi, 2008].

The latest scoring system developed by Bartlett et al.[2008] known as Basic Erosive Wear Examination (BEWE), has a 4-point scale ranging from 0 to 3, dividing the mouth into sextants and providing a cumulative score to guide practitioners in managing the condition: 0: no loss of the surface, 1: initial loss of enamel surface- slight wear, 2: hard tissue loss less equal or more than 50% of the surface- dentine is frequently involved, 3: hard tissue loss more than 50% of the surface-dentine is frequently involved. The advantages of this index are ease of use and has been reported to have validity, reliability, sensitivity and specificity required for a tooth wear index [Holbrook et al., 2014; Mulic et al., 2010; Olley et al., 2014]. It also provides examiners the ability to measure the severity as well as the risk level providing suggestions for managing the condition from eliminating aetiological factor, prevention and monitoring to clinical interventions. The BEWE index is now the most commonly used index for ETW internationally in over 34 countries and has been used in 96 peer review publications [Bartlett et al., 2019].

1.4.2 Profilometry

One of the most commonly used methods for tooth wear measurement in in-vitro studies are profilometry [Passos et al., 2013; Rodriguez et al., 2012; Rodriguez and Bartlett, 2010; Schlueter et al., 2011b]. Surface profilometry measures the loss of tooth tissue on a treated (eroded) surface compared to a non-treated tooth surface providing two or three-dimensional profiles [Attin et al., 2009]. Two types of profilometry are available: contact (CSP) by a stylus probe or non-contact (NCSP) by a laser light. Both consist of a stage for sample placement and a detector for collection of data points.

1.4.2.1 *Contact surface profilometry (CSP):*

CSP consists of a metal or diamond stylus, which traverses the eroded surface with a load force and measures at a rate of 10 mm/min, the radius of the stylus is about 2-100µm that moves along the surface to detect the height. The surface profile is then measured by the physical resistance of the surface, hence the slow progress compared to optical profilometry. The disadvantages of this method are that it could potentially damage the surface of the sample, causing an overestimation of early erosion depth and contamination of the stylus by direct contact. These limitations are overcome by the use non-contact surface profilometry (NCSP) [Ganss et al., 2009; Heurich et al., 2010].

1.4.2.2 *Non-contact surface profilometry (NCSP):*

NCSP provides an image of a scanned surface without the use of a physical stylus. It consists of a sensor and a reflector; deviation of the light sensor is picked up and digitised to produce data. The analysis of the reflected light is done through a charged coupled device or a spectrometer. The data are presented as a plot on a digital grid. The sensor in addition to a controllable stage platform can create 3-D topographical maps of the scanned sample [Austin et al., 2015; Leach, 2014]. The type of sensors that have been used previously in erosion studies includes: white light [Mistry et al., 2015b], confocal laser [Mullan et al., 2018a]and triangular laser [Rodriguez et al., 2012; Rodriguez and Bartlett, 2010].

NCSP is considered the "gold standard" measurement of tooth tissue loss in in-vitro research [Hall et al., 1997]. It measures the average change in height (a step height) of acid exposed and unexposed enamel surface from 2 reference areas. Previous studies using NCSP profilometry for characterising in-vitro and in-situ surface loss have reported the ability to characterise enamel surface changes under ETW conditions on both natural and polished samples [Hove et al., 2006; Hove et al., 2008; Mistry et al., 2015a; Mullan et al., 2017a; Mullan et al., 2018b; O'Toole et al., 2016; Rodriguez et al., 2012].

Previous studies have reported multiple factors that could affect the accuracy and uncertainty of NCSP [Austin et al., 2016a; Mullan et al., 2017b; Mullan et al., 2018b; Mylonas et al., 2018]. Austin et al.[2016a] demonstrated that NCSP is an effective quantitative measurement of the minute surface changes on polished enamel samples after citric acid exposure in-vitro. Leech [2014] observed that anything affecting the reflection of light back to the sensor can impact the certainty of NCSP. In addition, both NCSP and CSP are affected by the width of the probe tip, the wider the stylus or the laser

spot size the less accurate the measurement since it would be harder to penetrate through surface troughs. Increasing data points is more time consuming for the analysis [Rodriguez et al., 2012]. However, one of the greatest advantages of NCSP is that it does not directly contact the surface, and therefore causes no damage [Austin et al., 2015; Barbour and Rees, 2004]. In addition, NCSP is time efficient as a batch of samples could be programmed and scanned at once which is not possible when using CSP.

Although natural enamel surfaces have been used recently with promising results, there are some difficulties. The main limitation of surface profilometry accuracy and detection is that bulk surface loss is required in order to reliably detect any changes and a flat surface required for accuracy of step height profile [Austin et al., 2011]. A study comparing the effectiveness of three techniques in detecting early enamel changes after an erosive challenge on bovine enamel samples (Contact Surface Profilometry CSP, Non-Contact Surface Profilometry NCSP and Confocal Laser Scanning Microscopy CLSM) concluded that all three techniques produced similar statistical significance in measuring the step height, the data showed very high agreement between techniques in assessing erosive tooth wear [Paepegaey et al., 2013]. Other methods are not as well established or semi-quantitative rather than quantitative, such as the quantitative light-induced fluorescence (QLF), which has resulted in variable outcomes when tested for its suitability for erosion research in-vitro [Ablal et al., 2009; Nakata et al., 2009; Pretty et al., 2003].

1.4.2.3 *Techniques and analysis methods*

Various measurement techniques and analysis methods have been used to characterise enamel loss when using profilometry. The measurement techniques include the number of readings, profile extraction and superimposition alignment.

A single scan is the most commonly used measurement technique in in-vitro erosion studies, it is often taken at a mid-point. It has been used more on polished enamel samples compared with natural enamel samples, due to ease of barrier isolation and lesion characterisation [Mistry et al., 2015b; O'Toole et al., 2015b; Young and Tenuta, 2011]. Attin et al.[2009] measured vertical loss using a single profile technique from the mid-point. Rodriguez and Bartlett [2010] compared 2-D step height measurement using a single point technique with a 3-D step height measurement calculating the wear over a whole surface area on enamel samples after an in-vitro erosive challenge. They suggested an average of several readings decreases the risk of non-representative data compared with a single point measurement.

Another measurement technique is the use of profile extraction, which is by subtracting the data set from before and after erosion scans producing an image to calculate enamel loss [Stenhagen et al., 2010]. Stenhagen et al.[2010] applied the profile extraction method to calculate a step height formed on polished enamel samples. They scanned the samples prior to erosion with (0.01 M) HCl for 6, 12 minutes and after each erosion time point. The scanning was done using white light NCSP, images were aligned and subtracted from baseline. The step height calculation was done following Holme et al.[2005]. Results showed that the profile extraction method allowed for calculating a step height of 5.1 (1.1) and 10.4 (1.9) um after 6 and 12 minutes of immersion respectively. Profile extraction has been used in other studies showing promising results in determining enamel loss in natural enamel [Rodriguez et al., 2012; Rodriguez and Bartlett, 2010].

Superimposition alignment is a 3-D measurement technique provided by either NCSP or digital intra-oral scanners. Rodriguez and Bartlett [2010] applied superimposition

alignment in in-vitro erosion-abrasion models. They compared the effectiveness of the 2-D and 3-D techniques in measuring changes to human polished enamel samples. The study found no statistical difference between samples measured using 2-D technique, whereas using 3-D technique it was found that two of the experimental solutions (packed orange juice and passion fruit juice) produced more wear compared to the others. They concluded that 3-D measurements were more accurate. Kumar et al.[2019] measured enamel loss in-vitro using both maximum loss in surface profile and average surface profile loss. Both measurements increased after immersion of natural enamel in dietary acid for 120 seconds. Significant correlation was reported between both measurements (maximum profile loss and average profile loss) and increasing acid exposure ($r=0.88$; $p<0.001$) ($r= 0.63$; $p=0.019$) respectively. This method required prolonged immersion times to be able to measure enamel surface loss and which is not clinically representative. It was also difficult to produce a reference area on a natural enamel surface,

Analysis methods for calculating step height could be characterised using either ISO 5436-1 or non-ISO. ISO 5436-1 measures the step height from the eroded region subtracted from the average height of the non-eroded region [Iso, 2000]. Calculation could be done using a single line mid-point step height , which measures the difference in height from one profile line across the samples [O'Toole et al., 2015b], or using a mean single line mid-point step height, measures the difference from multiple profile lines across the sample. This has been previously applied by calculating 5 profile readings [Mistry et al., 2015a] or 10 [Mutahar et al., 2017b].

This is the most commonly used analysis method in in-vitro erosion studies, as it is able to measure step height of polished enamel samples. However, this method has not been utilised for natural enamel samples due to the complexity of the surface.

Non-ISO step height measurements have been used by [Ganss et al., 2000]. The study investigated the effect of 3 hours citric acid on natural and polished enamel samples. For polished enamel, it was defined as a vertical distance between the highest and lowest points on any given region, the mean reading of six tracings recorded. For natural enamel, it was defined as the highest trough on the non-eroded region and the lowest point on the eroded region within the first 0.3 mm of the regression line. This study did not consider the reproducibility and repeatability of this method. Lin et al. [2017] defined it as the vertical distance between the highest point on the non-eroded region and the lowest point on the eroded region within a defined erosive area. However, this study did not provide an elaboration on how to define the region of interest.

{Figure 1-1} shows an example of a step height formed on polished human enamel samples after an acidic challenge, measured as a vertical loss between treated and reference areas. The analysis was done using Boddies© software (Taicaan, Southampton, UK). {Figure 1-2} shows an example of a step height formed on a polished human enamel sample after an a cidic challenge, measured as a vertical loss using automated MountainsMap software (MountainsMap®, Digitalsurf, France) - ISO 5436-1 standard (ISO, 2000c).

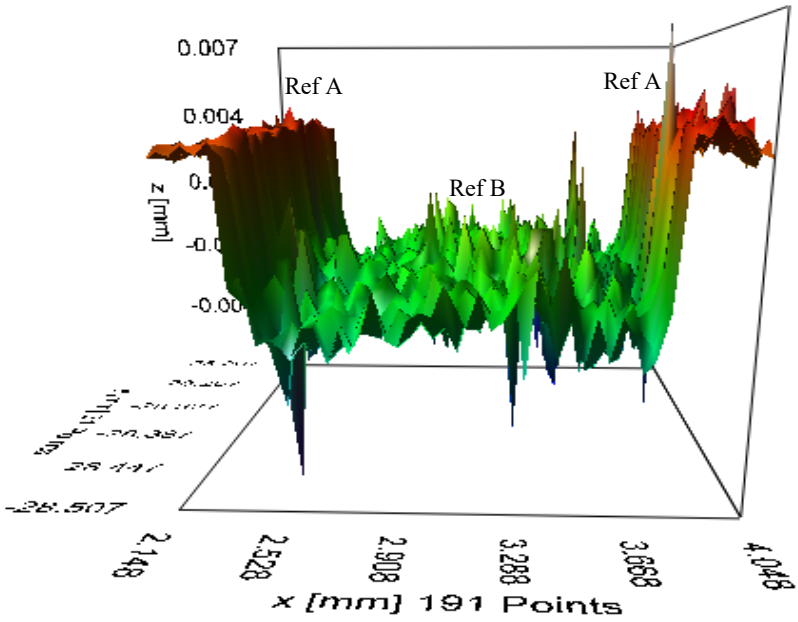

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Figure 1-1: Example of step height measured using Boddies© software. Ref (A) represents the reference non-eroded regions and Ref (B) represents the eroded region.

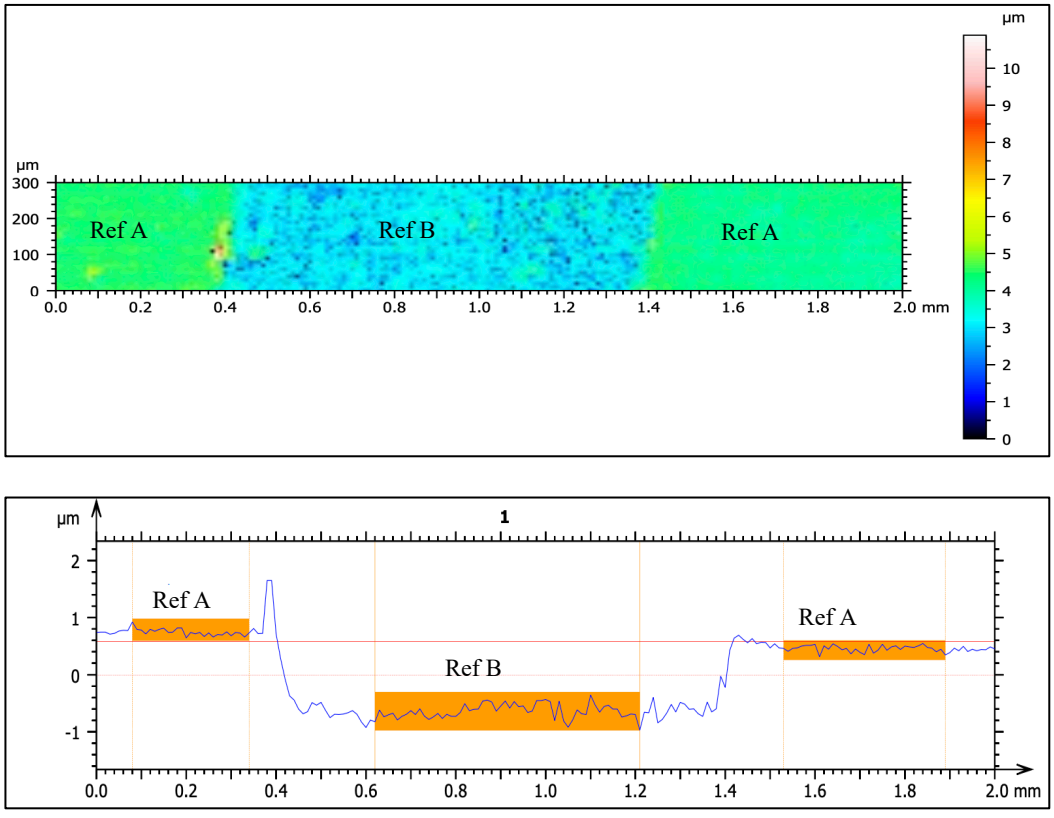


Figure 1-2: Example of step height measured using automated MountainsMap software. Ref A represents the reference non-eroded regions and Ref B represents the eroded region.

1.4.3 Microhardness

Hardness is described as the resistance of a material to permanent deformation under a given load. Elastic modulus is described as the resistance of a material to deform elastically quantifying the stress magnitude and degree of deformation ratio. Changes in both parameters (hardness and elastic modulus) result in mineral loss in mineralised tissue. However, hardness measurements have been recommended for characterising the mechanical behavior on a contact zone [Park et al., 2008].

To measure enamel softening, microhardness is considered the most useful method. The principal components of the hardware are a diamond tip (indenter) and a microscope to view the samples. The surface microhardness technique measures the surface hardness of the sample before and after intervention by providing data about the surface texture when the indenter penetrates. The diamond tip indents the tissue for a given load and a specific time. There are different types of indenters including nanoindeters and mirohardness indenters. They are both similar in the principle of action but nanoindentors are at a smaller scale and they penetrate less deeply than microhardness indentors (150- 500 nm) [Finke et al., 2000]. Knoop indenters and Vickers diamond indenter are rhomboidal and tetra pyramidal in shape {Table 1-2}.

There is no consensus on which type of indenter is suitable for ETW studies. Knoop indenters are shallow and elongated causing less cracks or crazing, hence they are considered higher in sensitivity and more suitable in assessing early ETW [Austin et al., 2011]. Knoop indenters are smaller than nanoindentations (~200nm) and create a 1.5 µm depth of indentation. Whereas Vickers indenters penetrate deeply in softened enamel reaching a depth of 5 µm, which is considered better for analysis of

surface properties. However, the indentation depth might be influenced by the sound underlying enamel [Schlueter et al., 2011b]. Vickers indenters have been used in studies characterising surface changes under the effect of remineralising agents on deciduous and permanent enamel surfaces [White et al., 2001]. They have also been used in studies investigating the anti-erosive effect of AEP, comparing the effect of different citric acid concentration and different immersion times on human enamel samples [Bajaj and Arola, 2009; Nekrashevych and Stosser, 2003b].

Knoop microhardness has been used previously in numerous ETW studies assessing changes on enamel surface. Mylonas et al.[2018] assessed the capability of microhardness measurement to characterise changes in both natural and polished enamel surfaces under early erosive challenges. They reported that it was not possible to obtain measurable knoop indentations on natural surfaces of enamel samples, due to the curvature of the enamel surface and discrepancy in surface profile and topography. Nekrashevych et al.[2004] investigated the effect of different acid exposure times using microhardness change. They reported that when acid exposure in enamel increased, microhardness measurement decreased, and mineral dissolution increased.

Austin et al.[2011] used Knoop microhardness measurement to characterise changes in polished enamel surfaces under an acidic exposure by HCl and toothbrush abrasion followed by fluoride varnish. The results showed no significant reduction in KHN ($p>0.05$) concluding that fluoride varnishes have limited protection against erosion. Furthermore, Knoop microhardness measurement has been used for measuring the anti-erosive properties of AEP, Sieber et al.[2019] investigated the effect of AEP with and without casein and mucin and showed significant protection by modified AEP with casein and mucin compared to unmodified AEP.

Microhardness and profilometry were used by Hara and Zero.[2008] to assess changes on enamel samples, they concluded that microhardness was able to detect initial stages of erosive tooth wear, whereas profilometry was not as sensitive in detecting surface changes in initial stages. However, microhardness showed limited ability to analyse advanced stages of erosive tooth. Other studies have also reported similar results [Stenhagen et al., 2010] [Jaeggi and Lussi, 1999].

Test	*Indenter*	*Side view*	*Top view*	*Load*	*Hardness number*
Vickers	Diamond pyramid			1-120 kg	$HK = \frac{1.854P}{L^2}$
Knoop	Diamond pyramid			25 g- 5 kg	$HK = \frac{14.2P}{L^2}$

Table 1-2: Comparison of Knoop and Vickers indenters

1.4.4 Inductively Coupled Plasma- Mass Spectrometry (ICP-MS)

ICP-MS is a quantitative elemental technology which measures the total amount of an element of interest within a liquid solution. It is a reliable and sensitive technique for analysing calcium and phosphate for studies modelling erosion [Grenby, 1996] It can detect very low concentrations (part per million 'ppm') and small volumes of metal and non-metal ions in a liquid sample [Schlueter et al., 2011a].

The advantages of ICP-CMS are it has high precision, sensitivity, speed, simple spectra, low detection limits. Previous erosion studies have used ICP-MS to analyse the mineral content of solutions as well as the mineral content of AEP [Carpenter et al., 2014; Mita et al., 2013]. ICP-MS was used to investigate predictors of erosive potential of beverages [Hara and Zero, 2008]. They analysed the calcium ion concentration, pH and titratable acidity in commercially available beverages. This study found lower levels of enamel demineralisation in beverages with the highest calcium concentrations and concluded that beverages with calcium supplements had reduced capacity to cause erosion.

The major limitation of ICP-MS is the matrix component, which requires low concentration of solids within the solution to prevent blockage of the nebulizer and sampler. However, these limitations can be avoided with the appropriate method application and calibration.

1.4.5 Scanning Electron Microscopy (SEM)

Scanning electron microscope (SEM) is an electron microscope that uses a beam of electrons to produce an image of a scanned surface. The surface topography is then produced by the electron interaction with atoms on variable depth of the surface. The

components of SEM are illustrated in {Figure 1-3}. There are two types of SEM: conventional and environmental, conventional SEM uses a high vacuum on samples, which produce artefacts and requires a metal coating on the surface. Whereas environmental SEM uses lower vacuum and is able to scan wet surfaces without the need for coating or drying the surface. The risk of artefacts is reduced by using environmental SEM; however they do not have a good resolution.

SEM has been used in in-vitro erosion studies for qualitative measurements, producing highly detailed surface morphology on a nano or micro scale [Joshi et al., 2016]. It has been used in studies characterising the differences on surfaces such as bovine enamel and polished and natural human enamel [Field et al., 2014; Field et al., 2017]. Meurman and Frank [1991] studied in-vitro the morphology of bovine enamel samples immersed in cola beverages with and without the presence of AEP using SEM. The study revealed the capability of SEM to visualise the biological variations in the surface morphology. They were able to visualise the difference in AEP layers on enamel samples as a thin film-like structure that provided protection against acidic attack.

SEM was used in an in-situ study to evaluate the influence of salivary flow rate on the pattern of demineralisation of human and bovine enamel samples in an erosion/abrasion model [Rios et al., 2008]. The study showed that it was possible to visualise the distinct destruction on both human and bovine enamel samples. No changes were seen in samples coated in nail varnish, whereas uncoated surfaces on samples subjected to erosion only showed enamel prism core dissolution. Those subjected to both erosion and abrasion showed removal of the superficial prism layer and had a more homogenous enamel surface.

SEM has many advantages including the ability to use on polished and non-polished surfaces. It can be used for imaging high mass/volume of material and produces an image of less than 1 nm size (reaching 0.4 nm). However the main limitation of SEM is that it requires gold coating to insure image precision, which causes irreversible damage to the sample surface.

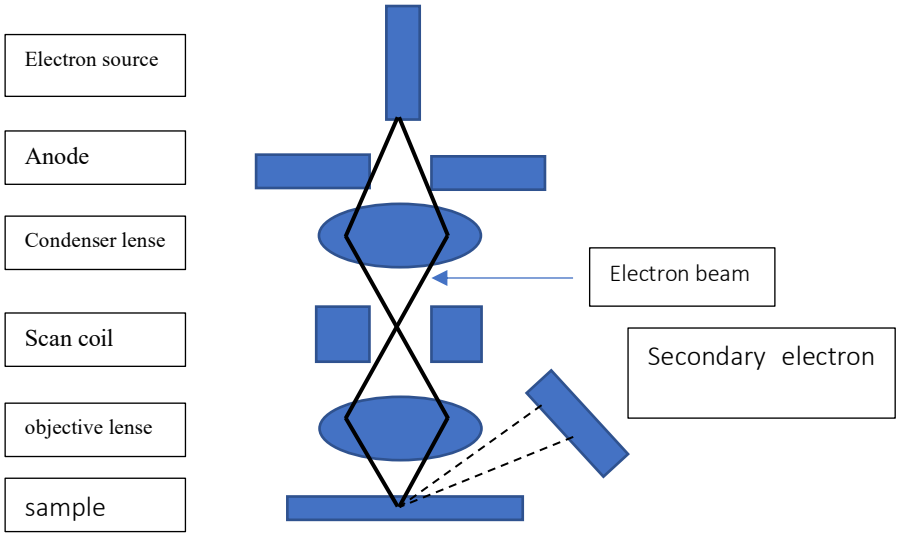


Figure 1-3: Schematic diagram of SEM components

1.4.6 Protein Identification in AEP

Since the 1970s, there have been advances in techniques for detection, identification and quantification of total protein concentration in saliva and AEP. A description of the technique used in this PhD is given below:

1.4.7 Bicinchoninic Acid Assay (BCA)

Since Smith et al.[1985] introduced the BCA protein assay in 1985, it has become widely used for quantifying total protein. The principle of this method is based on two steps: first, the biuret reaction which is an oxidisation of cupric ion (cu2+) to spurious ion (cu1+), this results in a faint blue colour. The blue colour formed is due to the interaction between three amino acids in the protein (cystine, tyrosine and tryptophan) with the copper enhancing the reduction in the biuret reaction. Second, is colorimetric detection of cuprous cation (Cu1+) by chelation with BCA resulting in a purple colour, this purple colour can be quantified by a wavelength between 550 and 570 nm. The colour formation depends on the temperature of the incubator, reagents that chelate copper and the presence of certain amino acids; hence assay standards are key to obtaining accurate results and protein quantification. Accuracy and consistency in quantifying total protein in saliva and AEP samples depends on the protein standards [Cheaib and Lussi, 2013]. BCA assay are inexpensive and simple to use. it has been used in several studies quantifying total protein concentration in natural saliva and AEP [Baumann et al., 2016; Moazzez et al., 2014; Walsh et al., 2004].

1.5 Laboratory Models for Erosion and ETW

1.5.1 Type of sample

Human and non-human enamel and dentine samples have been used in ETW research. In a workshop on ETW research methodologies, it has been reported that human samples are the sample of choice [Shellis et al., 2011]. In most studies human enamel samples have been polished to allow for accurate measurements. Polished enamel samples offer many advantages including: standardisation, ease of characterising surface changes and ease of application of acid resistant barriers [Shellis et al., 2011; Young and Tenuta, 2011]. However, they are less acid resistant compared to natural enamel samples, due to the fact that outer enamel tissue is composed of more loosely packed enamel rods [Zheng et al., 2010]. Whereas deeper enamel tissue has reduced mineral concentration, increased porosity and solubility [Cuy et al., 2002]. Therefore, the polishing process results in less acid resistant enamel as it removes the outer enamel layer [Zheng et al., 2010]. Recently, studies have used natural (non-polished) enamel as it mimics the clinical circumstances more closely [Young and Tenuta, 2011]. Although natural enamel samples are more relevant to clinical situations, they have limitations such as individual variability and limitation in measurement techniques [Ganss et al., 2000; Young and Tenuta, 2011].

Ganss et al.[2000] assessed the difference between polished and natural (non-polished) human enamel samples under an erosive challenge, they reported that polished enamel loss was statistically higher compared to natural samples after immersion in citric acid for 3 hours, using profilometry measurement. Likewise, Lin et al.[2017] reported that enamel loss, microhardness change and calcium loss were

statistically higher in polished enamel samples compared to natural samples after a cycle of 6 times immersion in 1% citric acid (pH 3.6) for 1 minute. Mylonas et al.[2018] evaluated the capability of quantitative and qualitative measurements in characterising ETW in both polished and natural enamel samples. They immersed samples in 0.3% citric acid for 0, 10, 30, 60, 120 and 300 seconds. The reported results showed that step height measurement using profilometry was measurable for polished enamel samples at 60, 120 and 300 seconds, whereas it was not measurable for natural enamel samples at any time point. Similarly, surface microhardness measurement for polished enamel samples decreased significantly at all time points but it was unmeasurable for natural enamel samples. They concluded that quantitative measurements were not as accurate and standardised on natural enamel samples compared with polished samples. Overall, Schlueter et al.[2011a] suggested to use flat polished enamel samples in order obtain accurate results.

Non-human enamel samples have been utilised in previous ETW studies. Since collection of human samples can be difficult, expensive and time consuming, enamel samples from bovine, ovine or hydroxeapetite discs have been used as alternatives. However, conflicting results can be found in the literature with regards to the suitability of bovine enamel. Some studies have reported similarities to human enamel in their ability to mimic erosion [Barbour and Rees, 2004; Shellis et al., 2011], whereas others reported differences between the two [Field et al., 2017; White et al., 2010]. White et al. [2010] investigated early and late erosion on both human and bovine enamel samples. The study detected a significant decrease in human enamel microhardness after 2 seconds exposure to acid, and bovine enamel samples after 5 seconds. Whereas statistically significant decrease was detected in late erosion times at 20 minutes for

human enamel samples, and at 10 minutes for bovine enamel samples. They concluded that human and bovine enamel produced similar erosion characteristics in early erosion models, whilst with severe erosion models, bovine enamel demonstrated more rapid erosion compared to human enamel. They speculated that the difference is due to the greater bovine enamel porosity and larger crystallites.

1.5.2 Sample preparation

Samples irrespective of their origin should follow a cleaning and preparation regime in order to ensure they are standardised and sterilised to be used in-vitro or in-vivo. The processes include ultrasonication, chemical and mechanical processes.

Polishing of enamel samples produces a smear layer on the enamel surface. To ensure consistency and cleanliness prior to acid treatment, this layer needs to be removed [Young and Tenuta, 2011]. The reported process includes ultrasonication of samples in deionised water for 15 minutes which successfully removes the smear layer [Austin et al., 2016b; Mistry et al., 2015b; Mullan et al., 2018a; Mullan et al., 2017a; O'Toole et al., 2016].

Sodium hypochlorite (NaOCl)[Lippert et al., 2004a; Lippert et al., 2004b], thymol solution [Shellis et al., 2011] and chloramine [Field et al., 2014; Field et al., 2017] have been used as cleaning agents without affecting the physiochemical properties of the enamel surface. Mechanical cleaning processes include the use of a scaler to remove any surface contaminants [Klimek et al., 1982] and the use of toothbrush and toothpaste to clean samples before polishing [Amaechi et al., 1999b]. West et al.[2000] described a chemo-mechanical cleaning process using 50% NaOCl and scraping the debris manually,

followed by washing in deionised water and sonication in 70% ethyl alcohol followed by washing in deionised water.

1.5.3 Type of acid

In-vitro studies of ETW have used acids depending on whether they were modelling extrinsic (dietary) or intrinsic (gastric) conditions. For modelling extrinsic acids, citric acid is most commonly used to stimulate dietary intake and mostly at 0.3% concentration with a ranging pH between 2.6 to 3.8 [Cheng et al., 2009c; Hjortsjo et al., 2010; Shellis et al., 2013; Young and Tenuta, 2011]. Some studies have used fruit juices and other soft drinks to more closely mimic dietary intake. Commercially produced drinks may have other added substances and there may be variations in different batches and therefore standardisation is more difficult [Shellis et al., 2011]. Other studies have investigated malic, lactic and acetic acids [Hughes et al., 2000].

For modelling intrinsic acids, hydrochloric acid (HCl) is most commonly used simulating gastric refluxate [Young and Tenuta, 2011]. However, other studies have used artificial gastric juice as well as a combination of pepsin, trypsin and gastric enzymes to mimic the clinical situation. Schlueter et al.[2010] investigated the effect of HCl, pepsin, trypsin and the combination of pepsin and trypsin on human dentine samples under an erosive cycle over six days. They assessed the mineral content and matrix degradation using microradiographs and hydroxyproline analysis and found that the combination of both pepsin and tryspin led to significantly increased mineral loss compared to others. In addition, Mann et al.[2014] assessed qualitatively (using scanning electron microscope) the effect of short repetitive erosive challenges on human enamel samples with HCl. Samples were immersed in HCl of different pH levels (1.5 and 3.0) for 30 seconds, 60

seconds and 120 seconds. They concluded that enamel is influenced by both pH concentration and acid exposure duration.

1.5.4 Acid treatment (Duration, Cycles, Agitation)

Erosion regimes, in in-vitro studies, vary depending on acid exposure time and number of erosive cycles and stirring [Young and Tenuta, 2011]. The exposure time of an erosive challenge depends on the pH of the erosive agent and the constancy of the solution composition determined by the clinical model being investigated [Shellis et al., 2011]. Variable acid exposure times have been reported in the literature, early erosion models reported periods ranging between several seconds and 120 minutes [Baumann et al., 2016; Mann et al., 2014; Mistry et al., 2015a; O'Toole et al., 2015a].

Mylonas et al. [2018] investigated the effect of an early erosion model on polished human enamel using profilometry and reported detection of enamel loss after 60 seconds exposure to citric acid. Surface roughness changes could be detected as early as 10 seconds of citric acid exposure. Field et al. [2017] observed the earliest time point to detect changes on polished human, bovine and ovine enamel was after 30 seconds of exposure to citric acid. Likewise, Austin et al. [2016a] observed similar results when exposing polished human enamel samples to citric acid. They observed that enamel loss was detected as early as 30 seconds of acidic challenge.

Late erosion models have been applied in in-vitro studies mainly to assess the effect of AEP, oral care products and the effect of acidic exposure to natural human enamel tissue. These models include time points greater than 5 minutes. Mullan et al. [2017a] investigated the effect of three cycles of orange juice (pH3.2) for 15 minutes on polished and natural human enamel. The results demonstrated that compared to baseline, polished enamel samples showed significant increase in median (IQR) roughness at 15,

30 and 45 minutes (+0.17 (0.13), +0.12 (0.09), +0.18 (0.15)) respectively. Whereas natural enamel samples showed a significant decrease in median (IQR) roughness only after 45 minutes erosion (-0.14 (0.34)). The study supported the concept that natural enamel requires increased erosion time in order to detect changes quantitatively.

Cycling models of erosive challenges are applied either to assess the effect of storage in a remineralising solution or to assess the effect of various exposures to acidic challenge. Cycling is done either manually transferring samples between media or by an automated system called "artificial mouth model". Many factors should be considered when using a cycling model including the length of each challenge, number of cycles, timing of each cycle and the time of each solution [Shellis et al., 2011]. Mutahar et al.[2017b] investigated the effect of AEP on polished human enamel under an acidic challenge using five cycles of citric acid exposure for 10 minutes, with immersion in pooled human whole mouth saliva for 30 minutes in between each cycle. The authors concluded that the presence of AEP resulted in less enamel loss but greater surface microhardness change.

Agitation of the acid solution during the erosion challenge is one of the factors that influences the amount of erosion in-vitro [Barbour and Rees, 2004; Shellis et al., 2005]. Some studies have assessed erosion caused by acids under static (un-agitated) conditions [Cheng et al., 2009a; Levy et al., 2012], whereas others have used agitation at various velocities [Lussi et al., 2000; Shellis et al., 2005]. A study by Mistry et al.[2015a] investigated the effect of using three most commonly used stirrers for agitation at four speeds (30, 40, 60 and 70 rpm), Orbital shaker (Stuart Orbital Shaker SS1; 2D circular orbital motion), See-Saw rocker Stuart See-Saw rocker SSL4, 3D; up and down rocking action from a central pivot) and Gyro rocker (Stuart 3D gyratory rocker SSL3; 3D up and

down, circular motion from a central point). Samples were eroded by 5 cycles of 0.3% citric acid (pH3.2) for 10 minutes. They measured step height and surface microhardness change and reported that agitation increased the step height, and that type of stirrer and speed influenced enamel loss. The orbital shaker produced the lowest mean step height compared to the other stirrers.

Their results were supported by others reporting that erosion depth is increased with increased flow rate of the acid and increased speed of agitation [Attin and Wegehaupt, 2014; Shellis et al., 2005]. At a high speed of agitation, the solution could physically remove the dissolved tissue resulting in greater tissue loss. It could also replace fresh ions on the dissolving solution increasing tissue loss. Therefore, it was recommended by Shellis et al.[2011] to control the flow rate of solution in a reproducible manner either by using a calibrated stirrer [Hemingway et al., 2008] or a pumping chamber at a known rate [Attin et al., 2003]. In general, agitation constantly renews the solution in contact with the enamel surface hence it enhances the dissolution process.

Overall, early and late erosion models, both single and multiple cycles of acidic exposure should fall within a clinically relevant situation being modelled, it should also be adjusted to suit the sample type being used.

1.5.5 Saliva and AEP variables

1.5.5.1 *Natural vs artificial*

Studies investigating saliva and AEP in relation to ETW have utilised a variety of methods and techniques for saliva and AEP collection, storage and experimental design, which makes standardisation and comparison difficult.

The type of natural saliva used in ETW studies includes whole mouth saliva, or saliva from specific glands. Human natural saliva exhibits many issues, such as diversity, saliva quality and quantity. These are dependent on many factors such as diet, medical condition, medications, age, gender and collection time of the day [Greabu et al., 2009; Humphrey and Williamson, 2001]. Thus, collection of whole mouth saliva should be standardised.

Furthermore, natural saliva is commonly replaced by artificial saliva due to difficulty in collecting and storing natural saliva, degradation and consistency in salivary components [Hara et al., 2008; Schipper et al., 2007a]. There is no consensus on the formulation of artificial saliva in the literature, however artificial saliva should simulate natural saliva as far as possible with regards to lubrication, protection and remineralisation potential [Austin et al., 2016b; Batista et al., 2016].

Many in-vitro studies have assessed and compared the use of natural and artificial saliva. Artificial saliva lacks the protein component which is an important factor for the anti-erosive property of saliva [Tschoppe et al., 2009]. Cheaib and Lussi [2011] and Hannig and Joiner [2006b] observed that protein components in natural saliva contribute to the formation of AEP and hence have an enhanced protective effect against ETW [Cheaib and Lussi, 2011; Hannig and Joiner, 2006a; Hellwig et al., 2013; Mutahar et al., 2017a]. In addition, Mutahar et al.[2017b] investigated the effect of

immersing human polished enamel samples in natural saliva, artificial saliva and deionised water for 24 hours against an erosive challenge. The study measured the enamel loss using profilometry and surface microhardness change after exposing samples to five erosive cycles. They demonstrated that natural saliva resulted in significantly less enamel loss but greater surface microhardness change and provided better protection against citric acid attack compared to artificial saliva and deionised water.

1.5.5.2 *Stimulated vs resting saliva*

Different methods have been reported in the literature for collecting resting or stimulated natural saliva. Resting saliva collection methods include the use of filter paper swabs, suctioning, draining by dripping saliva from the lower lip and spitting by expectorating saliva into a test tube [Navazesh, 1993; Navazesh and Christensen, 1982]. Stimulated whole mouth saliva is generally collected by asking the patients to chew on a piece of gum or paraffin wax or sucking a candy prior to collection [Jensdottir et al., 2005a; Turner and Sugiya, 2002]. Stimulated whole mouth saliva has been used in in-vitro studies more commonly than resting saliva; as it is faster and easier to collect, has a higher pH, higher buffering capacity and is more lubricating [Schlueter et al., 2011b].

A study by Michishige et al. [2006] compared the effect of different saliva collection methods and reported saliva volume was 2 folds greater when collected by suction compared to spitting and swab. They also investigated the protein components in saliva using those three different methods and found the total protein concentration was the same when saliva was collected by suction and spitting but it was lower when collected by using the swab method.

Most of the proteins found in resting saliva are derived form parotid glands, whereas in stimulated saliva 75% of proteins are derived from parotid glands and 25% from submandibular and sublingual glands [Carpenter, 2013; Rantonen and Meurman, 2000]. Comparing the levels of proteins between resting and stimulated saliva, statherin was found in high levels in stimulated saliva, whereas mucin5b (MUC5B) levels were higher in resting saliva [Rayment et al., 2001].

1.5.5.3 *Pooled vs single donor*

There is a wide variation in the literature regarding using saliva from a single donor or pooled from various donors [Faller et al., 2011; Hellwig et al., 2013; Mutahar et al., 2017a; Schlueter et al., 2011a; Wetton et al., 2007]. Single donor saliva has more consistency, whereas pooled saliva compensated for individual variations. Wetton et al.[2007] investigated the levels of saliva protection from 14 subjects on human enamel samples after an erosive challenge with citric acid. The study resulted in a significantly decreased enamel loss on profilometry when pooled saliva was used, whereas samples immersed in saliva from a single donor had significantly increased enamel loss when compared to the control. They concluded that the AEP protective effect in-vitro varies between indivduals.

1.5.5.4 *Fresh vs frozen*

In-vitro AEP has been formed either by using fresh saliva collected daily [Batista et al., 2016; Faller et al., 2011] or freezing the collected saliva at -80 °C and thawing prior to use, since using fresh WMS on daily basis is not always practical [Brevik et al., 2013; Hellwig et al., 2013]. The protective effect of these two different methods has been studied and no significant difference in protection against erosion was found [Hemingway et al., 2010]. The collected natural saliva is generally stored on ice for short term usage, and in a -80°C freezer for longer storage periods [Schipper et al., 2007b].

1.5.5.5 *AEP collection*

Research on the role of AEP on erosion and ETW has included formation of AEP in i-vitro and in-situ models as well as collection of AEP in-vivo. In-vitro models generally

involve placing samples in whole mouth saliva, glandular secretions or mixed purified proteins. This method is more convenient to collect vast amount of proteins, providing information of proteins interactions and affinity to hydroxyapatite. However, in-vitro models lack the dynamic conditions of the mouth, such as salivary flow and clearance. In in-situ models, either bovine or human enamel samples have been used. Most have used samples mounted on an intra-oral appliance [Carpenter et al., 2014]. The advantage of in-situ models is the ability to collect AEP in standardised models within the oral cavity [Hannig et al., 2004a; Hara et al., 2006a]. AEP collection through in-vivo models are challenging, since the process is technique sensitive. However, key proteins such as histatin, MUC5B, statherin, carbonic anhydrase, lactoferrin, cystatin and lysozyme were detectable in in-vivo models [Mutahar et al., 2017c; Yao et al., 2003].

1.5.5.6 *Length of AEP formation*

Studies have reported that AEP forms within moments of brushing and reaches saturation between 30 minutes and 2 hours [Ash et al., 2014; Hannig et al., 2004c], others have suggested a longer period of maturation ranging between 24 hours and several days [Hannig et al., 2004a; Hannig and Hannig, 2014]. Hannig et al.[2003a] investigated the effect of in-situ formed AEP ranging from 2 to 24 hours. They measured the protective effect based on the amount of dissolved calcium after immersion of enamel samples in 1% citric acid for 60 seconds. They found no significant difference in calcium release between different AEP formation times and therefore concluded that 2 hours formed AEP provided protection. Other studies have shown that 60 minutes in-vitro or in-situ formed AEP provides an equal protective effect against erosion when compared to AEP formed over longer times and the effect of ETW is not reduced further at any longer

periods [Hannig et al., 2003b; Wetton et al., 2006]. In addition, Hannig et al.[2004b] reported no difference in protection and inhibition of demineralisation when comparing in-situ AEP formation times at 30, 60 and 120 minutes, or when comparing AEP formation times at 2, 6, 12 and 24 hours. Hannig et al.[2004b] investigated the protective effect of in-situ formed AEP over periods of 30 minutes, 1 and 2 hours. They showed that there was no difference in the protective effect of 3 minutes and 2 hours.

However, others reported that maturation time affects the level of protection offered by AEP [Hannig and Hannig, 2014]. Amerongen et al.[1987] and Featherstone et al. [1993] reported that the longer the formation time of in-vitro AEP the better the protection. In addition, Mutahar et al.[2017a] found that 24 hours formed AEP provided better protection compared to 30 and 60 minutes.

1.5.5.7 *Number of cycles*

In terms of the number of times samples are immersed in natural saliva to form AEP, some studies have used a single immersion method [Brevik et al., 2013] whereas others have used multiple immersions. Multiple immersions results in re-formation of new protein layers which covers the enamel surface building new protective barriers [Hannig, 2002].

1.5.5.8 *AEP harvest and recovery: In-vivo*

In-vivo AEP can be harvested and analysed in the laboratory. It provides details such as total protein concentration and details about individual proteins. There are however challenges including difficulty in achieving standardisation due to individual variabilities as well as the difficulty in standardising the surface area from which AEP is collected. A larger surface area could result in different amounts of sample collected leading to greater amount of proteins captured.

Many methods and techniques have been introduced for AEP collection. Sōnju et al.[1997] was the first to introduce in-vivo AEP collection, their technique used a mechanical scaler on a tooth surface. Later in 1989, Alhashimi and Levine [1989] found a more effective way by using hydrophilic polyvinylidene difluoride membrane with scaling. Other techniques included mechanically scrubbing Whatman paper, filter papers [Svendsen et al., 2008] and filter pellets all soaked in sodium dodecyl sulfate (SDS) [Carlen et al., 1998; Hannig et al., 2005a]. The use of combined mechanical and chemical means of collecting AEP, has been found to improve efficiency of AEP collection [Hannig et al., 2005a; Li et al., 2004].

Different chemical agents have been applied for removal of AEP, such as sodium hypochlorite, sodium phosphate and hydrochloric acid [Hannig and Balz, 1999; Hannig et al., 2005b; Mayhall, 1970; Taira et al., 2018]. However, SDS has been frequently used to elute AEP at different concentrations. SDS in combination with mechanical rubbing of human enamel surface has been shown to completely remove in-vivo formed AEP [Svendsen et al., 2008]. SDS interacts with the enamel surface through different mechanisms, depending on surface properties, film thickness, time of protein adsorption

and SDS concentration. SDS binds and forms complexes with the adsorbed proteins [Svendsen et al., 2008].

1.6 Gastro-oesophageal Reflux Disease (GORD)

GORD is defined by the Montreal consensus as "a condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications" with oesophageal/typical and extraoesophageal/atypical symptoms [Vakil et al.,2007b]. GORD can occur daytime and/or during sleep (nocturnal). Around 40-81% of patients suffering from GORD report that their symptoms occur during sleep [Orr, 2010].

GORD is a highly prevalent disease, affecting 8-33% of the worldwide population [Gyawali et al., 2018; Savarino et al., 2009]. In the United States, 40% of the adult population suffers from GORD, ranking it the 4th most prevalence disease within the gastrointestinal field [Chiocca et al., 2005]. The estimated prevalence in the middle east is 8.7-33.1% [Boeckxstaens et al., 2014], and 21% of the Argentinean adult population, [Cho et al., 2005; Fujiwara et al., 2005]. In Europe, 31% of the Norwegian population and 21% of the Finnish population reported at least monthly heartburn [Isolauri and Laippala, 1995; Nilsson et al., 2004]. In the United Kingdom, a survey on 2000 households showed that 20% of the adult population in the UK experience symptoms of GORD at least twice a week [Hershcovici and Fass, 2011].

1.6.1 Physiology of The Oesophagus

The oesophagus is approximately 25cm long hollow muscular tube with a diameter of 2.5 cm. It extends from the level of the 6th cervical vertebra around the upper oesophageal sphincter (UOS) to about 2 cm below the lower oesophageal sphincter (LOS) where is joins the stomach. The upper third of the proximal oesophagus is composed of striated muscles (cervical oesophagus) and the distal lower third is smooth muscles

(thoracic and abdominal oesophagus), this differentiation in the muscular arrangement controls the pace of the motor waves from the upper part to the lower part where it slows down [Richter and Castell, 2011].

The gastro-oesophageal junction is a key anti-reflux barrier, located at the lower 4 cm of the oesophagus where it passes through a tunnel between the diaphragmatic crura and enters the upper part of the abdominal cavity and ends at the proximal stomach. Any incompetence of this junction causes retrograde movement of the stomach content [Richter and Castell, 2011].

The LOS is an anti-reflux barrier, it maintains the oesophageal average pressure to about 20 mmHg. Within 1.5 to 2.5 seconds of a swallow, the pressure of the LOS drops and remains low. Peristalsis is the sequential simultaneous muscle contraction producing a wave that travels through the oesophagus all the way to the stomach.

1.6.2 Aetiology of GORD

Any incompetence of the anti-reflux barriers can lead to episodes of retrograde movements of stomach contents into the oesophagus, mostly after a meal. These episodes are diagnosed when the oesophageal pH drops below 4 and lasts at for least 30 seconds [Orr, 2005].

The main mechanism for reflux episodes is transient lower oesophageal sphincter relaxation (TLESR), commonly affected by the consumption of alcohol, caffeine, nicotine, medications such as non-steroidal anti-inflammatory drugs, which delay the emptying of the gastric contents and increase acid secretion. The presence of a hiatus hernia is a predisposing factor in developing GORD as it weakens the LOS and stops the role of preventing the retrograde movement of stomach contents [Bredenoord, 2012]. Moreover, a hiatus hernia is associated with decreased acid clearance, decreased LOS pressure and increased reflux [Sifrim, 2006], it has been reported that as the hernia size increases reflux increases [Jones et al., 2001; Record Owner]. Additionally, conditions like pregnancy, straining, bending and obesity lead to an increase in the intra-abdominal pressure overcoming the LOS pressure and leading to GOR. Heavy meals and acidic/spicy beverages and food can also increase gastric volume which is a risk factor for developing GORD [Dodds et al., 1982]. {Table 1-3} summarises a list of possible predisposing factors in developing GORD [Diamant, 2006].

Symptoms related to oesophageal motility disorders are mostly related to the distal oesophagus rather than the proximal oesophagus. Smooth muscles disorders of distal oesophagus can be divided based on the type of innervation into those related to inhibitory innervation and those related to excitatory innervation. Conditions that may develop due to defective Inhibitory innervation include achalasia (a type of motility

disorder that is caused by the loss of oesophageal contractions and peristaltic sequence causing symptoms like regurgitation, dysphasia and chest pain), transient lower oesophageal relaxation (TLESR) and diffused oesphageal spasm. Those associated with a defective excitatory innervation include peristalsis, hypo/hypertensive LOS and decreased LOS contraction [Paterson et al., 2006].

Physiological and structural incompetance of the gastro-oesophageal junction (LOS, diaphragm, etc.)
Defective Transient LOS relaxation TLESR
Lack of Oesophageal clearance deficiency (motility, salivary)
Defective Mucosal integrity
Sensory mechanism
Irritant drugs (NSAIDs, antibiotics)
Drugs affecting oesophagus, motility of gastric content and LOS (Alcohol, caffeine, nicotine)
Other factors (genetic, psychological)

Table 1-3: Important factors in GORD development [Diamant, 2006]

1.6.3 Symptoms of Gastro-oesophageal Reflux Disease

When gastro-oesophageal reflux (GOR) progresses to the level that it produces gastric symptoms it becomes a disease. The first line of treatment of GORD in the UK is prescription of proton pump inhibitors (PPI). If the patient symptoms do not respond to PPI treatment for 12 weeks or more, then the condition is referred to as “refractory GORD” and symptoms considered as “refractory symptom”[Sifrim and Zerbib, 2012]. A global classification of GORD symptoms has been developed by the Montreal consensus

group based on evidence from 18 countries and has been divided as illustrated in the flow chart in {Figure 1-4}:

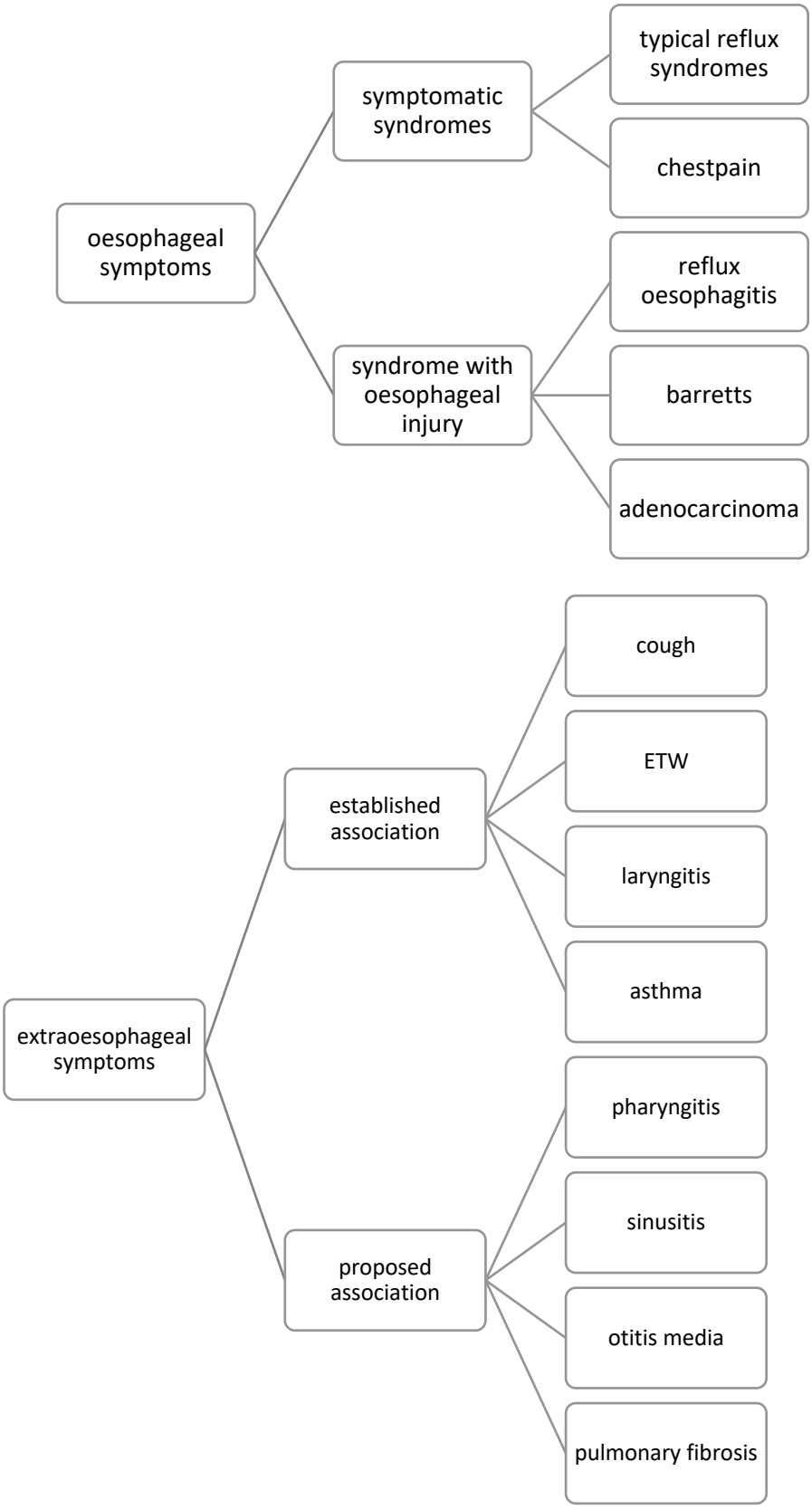


Figure 1-4: Flow chart of GORD symptoms classification [Vakil et al., 2007b]

1.6.3.1 *Oesophageal Symptoms*

Heartburn

Heartburn is defined as a retrosternal burning sensation along the length of the oesophagus and is considered a classical symptom of GORD. It is a very common symptom in western countries. Around 20% of the population complain of heartburn compared with 5% in Asia [Dent et al., 2005]. A study carried out in the UK showed that half of the British population experienced heartburn at least once a month [Bennett, 1991]. There are reports that between 72-99% of patients with pathological oesophageal acid exposure suffer from heartburn [Costantini et al., 1993; Vakil et al., 2007a; Wiklund et al., 2006]. Broderick et al.[2019] more recently investigated the presence of GORD symptoms including (heartburn, regurgitation, epigastric pain, respiratory symptoms, epigastric pain) in 1,031 GORD patients. The study methodology included the use of 3 types of questionnaires: self-reported, free form self-reported and nurse solicitated. They reported that heartburn and regurgitation were the most common prevalent symptoms (82.4% and 58.8% respectively).

Regurgitation

Regurgitation is defined as the involuntary movement of stomach contents into the oesophagus reaching the pharynx and UOS. Regurgitation is another classical symptom of GORD along with heartburn, with high prevalence ranges between 33-86% [Benz et al.; Costantini et al., 1993; Vakil et al., 2007b]. If there is an impairment in the anti-reflux

mechanism, such as incompetent UOS, the stomach contents could reach above the UOS entering the respiratory airways or the oral cavity.

Dysphasia

Dysphagia is defined as difficulty swallowing due to ineffective oesophageal transport caused by impaired LOS relaxation or non-peristaltic oesophageal contractions. It primarily is related to solids but in a supine position it can also occur with liquids as well [Paterson et al., 2006].

Belching

Belching or burping is a common condition in healthy adults, but when the symptom becomes excessive it might be as a result of LOS relaxation or GORD. Belching events have been correlated with pathological GORD [Barham et al., 1993]. In a study investigating a group of patients suffering with dyspepsia and a group with GORD, belching was related to acid reflux episodes more frequently in GORD patients compared with dyspeptic patients [Lin and Triadafilopoulos, 2003]. However other studies have suggested that acid reflux may be a consequence rather than a cause of belching [Sifrim et al., 2001].

Epigastric pain

Epigastric pain is defined as chronic pain or discomfort in the centre of the upper stomach, which may occur as an ulcer-type pain when it is localised, or reflux-like symptoms when associated with other symptoms such as heartburn, or a dysmotility-like symptom when associated with bloating. It has been reported that patients may have

epigastric pain even when the total distal acid exposure time is normal but would be as a result of the presence of acid in the oesophagus [Vakil, 2003]. Furthermore, 12-67% of patients with pathological acid reflux exposure reported epigastric pain [Costantini et al., 1993; Vakil et al., 2007b].

Chest pain

Chest pain is defined as recurring squeezing or pressure feeling behind the breastbone, it is of a non-cardiac origin and can be confused with the cardiac chest pain, if not diagnosed by proper history and physical examinations as both have similar description and can be improved by similar medications (nitro-glycerine). Chest pain could be caused by oesophageal motility disorders, such as, diffused oesophageal spasm and nutcracker oesophagus, hence patients suffering from chest pain after treatment for GORD should undergo oesophageal motility testing to rule out these disorders [Saritas Yuksel and Vaezi, 2012]. However, the most common cause is the direct contact of oesophageal mucosa with the refluxate contents such as pepsin and acids. A study by Fass et al. [1998] found 50-60% of patients with chest pain had GORD and that the most common cause of chest pain was GORD [Fass and Navarro-Rodriguez, 2008]. Furthermore, a study conducted on 52 patients with chest pain reported that 58% of them suffered from GORD [Karlaftis et al., 2013].

1.6.3.2 *Extra-Oesophageal Symptoms*

Extraoesophageal symptoms can occur as a consequence of either direct exposure of the oesophageal lining to gastric refluxate (gastric acid, bile, pepsin, etc) which could lead to a cough reflux, or by an indirect mechanism [Shaker, 2000]. There is also an indirect mechanism in which the refluxate interacts with acid sensitive receptors in the distal oesophagus stimulating vagally mediated reflux causing bronchoconstriction [Tokayer, 2008].

Data for extraoesophageal symptoms varies widely in the literature and they can be divided into symptoms with ascertained association such as: cough, laryngitis, asthma, hoarseness and ETW, and those with a proposed association such as: pharyngitis, sinusitis, pulmonary fibrosis and otitis media.

Cough

Chronic reflux cough is defined as persistent coughing events for more than 8 weeks period, the most common causes of this condition is postnasal drip syndrome (PNDS), bronchitis, asthma and GORD. Cough related to reflux should be solely present without signs of asthma , PNDS or due to cough inducing medications [Irwin, 2006]. GORD is the third leading cause of cough and is the primary cause in 10% of patients [Irwin et al., 1981]. A wide range between 30-50% of GORD patients have been reported to suffer from chronic cough symptom [Ludviksdottir et al., 1996]. Moreover, Sifrim et al.[2005] reported a high correlation between weekly acid reflux episodes and cough events. They found that 52% of reflux-related cough patients show abnormal acid exposure on pH monitoring test [Alhabib et al., 2007]. However, 75% of patients suffering from cough

related to GORD do not show any classic GORD symptoms such as heartburn and regurgitation [Everett and Morice, 2007].

Hoarseness

The vocal cords are two muscular bands within the larynx, which is part of the respiratory tract, these bands vibrate to produce sound. Hoarseness is defined as an abnormal change in voice ranging from deep, harsh to husky or hoarse like voice, it is uncertain whether the irritation to the vocal cord is due to acid insult or chronic throat clearance by coughing. It can be caused by other conditions such as: allergies, smoking, thyroid problems, upper respiratory tract infection, trauma to vocal cord or larynx and overuse of vocal cords. The association of hoarseness and GORD has been recognised since 1989 and in a study by Wiener et al. [1989], 79% of patients who had undergone pH monitoring for assessing the chronic hoarseness were diagnosed with severe GORD. Vaezi et al. [2003] reported that 10% of patients suffering from GORD had hoarseness. Moreover, it was estimated that hoarseness was caused by GORD in 10% of patients attending clinics [Vaezi et al., 2003]. In addition, 55% to 79% of patients suffering from hoarseness had acid reflux when they underwent 24 hours pH-monitoring [McNally et al., 1989].

1.6.4 Diagnostic Tools For GORD

Indications for clinical testing of GORD includes failure of proton pump inhibitors (PPI) to improve symptoms, uncertain diagnosis of the condition, refractory symptoms and GORD complications.

The primary focus in testing is to detect the acid reflux causing pathological GORD, combining diagnostic tools aiding to identify the symptoms and the disease pathophysiology. The initial diagnostic approach when reflux is suspected is clinical history and trial of proton pump inhibitors, when there is a lack of proton pump inhibitors response subsequent approaches are utilised. There are many proposed techniques available such as symptom questionnaires, microscopy of mucosal biopsies, narrow band imaging, barium radiographs, endoscopic measurement of intragastric pressure, bile monitoring, upper endoscopy, ambulatory reflux monitoring and adjunctive metrices including (baseline impedance, post-reflux swallow-induced peristaltic wave (PSPW), mucosal integrity, high resolution manometry, salivary pepsin concentration and association with supra-gastric belch. However, most of these techniques can only be used to clarify the pathophysiology of GORD and are not diagnostic tools. The advantages and disadvantages of the most commonly used clinical diagnostic techniques for reflux are listed in {Table 1-4}.

Technique	Advantages	Disadvantages
Endoscopy	• Easy visualisation of oesophageal erosion and damage of mucosa	• Requires sedation • Low in sensitivity and specificity • Costly
pH-metry	• Easy to apply • 24 hours monitoring • Non-invasive technique	• Approximately 30% false negative rate • Cannot predict response to extraoesophageal reflux • Catheter based
Impedance monitoring	• Easy to apply • 24 hours monitoring • Non-invasive technique • Differentiate acidic, liquid and gaseous reflux	• false negative rate unknown but might be as pH-metry • unknown response to extraoesophageal reflux • Catheter based • Unknown clinical implications in cases of abnormal PPI response

Table 1-4: Advantages and disadvantages of Clinical diagnostic tools [Richter and Castell, 2011]

1.6.4.1 *Questionnaires*

Questionnaires have been used for decades in clinical trials and chronic disease diagnosis [McColl et al., 2005]. The types of questionnaires are self-administrated or interviewer-administrated, both are inexpensive and easy to use. The first has an advantage of preserving confidentiality and is convenient as it is done by the respondents at a convenient time. However, a lack of motivation could result in lower response rate and/or questions could be wrongly filled as it can be misunderstood without the interviewer's help. In addition, it can be time consuming as it would take time between sending and collecting. Self-administered questionnaires can be web-based or mailed, wed-based questionnaires have been used more recently, as they can be easily set up and sent via a link to a large number of people. They can potentially result in a higher response rate compared to mailed ones. Also, they are less time consuming since investigators could collect the data without entering the data manually.

Interviewer-administered questionnaires are carried out face to face or via telephone. The advantages of this type of questionnaires are that the presence of the interviewer helps the respondent by clarifying ambiguous questions and they can beused for illiterate people. However, the effect of interviewers' perception might introduce bias. Another disadvantage could be the need for more than one interviewer in cases of large surveys, which would result in lack of consistency and increase in resources.

Questions can be open or closed. Open questions are mostly applied when generating a hypothesis in qualitative research, but they are more subjective to interviewer bias. Closed questions are more simple and quicker to answer, easier to code and analyse, simpler to compare and report. However, they are limited by the number of

answers [Ritchie and Lewis, 2003]. Closed questions can be in used in formats such as; single choice, checklist, rating scale and Likert scale.

Validation of questionnaires is essential. A questionnaire is considered validated when it represents high reliability (produces the same answers when applied to the same population) and high internal consistency (analysing the responses to questions assessing the same concept). Validated questionnaires are often used when a large number of questions are required, such as in chronic diseases and quality of life studies [Silman and Macfarlane, 2002]. Questionnaires should be validated for the population of interest as differences exist between ethnic groups in regard to symptoms [Holloway, 2009].

Questionnaires are either discriminative (focusing on diagnosis), predictive (indicating the likelihood of developing a disease) or evaluative (concentrating on the severity and magnitude of a symptom over time).

A wide variety of questionnaires have been developed, validated, translated, evaluated for clinical application to aid assessing GORD [Bolier et al., 2015]. Multiple dimensions should be considered when diagnosing GORD using a questionnaire, these include: considering typical and atypical symptoms, treatment response measured by changes in recorded frequency and intensity of symptoms, diagnosis by discriminating GORD from other diseases and effect of GORD on quality of life [Mouli and Ahuja, 2011]. Besides these dimensions, questionnaire differ in assessment characteristics such as disease-specific questionnaires, gastrointestinal-generic questionnaires and multidimensional questionnaires (cover all symptoms dimensions) for assessing GORD [Yacavone et al., 2001].

Diagnostic questionnaires, as short self-reported questionnaires, have been used in clinical practice to assess the diagnosis of disorders [Dent et al., 2010]. Questionnaires

such as reflux disease questionnaire (RDQ) [Shaw et al., 2001], gastro-oesophageal reflux disease questionnaire (GERDQ) [Jones et al., 2009] and reflux symptoms questionnaire (RESQ) [Rydén et al., 2013] and several others used to diagnose GORD should be as brief and use modified and tested words assisted by focus groups [Dent et al., 2010]. The GORD impact scale (GIS) was designed to assess the impact of symptoms suggestive of GORD rather than diagnosing the condition [Jones et al., 2007], whereas the RDQ was designed for those attending primary care to divide symptoms into those originating from the upper abdominal or lower retrosternal areas. The RDQ is the most prominent instrument used to assess frequency and intensity of individual symptoms constructed on general population suffering from GORD. The RESQ-7 was developed based on RDQ for GORD patients with partial response to PPI. It demonstrated good-to-excellent test-retest reliability results and high inter-item correlation indicating high internal consistency and reliability. When comparing RDQ and RESQ-7, RESQ-7 uses the 6 items in RDQ (burning feeling behind breastbone, pain behind breastbone, burning feeling in the centre of upper stomach, pain in the centre of upper stomach, acid taste in your mouth, unpleasant movement of material upwards from the stomach) as well as 7 additional items (burping, hoarseness, coughing, difficulty swallowing, a bitter taste in your mouth, stomach contents moving upwards to your throat or mouth, heartburn).

When comparing the accuracy of pH-metry and questionnaire-based (RDQ) diagnosis of GORD, pH-metry showed 70% sensitivity and 67% specificity, whereas the questionnaire had 62% sensitivity and 67% specificity [Dent et al., 2010].

1.6.4.2 *Proton pump inhibitors (PPI) trials/ Endoscopy*

A trial comparing PPI treatment with endoscopy and conventional pH-metry in diagnosing GORD in patients with heartburn, had 71% sensitivity and only 44% specificity compared to 67% sensitivity and 70% specificity of combined endoscopy and pH-metry [Dent et al., 2010]. A study by Bytzer et al.[2012] assessed the efficacy of proton pump inhibitors in diagnosing GORD in 308 patients with symptoms, in conjunction with other clinical tests. The study reported that proton pump inhibitors relieved symptoms in 69% of patients with oesophagitis, 35% of patients with normal endoscopy and pH-metry and 49% of patients with non-erosive reflux disease. Moreover, one of the limitations of using PPI trials is the low response rate to PPI in patients with atypical symptoms such as chestpain and chronic cough [Gyawali and Fass, 2018]. Thereby It was concluded that PPI therapy has limited ability to diagnose GORD.

Endoscopy provides visual examination of the oesophagus, indicated when patients do not respond to PPI therapy to detect complications and provide alternative therapies. However, it is the gold-standard for diagnosis of erosive reflux disease. Complications such as oesophagitis, Barrett's oesophagus and peptic ulcers can be visualised and used as confirmatory evidence for GORD [Roman et al., 2017b].

1.6.4.3 *High Resolution Manometry (HRM)*

In recent years, the clinical tests in monitoring oesophageal function has been rejuvenated with new technologies. High resolution manometry (HRM) and impedance monitoring are the latest in the field. Both swallowing and reflux can either have an antegrade or retrograde intralumenal flow, which is dependent on the intralumenal pressure facilitated within the bolus. The principle of HRM is to provide qualitative and quantitative measurements of pressure and peristalsis of the oesophagus. It accurately records the intra-oesophageal pressure and contractility using a topography plot to track areas of high pressure and assess oesophageal motility. The key advantages of this technology are the ability to visualise the oesophageal contractility and the ability to outline both oesophageal pressures [Kahrilas and Sifrim, 2008]. It has been applied to further explore the absence of contractility, loss of peristalsis contraction, oesophageo-gastric junction (OGJ) obstruction, ineffective oesophageal motility (IOM) and achalasia. The indications for this procedure are to diagnose the motility, prior to intralumenal device placement and preoperative assessment for monitoring.

The HRM is a catheter, composed of an assembly of 36 solid-state sensors with 1cm space, inserted trans-nasally to the stomach with sensors recording from the hypopharynx to 5 cm below the LOS with 5 intragastric sensors. The catheter is left in place for 5 minutes to assess the contractions while patient swallow 5 mL of water.

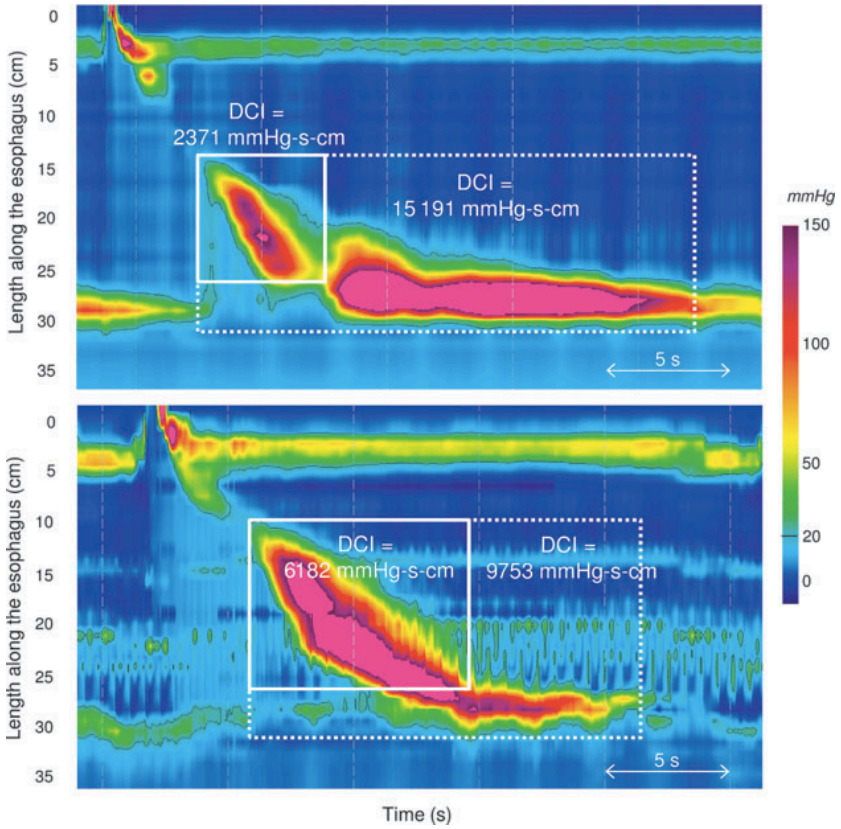


Figure 1-5: Colour topography of HRM illustrating normal oesophageal contraction [Kahrilas et al., 2015a].

High-resolution manometry (HRM) is illustrated by colour topography of pressure plots that is also referred to as Clouse plots (in honour of the key inventor) {figure 1-5}. Substantial work was undertaken to link the HRM pressure topography plots to the gut luminal physiology to develop an atlas that can distinguish between normal gastrointestinal (GI) motility and motility disorders. The international HRM working group attempted to reform this atlas of oesophageal motility disorders into a hierarchy of motility disorders which is called Chicago classification. The main objective of this classification is to use a standard matrix for categorizing patients with chest pain and non-

obstructive dysphasia [Kahrilas et al., 2015b]. The motility diagnosis provided by the latest version of Chicago classification (v.3) is illustrated in {Figure 1-6}.

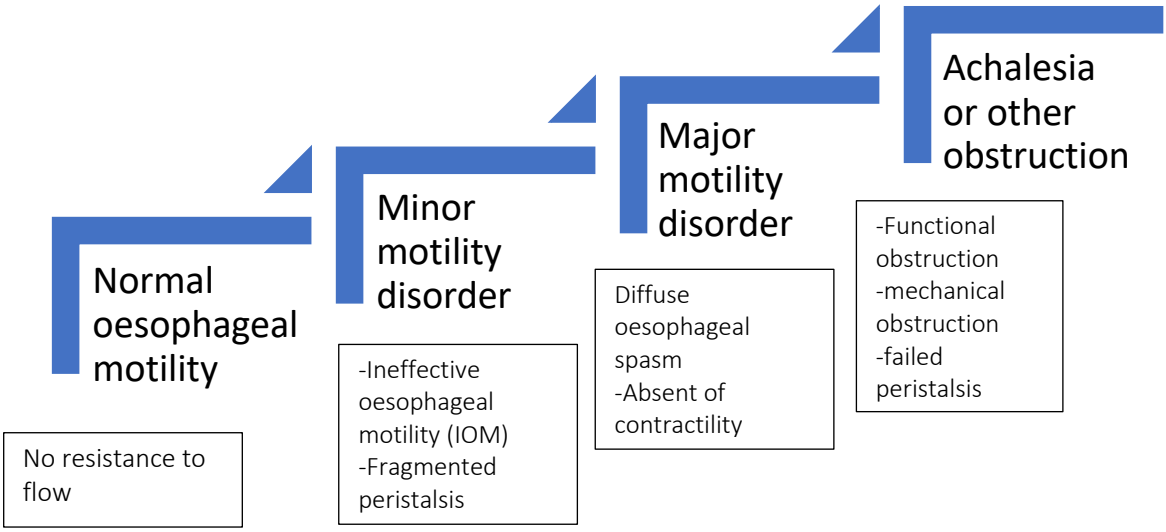


Figure 1-6: Hierarchy for characterising oesophageal motility using HRM with Chicago classification 3.0 [Bredenoord et al., 2012]

1.6.4.4 *Ambulatory Reflux Monitoring (pH-metry/ Intraluminal Impedance monitoring)*

Historically in clinical practice reflux was only identified if it triggered oesophageal inflammation and until the mid 1970's when prolonged reflux monitoring was introduced by Johnson and Demeester to expand the knowledge on GORD [Johnson and Demeester, 1974]. Reflux was then quantified in healthy volunteers and named as physiological reflux, whereas, reflux above this level was identified as pathological reflux. Pathological reflux is classified according to the body position as: upright, supine and combined (total). The normal pH of the oesophagus is considered to be pH 7, any sudden drop of pH level below 4 for at least 30 seconds requires monitoring. The reason behind this cut-off point is that symptoms such as heartburn have been reported in most GORD patients only when their oesophageal pH falls below 4. Another reason is that pepsin (the digestive enzyme) becomes active when pH is below 4 [Johnson and Demeester, 1974; Tuttle et al., 1961].

The frequently used parameters for diagnosis of GORD are: number of reflux episodes below pH 4, percentage time pH is below 4 divided by the total recording time. A full list of all the parameters considered of significance and their normal values are listed in {Table 1-5}. {Table 1-6} lists the cut-off value of acid exposure time (AET%) which is considered the most reliable parameter for the purpose of diagnosing pathological reflux according to the Lyon consensus [Gyawali et al., 2018].

The DeMeester score was first introduced by Johnson and DeMeester [1974] and used to diagnose GORD positive or GORD negative patients, although it has been criticised for the efficacy in defining pathological reflux. It is a calculated score based on 6 parameters obtained from the 24hr-pH monitoring, there parameters are:

1) Total number of reflux episodes

2) % total time pH<4

3) % upright time pH<4

4) % supine time pH<4

5) Number of reflux episodes for 5 minutes or more with pH<4

6) Longest reflux episode with pH<4.

When comparing DeMeester score and AET, a study analysed data from 25 asymptomatic healthy subjects and 25 patients with signs of increased acid exposure, it reported the sensitivity and specificity of DeMeester score to be 96% and 100% respectively, whereas for AET it was 96% for both parameters [Neto et al., 2019].

pH-metry

Oesophageal pH-metry is a recording of the distal oesophagus pH. The technique detects acidic reflux in the distal oesophagus allowing a quantification of the acidic episodes and hence diagnosis of GORD [Johnson and DeMeester, 1986]. In pharmacology studies, this technique is considered a gold standard diagnostic method for GORD [Kahrilas and Quigley, 1996]. The principal components of the pH monitoring are pH sensors and a data logger as a portable recorder. The sensors are calibrated using buffer solutions chosen by the manufacturer of the equipment. Patients are usually asked to fast for at least 4 hours (up to 12 hours) prior to the ambulatory procedure.

A pH catheter is inserted from the nostrils through the lower oesophageal sphincter (LOS) to the stomach with either single or double sensors. When a single pH sensor is used it is positioned 5 cm above the upper boundary of LOS. The position is considered by global consensus as an optimal depth for monitoring the distal oesophagus. The second sensor if used, is positioned 10 cm below the LOS to monitor the intragastric acid exposure. The pH sensors only measures acidity at the level of the sensor. Once the insertion and placement of the catheter is complete, taping is applied to limit the movement. The portable recorder is connected to the catheter and digitally registers the pH every 4 seconds for 24 hours [Kahrilas and Sifrim, 2008].

Intraluminal Impedance monitoring

Ambulatory reflux monitoring using pH-metry alone which only identifies acid reflux episodes was considered the gold standard technique in diagnosing GORD for many years. The evolution of Impedance-pH monitoring has superseded pH-metry for many reasons including the prolonged monitoring of oesophageal pH, detects the types

and composition of reflux as acid, non-acid or gaseous, the association of symptoms to reflux episodes along with a gastric pH [Nasi et al., 2018]. In addition, one of the most relevant advantages of pH-impedance over pH-metry is the ability to measure the proximal extension of refluxates. The proximal extension usually occur due to large volume of refluxated material rather than its acidity, causing symptoms such as chest pain, cough and regurgitation [Nasi et al., 2018].

The principal of this monitoring technique is based on measuring changes in resistance to electrical current flow between electrodes using an intralumenal probe. For instance, the air within the oesophagus has high impedance whereas antegrade or retrograde flow of liquid has low impedance. When the oesophagus is empty, the impedance measures the conductivity of oesophageal mucosa. The insertion procedure follows the same protocol as the pH monitoring system, but impedance/pH catheter is composed of 8 channels, two of which are pH electrodes and the other six impedance electrical resistance electrodes. The upper six channels detect the direction of pH changes and if the acid is from an external source (coming from the oral cavity) {figure 1-7} or an internal source (coming from the stomach) {figure 1-8}. Patients are instructed to reproduce their daily activities that trigger symptoms as much as possible and record their symptoms at the start and end of each meal and in relation to the body position (upright or supine) [Martinez et al., 2003; Nasi et al., 2018]. This technique is reported to have high sensitivity and accuracy in reflux monitoring and intra-oesophageal bolus movement [Kahrilas and Sifrim, 2008; Roman et al., 2017a].

Data obtained from intraluminal pH- impedance monitoring can be subdivided for the purpose of analysis into the following:

- pH data

- impedance data (bolus movement)
- relationship between pH data and impedance data
- relationship between symptoms and reflux episodes

Parameter	Proximal oesophagus	Distal oesophagus
Acid exposure time (AET)	<0.9%	<4.2%
Upright acid exposure	<1.2%	<6.3%
Supine acid exposure	<0.0%	<1.2%
Total number of reflux episodes	<73 episodes	
DeMeester score	<14.7	

Table 1-5: Parameters and values using catheter-based pH and impedance monitoring

Diagnosis	pH or pH-impedance monitoring
Conclusive pathological reflux	AET >6%
Inconclusive pathological reflux	AET 4-6% Reflux episodes 40-80
No pathological reflux- normal	AET <4% Reflux episodes <40

Table 1-6: Interpretation of pH and Impedance monitoring test [Gyawali et al., 2018]

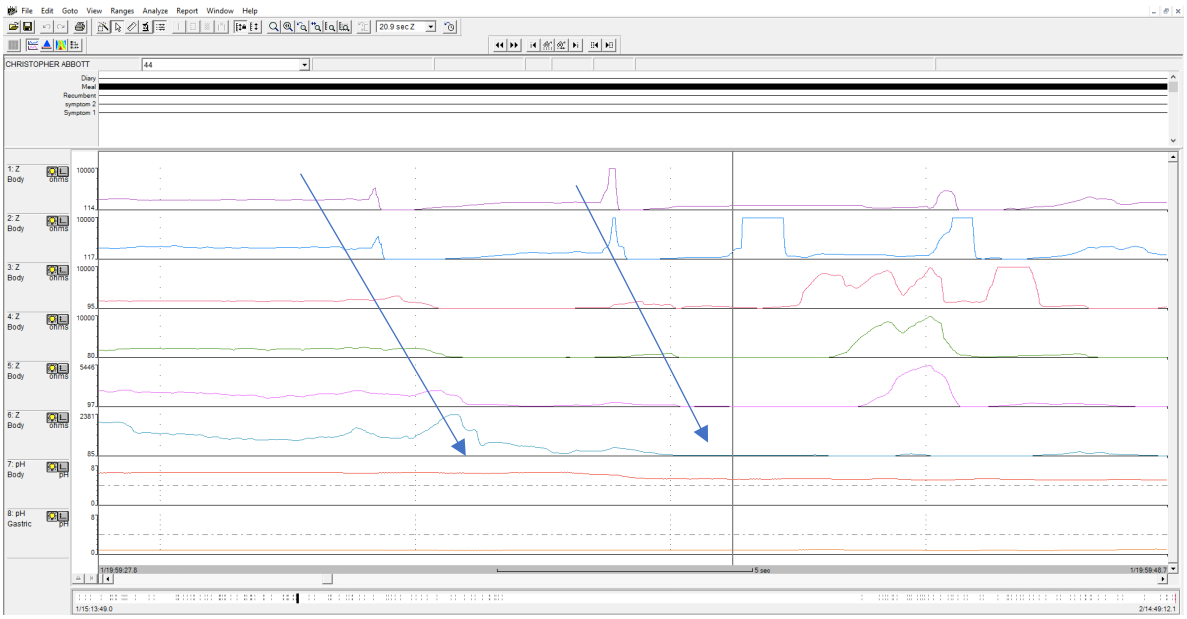


Figure 1-7: Antegrade movement of bolus: stepwise change in impedance as bolus transports to the stomach, a decrease drop in the pH as bolus reaches the distal oesophagus due to extrinsic acidic intake.

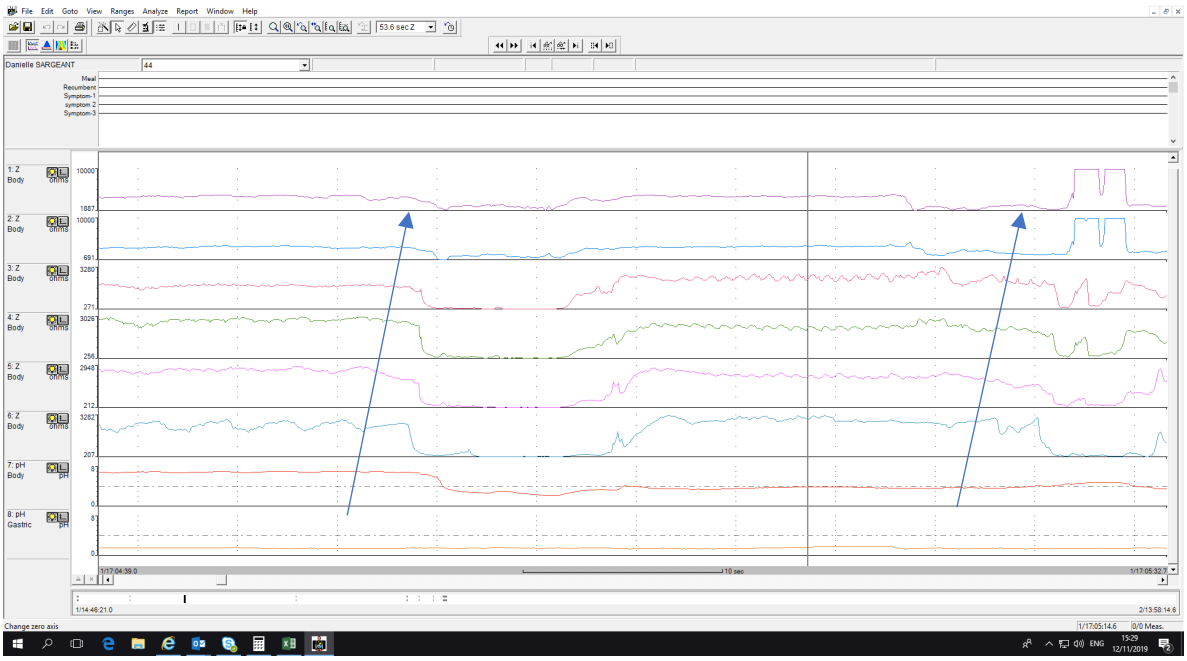


Figure 1-8: Retrograde flow of bolus: gastric content flow from the stomach to the oesophagus, starting from the distal oesophagus as low impedance can be seen, then mid and finally proximal with time.

1.6.4.5 *Reflux Symptoms Association*

In addition to the patients' reflux profile that is quantified by the pH-impedance monitoring test, the test provides the ability to assess the relationship between symptoms and the onset of reflux. It provides the ability to inspect the relationship through quantitative measures. Ward et al. [1986] were the first to propose an index to describe the relationship between reflux and symptoms.

Symptoms index (SI) is defined as the percentage of symptom events related to reflux. It is calculated by dividing the number of symptoms related to reflux episodes by the total number of symptoms x 100. It measures the effect size and the optimal threshold is considered to be 50% [Singh et al., 1993]. The disadvantage of this index is that it does not take into account the probability of reflux episodes being associated with symptoms by chance. Therefore, the symptoms associated probability (SAP) was introduced, which expresses the probability of association between reflux and symptoms events [Martinez et al., 2003]. It is calculated by a complex statistical software, measuring the "probability" and the optimal threshold is considered to be 95%.

A positive reflux symptom association is considered when SI is equal or more than 50% and SAP is equal to or more than 95%. The comparison between the two indices is not possible as they measure different parameters, but the combination of both positive SI and SAP presents an ideal clinical relevance of symptoms association to reflux episodes. However, when one is positive and the other is negative it is recommended to further clinically investigate the symptoms and other parameters [Roman et al., 2017b].

The degree of reproducibility of reflux symptom association analysis is high with SI being less reproducible compared to SAP [Hemmink et al., 2008]. The limitations of these

indices are the day to day individual variability of symptom occurrence during the monitoring test [Barriga-Rivera et al., 2013].

5. Wireless Catheter-Free Testing:

A wireless catheter free system, The Bravo® system (Medtronic Inc. Minneapolis, MN) is a capsule composed of an antimony pH capsule with an internal reference, a capsule delivery system, radiofrequency transmitter, battery and external receiver monitoring the pH level. The gastro-oesophageal mucosal separation is determined endoscopically (the gastro-oesophageal junction), the capsule is then positioned and pierced 6 cm above it. The pH is recorded and transmitted every 5 seconds to the external receiver. The parameters provided by this system to diagnose GORD are listed in {Table 1-7} with the threshold values.

This system is used when the catheter-based tests generate negative results in patients with high suspicion of GORD. Some of the advantages of this system over the catheter-based monitoring are improved patient comfort and tolerance, it as well provides prolonged monitoring of 48 to 96 hours, hence increasing the diagnostic yield. The limitations of this technique are the high cost, the possibility of an early detachment, the risk of losing data between transmission and receiving, the need of endoscopy and the need of multiple recording sites.

Parameter	Distal oesophagus
Total acid exposure (AET)	<5.3%
Upright acid exposure	<6.9%
Supine acid exposure	<6.7%

Table 1-7: Parameters and values using wireless monitoring [Ayazi et al., 2009]

1.7 Role of Saliva in GORD

GORD involves the exposure of oesophageal mucosa to abnormal concentrations of acid and proteolytic enzymes which are activated only when the pH is low. The integrity of the oesophagus depends on an equilibrium between the defence mechanisms and the aggressive factors (such as acids, bile, pepsin). Saliva is the fundamental component in the protection and clearance of acid exposure and along with the oesophageal mucus glands it facilitates neutralisation and restoration of the pH level to near-neutral within the oesophageal lumen. Upon swallowing a bolus, oesophageal contractions are induced which empties the bolus and clears the acid within the oesophagus. Saliva subsequently provides chemical neutralisation of any remaining acid [Helm et al., 1984].

It has been shown that patients with erosive oesophageal reflux disease (ERD) have a different proteomic profile of saliva and weaker oesophageal mucosa than patients with non-erosive oesophageal reflux disease (NERD). This is due to reduced cell proliferation, cell migration, response to stress and keratinized tissue of the oesophagus. This results in an impaired response to acid and pepsin attack [Calabrese et al., 2009]. A study reported that salivary analysis of patients with GORD, have the following differences when compared to controls: lower stimulated flow rate, higher pH, higher potassium concentration and lower sodium concentration [Calabrese et al., 2009]. The limitation in assessing the exact role of salivary secretion and alteration in the pathogenesis of GORD is due to the fact that GORD is a multifactorial condition.

1.8 The association between GORD and ETW

The association between ETW and GORD was published as a case report described by Hawden [1971], subsequently in later years ETW was recognised as epidemiologically associated with GORD. In 2006 the Montreal consensus stated: 'The prevalence of ETW, especially on the lingual and palatal tooth surfaces, is increased in patients with GORD'- statement no.48, which was the highest evidence associating GORD to extra-oesophageal manifestations [Vakil et al., 2007b].

A systematic review by Pace et al.[2008] assessed the relationship between GORD and ETW involving 17 studies and reported that the median prevalence of ETW in patients with GORD was 24% and the median prevalence of GORD in patients with ETW was 32.5% [Pace et al., 2008]. {Table 1-8} and {Table 1-9} summarise the prevalence of GORD in ETW patients and ETW in GORD patients.

The most recent systematic review on the association between ETW and GORD was done by Jordão et al. [2020], the meta-analysis included 27 studies composed of both subjective and objective measures to diagnose GORD. This review demonstrated a significantly increased odds of developing ETW in patients with objectively measured GORD (OR 4.13, 95% CI: 1.68-10.13) in comparison to subjectively measured GORD (OR 2.69, 95% CI: 1.13, 6.38). The meta-analysis concluded that objectively measured GORD was more reliable than subjective measures reported by patients. {Table 1-10} summarises the diagnostic measures used for diagnosis of both conditions GORD and ETW. There is therefore a strong association between GORD and ETW, and that the severity of ETW correlates with symptoms of GORD as well as the exposure of the oral cavity to an acidic pH [Pace et al., 2008].

Study	Number of patients with GORD	GORD diagnostic method	Prevalence
Munoz et al.[2003]	181 129 78	Symptoms 24 hours pH metry endoscopy	47.5%
Moazzez et al.[2004a]	18/31	24 hours pH metry	Not specified
Schorder et al. [1995]	20/30	24 hours pH metry	40%
Loffed [1996]	293	Endoscopy	32.5%
Meurman et al.[1988]	117	Symptoms	24%
Jarvinen et al.[1988]	35	Endoscopy	5%
Oginni et al. [2005b]	125	Symptoms	16%

Table 1-8:Prevalence of ETW in patients with GORD

Study	Number of patients with ETW	Method of diagnosing GORD	Prevalence
Bartlett et al.[2013]	36	24hours pH metry	64%
Gregory-Head et al. [2000]	20	24hours pH metry	50%
Gudmundsson et al.[1995]	14	24hours pH metry	21%
Schroeder et al.[1995]	12	24hours pH metry	83%

Table 1-9: Prevalence of GORD in patients with ETW

Study	GORD diagnostic measure	No. case/control	ETW diagnostic measure	No. case/control
[Silva et al., 2001]	endoscopy/ questionnaire	31/14	Eccles and Jenkins	1/0
[Correa et al., 2012]	endoscopy/ pH-manometry	30/30	Eccles and Jenkins	not reported
[Di Fede et al., 2008]	endoscopy/ pH-manometry	200/100	Smith and Knight	18/13
[Li et al., 2017]	endoscopy/ questionnaire	51/50	Smith and Knight	31/14
[Ramachandran et al., 2017]	endoscopy/ questionnaire	25/25	BEWE	22/8
[Roesch-Ramos et al., 2014]	endoscopy/ questionnaire	60/60	Eccles and Jenkins	46/2

[Yoshikawa et al., 2012]	endoscopy/ questionnaire	40/30	Smith and Knight	9/0
[Picos et al., 2020]	Endoscopy/questionnaire	141/122	BEWE	131/87
[Rauber et al., 2020]	Endoscopy/questionnaire		BEWE	55/180
[Alaraudanjoki et al., 2016]	questionnaire	1164/699	BEWE	561/323
[Antunes et al., 2017]	questionnaire	3/105	clinical examination	2/19
[Li et al., 2018]	questionnaire	68/658	Smith and Knight	33/191
[Milani et al., 2016]	questionnaire	143/2741	Smith and Knight	37/47

[Teixeira et al., 2017]	questionnaire	76/201	Eccles and Jenkins	73/200
[Wan Nik et al., 2011]	questionnaire	22/11	Smith and Knight	no reference
[Wei et al., 2016]	questionnaire	39/681	BEWE	35/568
[West et al., 2013b]	questionnaire	771/2145	BEWE	473/309

Table 1-10: List of studies with details on characteristics to diagnose ETW in patients with GORD symptoms

1.9 Summary and Aims of Research

Although the relationship between erosive tooth wear and gastro-oesophageal reflux disease is being continually investigated, there is lack of information regarding the mechanism of which saliva could increase erosive tooth wear under an intrinsic acidic challenge in-vitro and in-vivo. There are no evidence-based studies on the association between symptoms of gastro-oesophageal reflux and the presence of erosive tooth wear using the latest clinical investigation tools (pH-impredance monitoring test) in combination with self-assessment tool (questionnaire).

The investigations of this thesis occurred in three parts:

1. A laboratory investigation of dietary and intrinsic acidic challenges with and without acquired enamel pellicle.
2. A case-control study on 300 paricipants investigating the association of gastro-oesophageal reflux symptoms with erosive tooth wear.
3. An in-vivo study comparing the total protein concentration of teeth with and without erosive tooth wear from patients suffering from gastro-oesophageal reflux symptoms.

The null hypotheses for this thesis are:

1. There will be no difference in erosion effect comparing extrinsic and intrinsic acids on enamel samples.
2. There will be no difference in the protective effect of an acquired enamel pellicle on enamel samples before an acidic challenge.
3. There will be no association between erosive tooth wear and oesophageal motor function.

4. There will be no association between erosive tooth wear and gastro-oesophageal reflux symptoms.
5. There will be no difference between erosive tooth wear and frequency and intensity of gastro-oesophageal reflux symptoms.
6. There will be no difference in total protein concentration between eroded and un-eroded surface from patients with gatrsoesophageal reflux symptoms.

2 Chapter 2: The Effect of Citric Acid, Hydrochloric Acid and Artificial Gastric Juice on Human Enamel: In-Vitro

2.1 Introduction

Patients suffering from GORD are at risk of developing erosive tooth wear. GORD is a highly prevalent disease affecting 8-33% of the world's population [Gyawali et al., 2018; Savarino et al., 2009] and Erosive tooth wear (ETW) is the most common oral manifestation of GORD [Ruff et al., 1992; Wolcott et al., 1984]. ETW is a chemical-mechanical process resulting in a cumulative loss of hard dental tissue not caused by bacteria, originating from either extrinsic or intrinsic sources.

Enamel is a crystalline structure made of hydroxyapatite that may be dissolved when in contact with solutions with pH level below 5.5 [Gudmundsson et al., 1995]. Gastric juice has an acidic pH range between 0.9 to 1.5 and if it reaches the oral cavity, can result in ETW in some patients [Carvalho et al., 2015]. As well as the acids, it contains proteolytic enzymes such as pepsin, bile, rennin and hydrochloric acid (HCl) [Bartlett and Coward, 2001a; Newton et al., 2004]. Pepsin is a proteolytic digestive enzyme found in the cell lining of the stomach, it is secreted in the form of pepsinogen, which is transferred into an active form of pepsin in the presence of HCl. Pepsin is considered the main digestive component of gastric juice and the digestion process is impossible without the presence of pepsin. Pepsin is not present in the oral cavity under normal conditions, but in patients with GORD or chronic vomiting it can reach the oral cavity within the gastric refluxate.

In-vitro studies have assessed various acids to try and mimic gastric reflux. Studies have utilised HCl [Hove et al., 2007; Schlueter et al., 2012; Stenhagen et al., 2013; West et al., 2001] enzymes such as pepsin and trypsin [Schlueter et al., 2012; Schlueter et al., 2010] and gastric juice on human dental tissue [Bartlett and Coward, 2001a; Davies et al., 2002]. Under in-vitro conditions, it has been reported that pepsin can degrade the organic matrix in dentine completely after immersing samples for three days [Tonami and Ericson, 2005]. Other studies have shown the capability of pepsin to partially degrade dentine matrix (25%) without an effect on the mineral loss [Schlueter et al., 2007]. In addition, the combination of both pepsin and trypsin has been shown to enhance erosive mineral loss on dentine samples [Imfeld, 1996]. However, little is known about the effect of these enzymes on human enamel surface.

Furthermore, studies mimicking intrinsic acids have used HCl or gastric juice. Immersion of enamel samples (human and bovine) in HCl for 30 seconds at pH 1.5 and pH 2.0 significantly increased surface roughness [Derceli Jdos et al., 2016; Mann et al., 2014]. The erosive effect of gastric juice has also been investigated. Braga et al. [2011] investigated calcium concentration loss on polished human enamel samples after immersion in aspirated human gastric juice. They found that compared to orange juice, the calcium concentration loss was significantly higher. However, the use of human aspirated gastric juice is a complex method and collection is difficult, therefore artificial gastric juice is used in some studies to mimic the clinical situation.

Studies mimicking extrinsic acids have used citric acid as it is the most common type of acid found in most dietary intakes [Mutahar et al., 2017b; Mylonas et al., 2018]. It is found in high concentration in fruit juices, soft drinks, citrus fruits, energy drinks, sport drinks, vitamin C products and alcoholic beverages, with a pH range between 2.6

to 3.8 [Cheng et al., 2009c; Hjortsjo et al., 2010; Lussi and Schaffner, 2000; Wang and Lussi, 2012; Young and Tenuta, 2011].

The erosive tooth wear process depends on chemical and biological factors. The chemical factors in relation to the acids include the titratable acidity, pH, buffering capacity, mineral components and chelation properties [Barbour et al., 2011]. Whereas the biological factors depends on several intraoral protective mechanisms, mainly saliva and acquired enamel pellicle (AEP) [Buzalaf et al., 2012b]. AEP is regarded as barrier against ETW and many in-vitro studies have correlated the barrier effect to the mineral content [Cheng et al., 2009b; Hjortsjo et al., 2010], whereas others have suggested the protein contents are more important in protection against ETW [Ireland et al., 1995; Meurman and ten Cate, 1996]. Although it has been shown that AEP provides a protective layer on the tooth surface inhibiting the direct contact between the erosive challenge and enamel, there are limited reports on the effect of in-vitro AEP against HCl and artificial gastric juice (AGJ) challenges [Moazzez and Bartlett, 2014]. Therefore, the aim of this in-vitro study was to investigate the effect of exposure of human enamel samples to dietary and gastric acids at various time points with and without the presence of AEP.

2.2 Aims, objectives and null hypothesis

Aims

1. To investigate the effect of exposure of polished human enamel to gastric acid.

2. To evaluate the protective effect of the presence of AEP on enamel samples exposed to dietary and gastric acids.

Objectives:

The objectives of this study were:

1. To compare the effect of citric acid and hydrochloric acids at 30, 60, 120 and 300s and artificial gastric juice at 120s and 300s on polished human enamel samples compared to deionised water DIW (control) using non-contact surface profilometry and microhardness with and without the presence of AEP.

2. To assess qualitative changes of the enamel samples using surface scanning electron microscopy (SEM) imaging of enamel samples after immersion in citric acid, hydrochloric acid, artificial gastric juice and DIW with and without the presence of AEP at 120s and 300s.

3. To measure calcium and phosphate concentration in artificial gastric juice using Inductively coupled plasma mass spectrometry (ICP-MS)

Null hypotheses

The null hypotheses were:

1. There will be no difference between the effect of exposure of human enamel samples to dietary and gastric acids at various time points.

2. AEP does not offer any protection on enamel samples exposed to dietary and gastric acids.

2.3 Materials and methods

2.3.1 Sample preparation

One hundred and twenty extracted human caries free molars were collected from the oral surgery department (23rd floor at Guy's hospital) after obtaining informed written consent from patients (approved by the National Research Ethics Service (NRES) in London – Bloomsbury (REC REF: 12/LO/1836) {PIS for teeth collection in appendix 7.1} {ICP for teeth collection in appendix 7.2}. The teeth were disinfected by immersion in a sodium hypochlorite solution (0.1 M) for at least 48 hours prior to use. The samples were embedded in impression compound (Impression compound, Kerr, Green, type 1, Peterborough, UK), attached to a copper tube as shown in {figure 2-1} and sectioned using a 4 inch blade (Diamond wafering blade XL 12205, Benetec Ltd, London, UK) attached to a cutting machine (Buehler Isomet GmbH, Düsseldorf, Germany) at a speed of 180 rpm with 1.0 N force applied at the cemento-enamel junction. Only the buccal surfaces were included and were sectioned into two halves, providing two samples per tooth (n=240) and stored dry.

Samples were embedded in a custom-made silicone mould (Metrosil silicone duplicating material part A and B, Metrodent Ltd, Huddersfield, West Yorkshire, UK) filled with Bis-acrylic composite (Protemp™4, 3M ESPE, Seefeld, Germany) with a size of 5 x 2.5 x 2mm as shown in {figure 2-2}. The polishing regimes followed previously published protocols [Ganss and Lussi, 2014; Mistry et al., 2015a; Mylonas et al., 2018], using a constant water-cooled rotating polishing machine (Meta-Serv 3000 Grinder-Polisher, Buehler, Lake Bluff, Illinois, USA) with a semi-automated polishing head (Vector LC Power Head, Buehler, Lake Bluff, Illinois, USA) using different grit polishing discs {figure 2-3}. All samples were polished in a sequence using a single specimen method with water

directed to the samples to lubricate, the sequence of polishing discs was as follow {Table 2-1}:

Grit size	500	1200	2000	4000
Grain size	30 μm	15 μm	10 μm	5 μm
Polishing time	5sec	0:25min	0:30min	1:00min
Force	15 N	15 N	15 N	20 N

Table 2-1 Polishing method used

Polishing discs with grit sizes 500, 1200, 2000 and 4000 were applied to the enamel surface to create flat samples, then randomised and numbered for identification. All samples were immersed in an ultrasonic bath (Nusonics GP-70, T310, Lakewood, USA) at 70Hz for 15 minutes filled with deionized water of pH 7, after which they were left to dry at least 12 hours. This was followed by taping the enamel samples using PVC adhesive tape leaving an exposed window of 2 mm width.

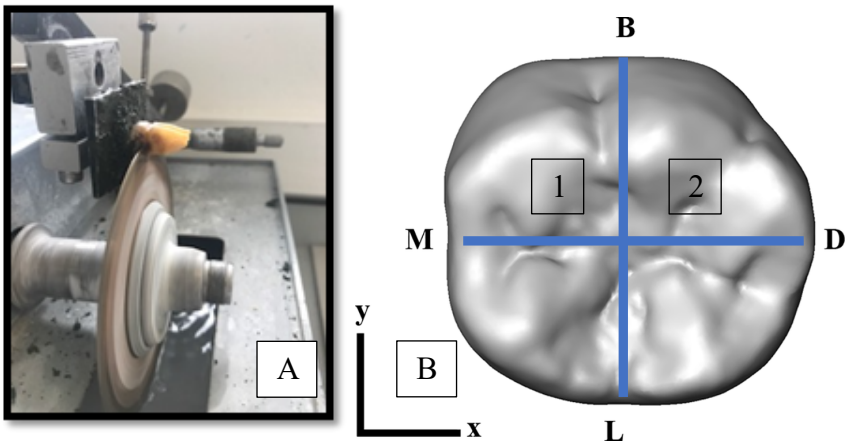


Figure 2-1: A- Image of tooth in impression compound during sectioning, B- Schematic image of sectioning protocol used (buccal surface included in the study providing two samples)

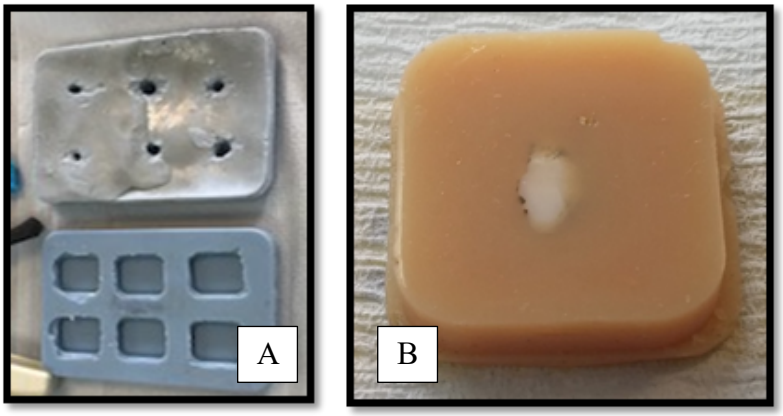


Figure 2-2: A- Image of mould lid with holes and the silicone holder for embedding teeth, B- image of sample in a size of 5 x 2.5 x 2mm

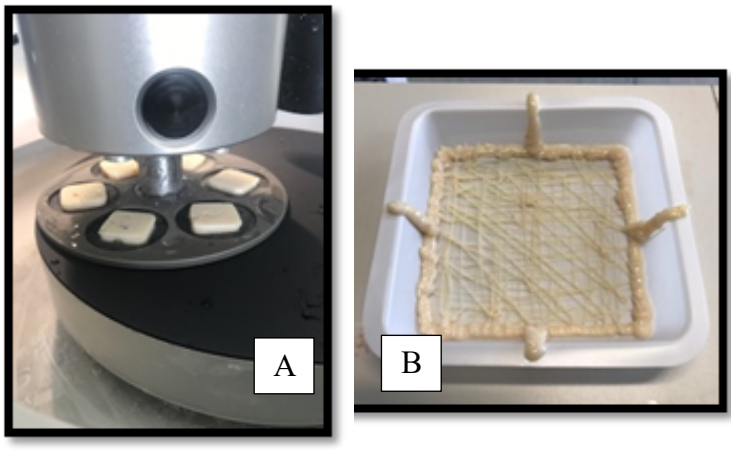


Figure 2-3: A- image of the polishing machine with water lubrication, B- image of Bis-acrylic composite net

2.3.2 Solution preparation

Experimental solutions were made as detailed in {Table 2-2}. Solids/powder of acid were weighed using an electronic analytical scale (Mettler Toledo, XS105 Dual Range Analytical Balance, Greifensee, Switzerland), whilst liquids were measured by a graduating measuring cylinder. The pH was adjusted by the addition of 0.1 M Sodium Hydroxide (NaOH) measured using a calibrated pH meter (Mettler Toledo, Greifensee, Switzerland). The titratable acidity was calculated as the volume of sodium hydroxide (0.1 M) required to increase the pH of the experimental solution to pH 7.0.

AEP was formed from stimulated whole mouth saliva collected from healthy volunteers after obtaining informed consent (approved by the National Research Ethics Service (NRES) in London – Bloomsbury (REC REF: 12/LO/1836) {PIS for saliva collection in appendix 7.3} {ICF for saliva collection in appendix 7.4}. The volunteers were asked to fast for two hours, then chew on a piece of paraffin wax and expectorate saliva for 5 minutes in a standard 20 ml sterile polypropylene universal tube, each tube was placed in an ice bucket for transfer to the laboratory and it was subsequently frozen in a -80 °C freezer until the time of use. Samples were thawed simultaneously at room temperature for four hours prior to time of use. In order to avoid loss of salivary protein components, thawed saliva was mixed vigorously using a vortex mixer (Bibby Scientific Limited, Staffordshire, UK) [Addy and Hunter, 2003; Francis et al., 2000]. Upon completion of each cycle, the remaining saliva samples were disposed of following the protocol submitted to the Ethics Committee and HTA guidelines. A total of 480 ml of pooled saliva was collected.

In-vitro AEP was formed following a previously published protocol [Mutahar et al., 2017b], by immersing each polished enamel samples in 8 ml of pooled natural saliva, for

24 hours, then agitated (62.5 RPM, Stuart mini-Orbital Shaker SSM1, Bibby Scientific, England) in deionised water for two minutes and left overnight at 22 °C±1. To achieve standarised immersion times, a net base holder was constructed from acrylic resin in order to facilitate the immersion and removal of samples from the same group simultaneously {figure 2-3}.

Deionised water (DIW)	pH 7.0
Citric acid (CA)	1L of deionized water added to 3 grams of 0.3% solid citric acid (Sigma Aldrich, Poole, Dorset, UK) adjusted to pH 3.2 using 0.1M of Sodium Hydroxide (NaOH) buffer.
Hydrochloric acid (HCl)	999.17 ml of deionized water added to 0.833 ml of 0.01M HCl (Sigma Aldrich, Dorset, UK) adjusted to pH 2.2 using 0.1M of Sodium Hydroxide (NaOH) buffer.
Artificial gastric juice (AGJ)	500 ml mixture of water (%99.46), concentrated HCl (%0.23), Sodium chloride (%0.21) and (1.0 gram) of pepsin given the pH of 1.1 (Ward's science, Rochester, NY, USA).

Table 2-2: Experimental solutions used

2.3.3 Experiment procedure

The samples were randomly allocated to seven groups as follows {figure 2-4}:

Non-AEP groups (DIW, CA, HCl, AGJ) – For each group (n=10 per immersion time) samples were stirred in 80 ml of the corresponding experimental solution at 22°C±1 for 30s, 60s, 120s or 300s using an orbital shaker (Bibby Scientific, Staffordshire, UK) at 60 rpm. Followed by agitating the samples for 2 minutes in DIW, they were then air dried for 15 seconds. Post erosion measurements were then obtained, as shown in {figure 2-5}.

AEP groups (CA, HCl, AGJ) – For each group (n=10 per immersion time), samples were immersed in 80 ml of human whole mouth saliva for 24 hours to form the AEP prior to exposure to the experimental solution, then agitated in DIW for 2 minutes. The same procedure as the non-AEP groups were then followed as shown in {figure 2-6}

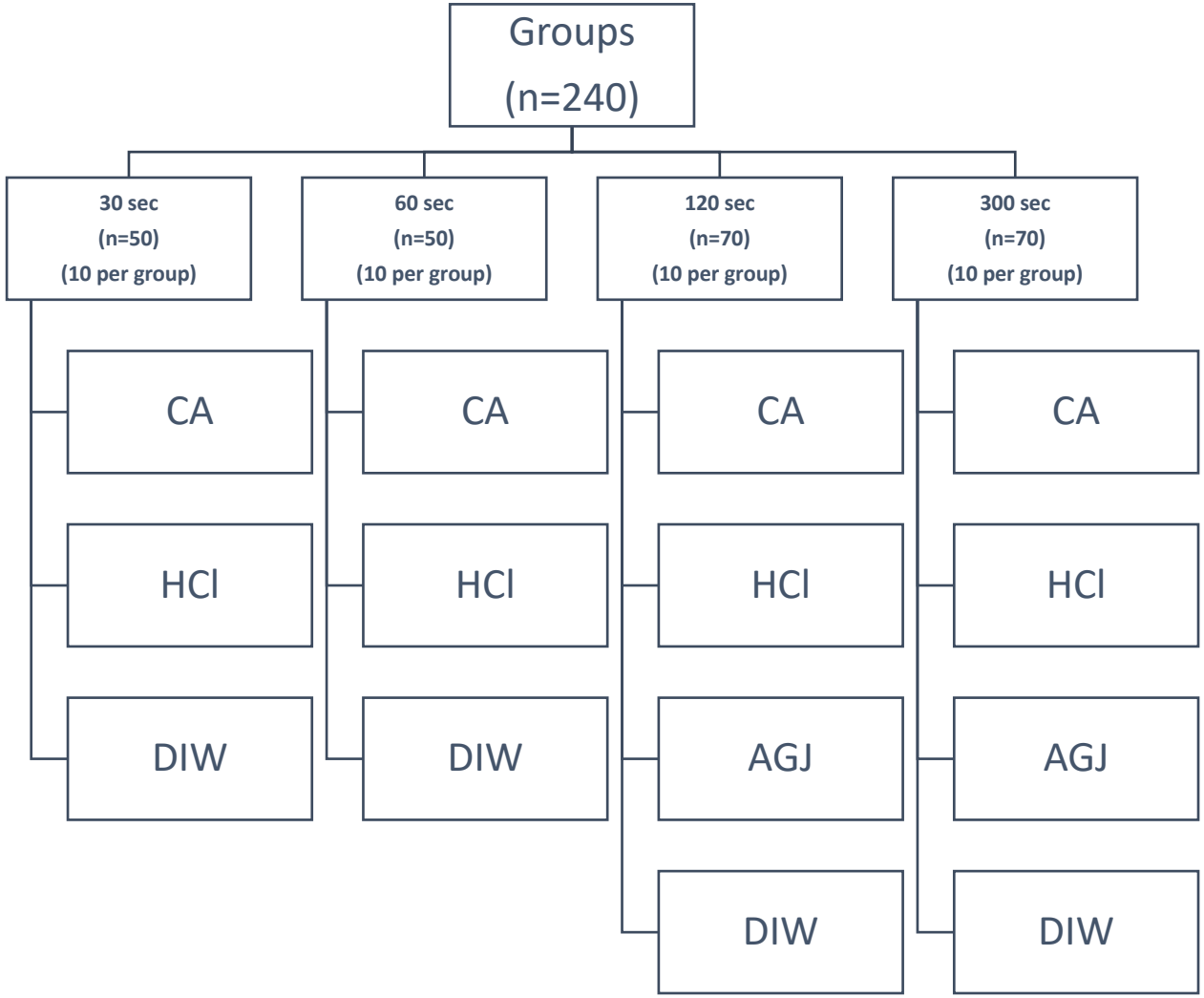


Figure 2-4: The various groups at different time points

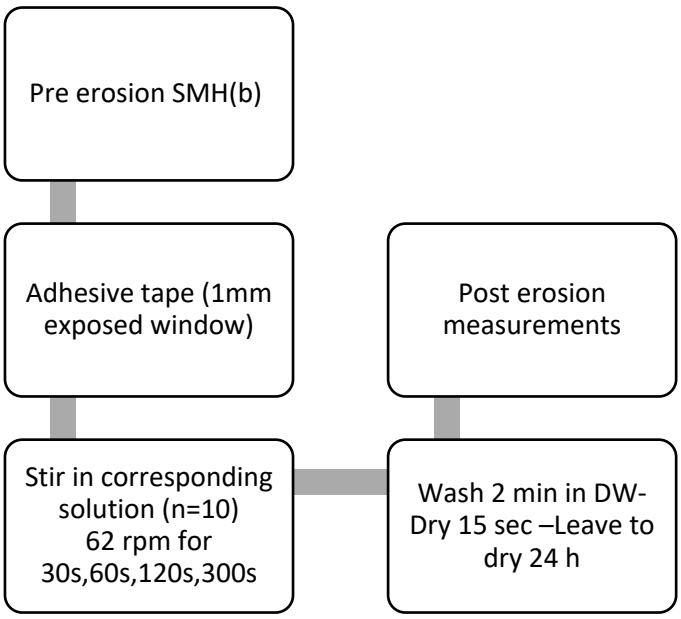


Figure 2-5: Experimental procedure for the Non-AEP groups

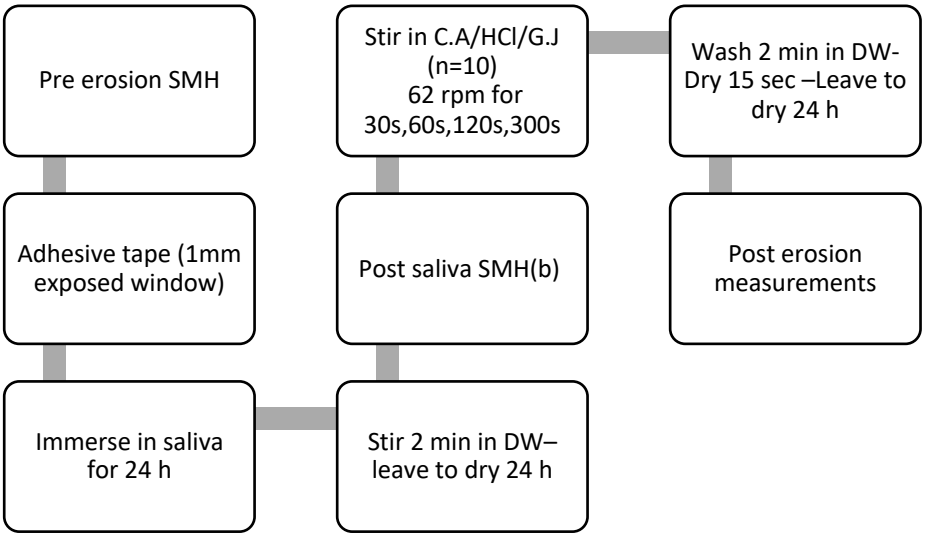


Figure 2-6: Experimental procedure for the AEP groups

2.3.4 Measurements

Profilometric measurements were carried out before and after immersion of samples in the solutions. 10% of samples were measured pre-erosion to assess the flatness tolerance (ranging between ±0.3 to ±0.6 mm) [Austin et al., 2016b; Mistry et al., 2015b; Mullan et al., 2018a; Mullan et al., 2017a] using a red light confocal non-contact laser profilometer (NCSP) with 655nm displacement sensor (Taicaan, XYRIS 2000, Southampton, UK) and a 0.01 µm resolution and laser spot sizes of 0.2 µm using a constant scanning dimension and 10 um of scanning interval. The profilometric measurement method and setting followed a previously published protocol [Mistry et al., 2015]. Reference points for co-localisation were determined on the bis-acrylic material on each sample by using an indelible pen. Analysis of 3D step height profile was conducted using a single scan measurement technique on Boddies© software (Taicaan, Southampton, UK) by manually extracting five lines on the Y axis from each sample, and a mean of five readings calculated, following a previously published methodology to decrease the risk of non-representative data [Rodriguez and Bartlett, 2010]. Post-erosion measurements were obtained by scanning from the non-treated (reference area) across the treated area (the exposed window) to the other side of the non-treated area (reference area).

The Knoop microhardness tester (Duramin 2, Struers, Germany) measured surface microhardness (KHN) as an average of three indentations at 100µm intervals from each other, with a load of 100 g and 10 seconds dwell time {Table 2-3}. If the microhardness measurement of the enamel samples fell within the range of 272 KHN to 440 KHN they were included in the study [Mylonas et al., 2018]. Baseline surface microhardness (SMHb)

for the non-AEP groups was obtained from the mean values of three indentations on the enamel surface. For the AEP groups, baseline surface microhardness (SMHb) was measured after immersion in saliva and formation of AEP.

Post-erosion surface microhardness (SMHe) was obtained from the treated enamel surface (the exposed window) after immersion in the experimental solution. Followed by measuring the change (SMHc) in microhardness of each sample by subtracting the baseline value from the eroded value (SMHb)– (SMHe) averaged over each sample [O'Toole et al., 2015b].

Parameters	Value
Time	10 seconds
Load	100 g
Indentation speed	<0.1 mm/s
Indentations	5 indentations within the area of test
Space between indentations	100 mm

Table 2-3: Settings used for knoop microhardness measurement

To characterise the surface structure on the enamel samples, surface scanning electron microscopy (SEM) was used (SEM Type: S4000; Hitatchi, Japan). Two samples from each experimental group immersed for 300 seconds were randomly selected. Samples were gold coated using a 10 nm gold film. Images were recorded using a secondary electron detector with the acceleration voltage set to 25 KV. The electron detector uses a Field Emission Gun (FEG), which is made of a single crystal of Tungsten

sharpened to a 100 nm sharp point. All samples were taken at magnifications of x1.0 K and x10.0 k.

Measurement of the concentration of Calcium (Ca) and Phosphorous (P) ions released into the artificial gastric juice solution were carried out by the Centre of Excellence for Mass Spectrometry, King's College London, Faculty of Life Sciences and Medicine. Inductively coupled plasma mass spectrometry was used (ICP-MS) (Perkin Elmer 'NexION 350D', Waltham, Mass., USA), with Cetac 'ASX520' autosampler and running Perkin Elmer's 'syngistix' software, v1.0). Samples were diluted in a ratio of (1:1000). External calibration method was used, by using a series of standard solutions and calibration blank. Calcium and phosphorous concentrations were measured from a plotted calibration line.

2.3.5 Statistical analysis

Data were logged into an Excel spread sheet (Microsoft®Office Excel®2016, Microsoft® Corporation,USA) and analysed using GraphPad Prism (GraphPad Prism Version 7.00 for Windows, GraphPad Soft-ware, La Jolla California USA,www.graphpad.com). The normality of the microhardness and profilometry data were checked using D'Agostino & Pearson normality test and they were normally distributed. The data is presented as means and standard deviations for each acid immersion group. P values <0.05 was regarded statistically significant. ONE-WAY and TWO-WAY ANOVA and Tukey's multiple comparison tests were applied.

2.4 Results

2.4.1 Non-contact surface profilometry (NCSP)

Mean and standard deviation (SD) for all groups at all immersion times from the NCSP are displayed in {figure 2-7, 2-8} and {Table 2-4}.

Comparison of mean (SD) between CA, HCl and AGJ (with and without AEP) with DIW (control) shows the following:

Comparison of CA and DIW

Mean (SD) step height for samples immersed in CA without AEP at 30s, 60s, 120s and 300s were: 0.16 (0.11) um, 0.94 (0.49) um, 2.23 (0.84) um, 2.21 (0.94) um respectively and were statistically significantly greater than those for DIW (0.06 (0.01), 0.06 (0.03), 0.05 (0.02); $p<0.0001$) respectively. Mean (SD) step height for samples immersed in CA with AEP at 30s, 60s, 120s and 300s were: 0.08 (0.04) um, 0.40 (0.16) um, 1.77 (0.80) um, 1.44 (0.46) um respectively and were statistically higher than those for DIW (0.06 (0.03), 0.05 (0.02); $p<0.001$) respectively.

Comparison of HCl and DIW

Mean (SD) step height for samples immersed in HCl without AEP at 30s, 60s, 120s and 300s were: 0.54 (0.21) um, 0.92 (0.38) um, 1.89 (0.41) um, 4.58 (0.83)um respectively and were statistically significantly greater than those for DIW at 60s, 120s and 300s (0.06 (0.01), 0.06 (0.03), 0.05 (0.02); $p<0.0001$) respectively. Mean (SD) step height for samples immersed in HCl with AEP at 30s, 60s, 120s and 300s were: 1.88 (0.98) um, 3.29 (0.43) um, 2.68 (0.52) um, 6.7 (0.58) um respectively and were

statistically different compared to step height of DIW at all time points (0.06 (0.02), 0.06 (0.01), 0.06 (0.03), 0.05 (0.02); p<0.001) respectively.

Comparison of AGJ and DIW

Mean (SD) step height for samples immersed in AGJ without AEP at 120s and 300s were: 16.6 (3.4) um, 27 (8.3) um and AGJ with AEP were: 19.3 (4.5), 36.3 (7.1) um respectively and those were statistically significantly greater than those for DIW at all immersion time points (0.06 (0.02), 0.06 (0.01), 0.06 (0.03), 0.05 (0.02); p<0.0001) respectively. Two-way ANOVA showed significant interaction between independent variables (time) and (acid); p<0. 0001. Hence Tukey's multiple comparisons test reported the following results:

At 30 seconds, there were no statistically significant differences in step height between any of the experimental groups when comparing to DIW except for HCl with AEP (1.88 um; p<0.0001) where the step height was significantly higher. When comparing HCl and CA groups, there were no statistically significant differences between CA and HCl without AEP (0.16 (0.11) um- 0.54 (0.21) um; p=0.5). However, step height was significantly higher when comparing HCl with AEP with CA with AEP (1.88 (0.98) um – 0.08 um(0.04); p<0.0001).

When comparing the groups with and without AEP, no statistically significant differences was observed.

At 60 seconds, there were no statistically significant differences in step height between any of the experimental groups when comparing to DIW except for HCl with

AEP (3.29 um; p<0.0001) where the step height was significantly higher. When comparing the HCl and CA groups, there were no statistically significant differences between CA and HCl without (0.94 (0.49) um - 0.92 (0.38) um; p=0.5). However, step height was significantly higher when comparing HCl with AEP with CA with AEP (3.29 (0.43) um -0.40 (0.16) um; p<0.0001) When comparing the groups with and without AEP, step height was statistically significantly higher in HCl with AEP compared to HCl without AEP (3.29 (0.43) um - 0.92 (0.38) um; p<0.0001).

At 120 seconds, there were no statistically significant differences in step height between any of the experimental groups when comparing to DIW except for AGJ and AGJ with AEP where the step height was significantly higher (p<0.0001). When comparing the AGJ, HCl and CA groups, step height was statistically significantly higher when comparing AGJ with HCl (16.6 (3.4) um -1.89 (0.41) um) and AGJ with CA (16.6 (3.4) um- 2.23 (0.84)um) (p<0.0001). Also step height was statistically significantly higher when comparing in AGJ with AEP with HCl with AEP (19.3 (4.5) um – 2.68 (0.52) um) and AGJ with AEP with CA with AEP (19.3 (4.5) um- 1.77 (0.80) um) (p<0.0001). When comparing the groups with and without AEP, no statistically significant difference was observed.

At 300 seconds, step height was statistically significantly higher between all experimental groups when compared to DIW except for CA and CA with AEP where there was no difference (p>0.05). When comparing the AGJ, HCl and CA groups, step height was statistically significantly higher when comparing AGJ with HCl (27.9 (8.3) um -4.58 (0.83) um) and AGJ with CA (27.9 um (8.3) um – 2.21 (0.94) um) (p<0.0001). Step height was statistically significantly higher when comparing AGJ with AEP with HCl with AEP

(36.3 (7.1) um -6.7 (0.58) um) and AGJ with AEP with CA with AEP (36.6 (7.1) um – 1.44 (0.46) um) ($p<0.0001$). step height was statistically significantly higher when comparing HCl with AEP with CA with AEP (6.7 (0.58) um -1.44 (0.46) um; $p<0.001$). However, there was no statistically significant difference between HCl and CA ($p>0.05$). When comparing the groups with and without AEP, step height was statistically higher only in AGJ with AEP compared to AGJ (36.3 (7.1) um- 27.9 (8.3) um; $p<0.001$).

	Step height			
IMMERSION TIME (S)	*30s*	*60s*	*120s*	*300s*
DIW	0.06 (0.02)	0.06 (0.01)	0.06 (0.03)	0.05 (0.02)
CA + AEP	*0.08 (0.04)*	0.40 (0.16)	1.77 (0.80)	1.44 (0.46)
CA	*0.16 (0.11)*	0.94 (0.49)	2.23 (0.84)	2.21 (0.94)
HCl + AEP	*1.88* (0.98)	3.29 (0.43)	2.68 (0.52)	6.7 (0.58)
HCl	0.54 (0.21)	0.92 (0.38)	1.89 (0.41)	4.58 (0.83)
AGJ + AEP			19.3 (4.5)	36.3 (7.1)
AGJ			16.6 (3.4)	27.9 (8.3)

Table 2-4: Mean (SD) of step height using NCSP (um)

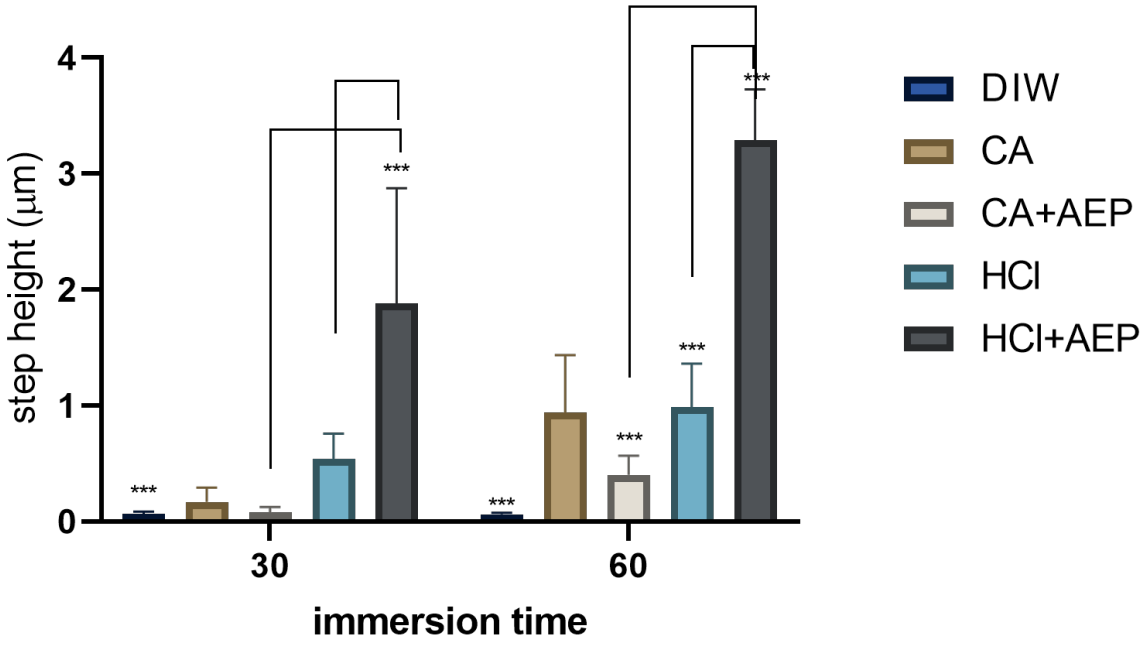


Figure 2-7: Mean (SD) NCSP measuring step height (mm) when samples immersed for 30s and 60s in the experimental solution, lines and (***) represent significant difference of (P<0.001)

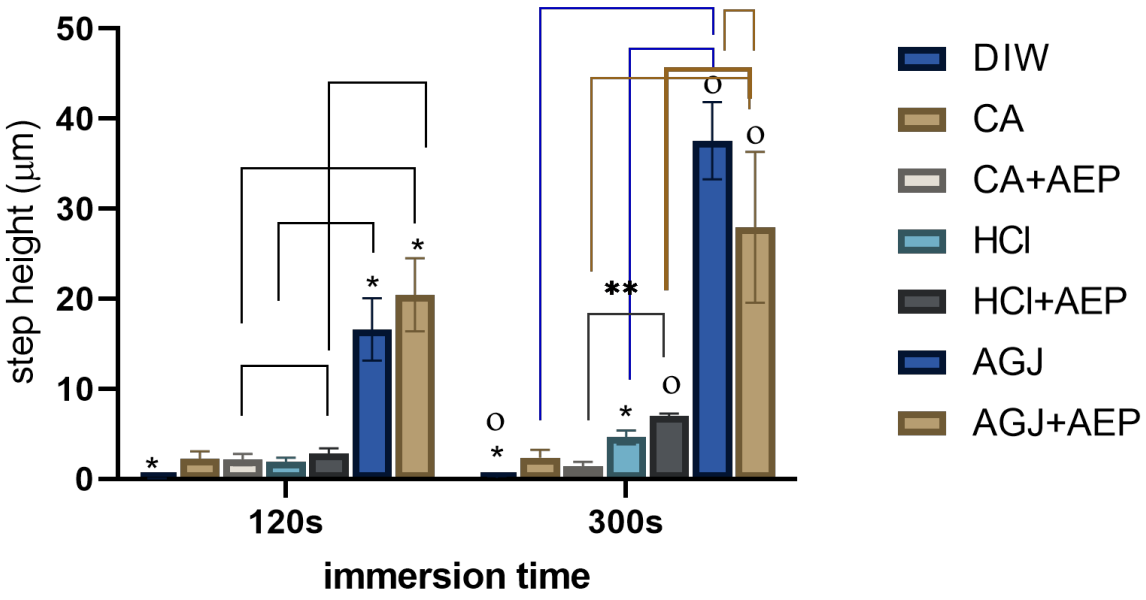


Figure 2-8: Mean (SD) NCSP measuring step height (mm) when samples immersed for 120s and 300s in the experimental solution, lines represent significant difference (P<0.001), (**) represent significance of (P<0.001), (*) and (o) represent significance of (P<0.05).

2.4.2 Microhardness

Microhardness baseline values for samples that were immersed in saliva were taken from post immersion in saliva to account for the action of AEP [Mutahar et al., 2017a].

Mean (SD) from the microhardness results are displayed in {Figure 2-9, 2-10, 2-11} {Table 2-5}.

In all experimental groups, the SMHc showed a significant increase with the increase of immersion time except for DIW ($p<0.0001$). Compared to DIW, SMHc for all experimental solutions were statistically higher at all immersion time points: Mean (SD) SMHc for CA at 30s, 60s, 120s and 300s were: 46.2 (2.8) KHN, 57.2 (4.2) KHN, 73.6 (3.5) KHN, 95.3 (2.8) KHN respectively and for CA with AEP were: 55.1 (2.9) KHN, 76.1 (3.6) KHN, 85.8 (2.8) KHN, 116.0 (5.5) KHN respectively.

Mean (SD) SMHc for HCl at 30s, 60s, 120s and 300s were: 52.5 (4.6) KHN, 75.4 (3.8) KHN, 85.1 (2.4) KHN, 109.5 (4.4) KHN respectively and for HCl with AEP were: 66.02 (2.3) KHN, 90.2 (4.6) KHN, 105.0 (4.8) KHN, 123.7 (3.4) KHN respectively. All were statistically higher when compared to DIW and increased with increase in immersion time. Mean (SD) SMHc for AGJ at 120s and 300s were: 204.7 (37.5) KHN and 152 (22.1) KHN and for AGJ with AEP were: 206.5 (43.1) KHN and 245.4 (39.8) KHN respectively. All were statistically higher when compared to DIW and increased with increase in immersion time.

Two-way ANOVA showed significant interaction between independent variables (time) and (acid); $p<0.001$. Hence Tukey's multiple comparison test reported the following results:

At 30 seconds, when comparing HCl and CA groups, SMHc was statistically significantly higher in HCl compared to CA (52.5 (4.6) KHN- 46.2 (2.8) KHN; $p<0.001$) and in HCl with AEP compared to CA with AEP (66.02 (2.3) KHN- 55.1 (2.9) KHN; $p<0.0001$). When comparing the groups with and without AEP, SMHc was statistically higher in CA with AEP compared to CA (55.1 (2.9) KHN - 46.2 (2.8) KHN; $p<0.0001$) and HCl with AEP compared to HCl (66.02 (2.3) KHN - 52.5 (4.6) KHN; $p<0.0001$).

At 60 seconds, the SMHc followed the same results as when samples were immersed for 30s. when comparing HCl and CA groups, SMHc was statistically significantly higher in HCl compared to CA (75.4 (3.8) KHN- 57.2 (4.2) KHN; $p<0.001$) and in HCl with AEP compared to CA with AEP (90.2 (4.6) KHN- 76.1 (3.6) KHN; $p<0.0001$). When comparing the groups with and without AEP, SMHc was statistically higher in CA with AEP compared to CA (76.1 (3.6) KHN – 57.2 (4.2) KHN; $p<0.0001$) and HCl with AEP compared to HCl (90.2 (4.6) KHN – 75.4 (3.8) KHN; $p<0.0001$).

At 120 seconds, when comparing AGJ, HCl and CA groups, SMHc was statistically significantly higher in AGJ compared to HCl (204.7 (37.8) KHN- 85.1 (2.4) KHN) and AGJ with CA (204.7 (37.8) KHN - 73.6 (3.5) KHN) ($p<0.001$). SMHc was statistically significantly higher in AGJ with AEP compared to HCl with AEP (206.5 (43.1) KHN- 105 (4.8) KHN) and AGJ with AEP with CA with AEP (206.5 (43.1) KHN- 85.8 (2.8) KHN) ($p<0.001$). When

comparing the groups with and without AEP, SMHc was statistically higher in CA with AEP compared to CA (85.8 KHN (2.8) - 73.6 (3.5) KHN; p<0.001) and HCl with AEP compared to HCl (105.0 (4.8) KHN – 85.1 (2.4) KHN; p<0.001)

At 300 seconds, the SMHc reported the same results as when samples were immersed for 120s. When comparing AGJ, HCl and CA groups, SMHc was statistically significantly higher in AGJ compared to HCl (152.9 (22.1) KHN- 109.5 (4.4) KHN) and AGJ with CA (152.9 (22.1) KHN -95.3 (2.8) KHN) (p<0.001). SMHc was statistically significantly higher in AGJ with AEP compared to HCl with AEP (245.4 (39.8) KHN- 123.7 (3.4) KHN) and AGJ with AEP with CA with AEP (245.4 (39.8) KHN- 116.0 (5.5) KHN) (p<0.001). When comparing the groups with and without AEP, SMHc was statistically higher in CA with AEP compared to CA (116.0 (5.5) – 95.3 (2.8) KHN; p<0.001) and HCl with AEP compared to HCl (123.7 (3.4) KHN – 109.5 (4.4) KHN; p<0.001)

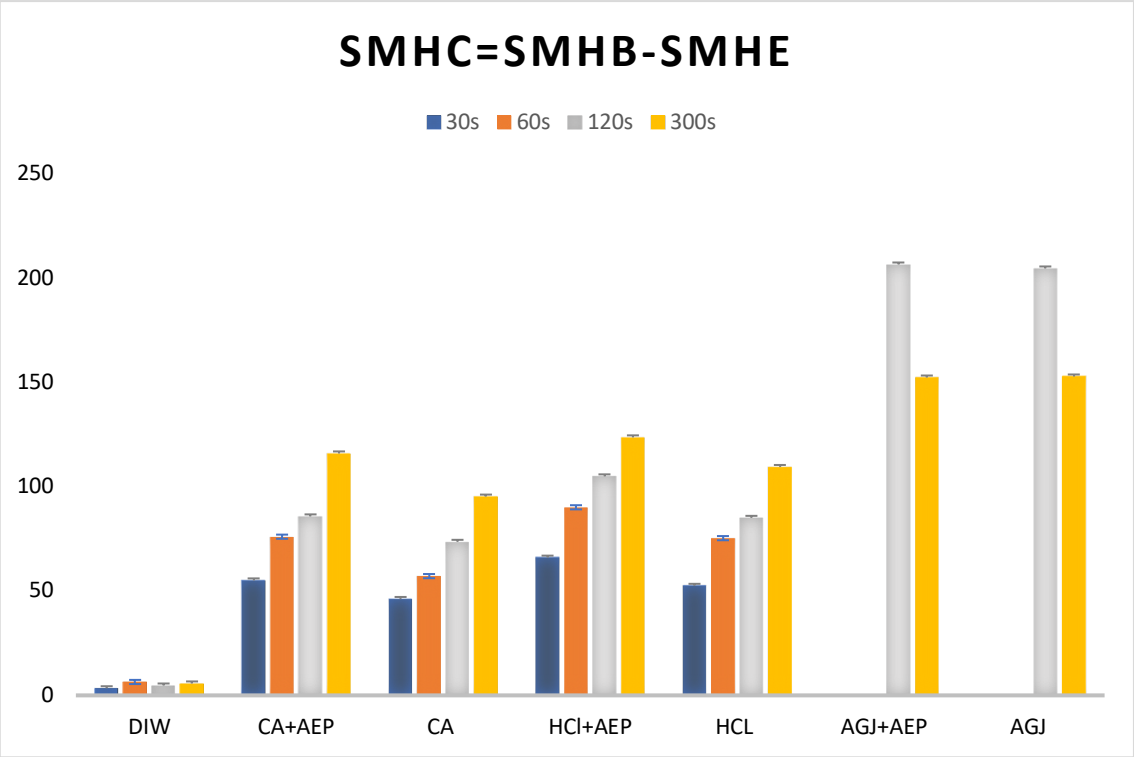


Figure 2-9: mean (SD) of microhardness change for all experimental solutions at all immersion times.

Table 2-5: Mean (SD) of surface microhardenss change measured using the formula (SMHc= SMHb-SMHe)

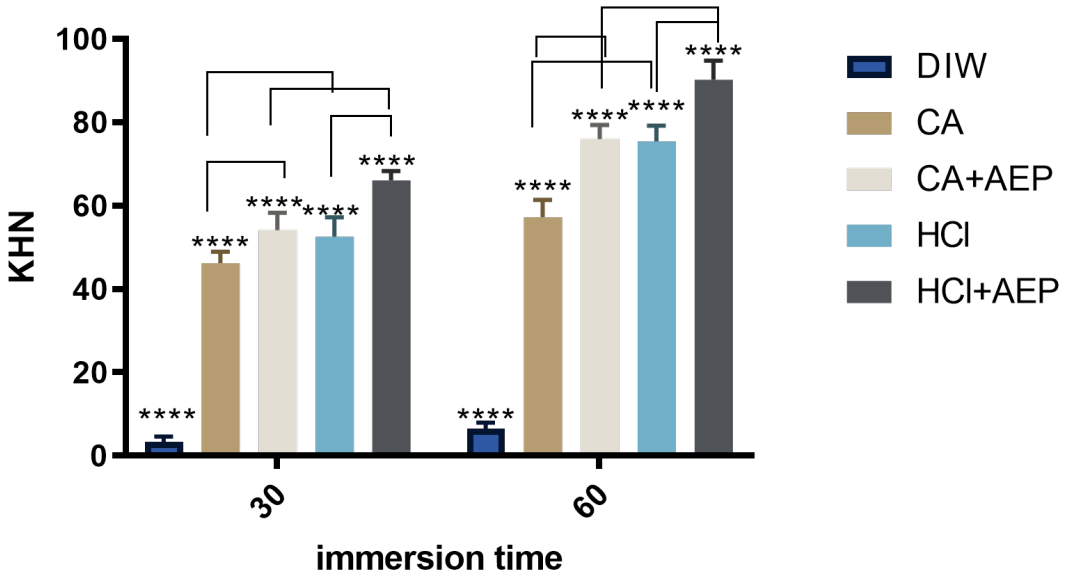


Figure 2-10: Knoop microhardness measuring SMHc (KHN) when samples were immersed for 30s and 60s in the experimental solution, lines and (***) represent significant difference of(P<0.001)

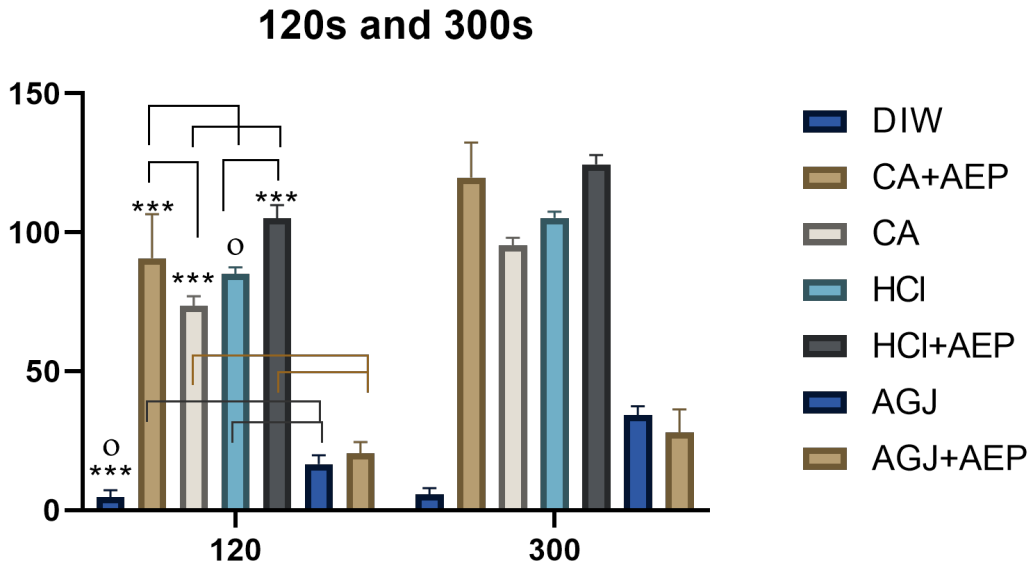
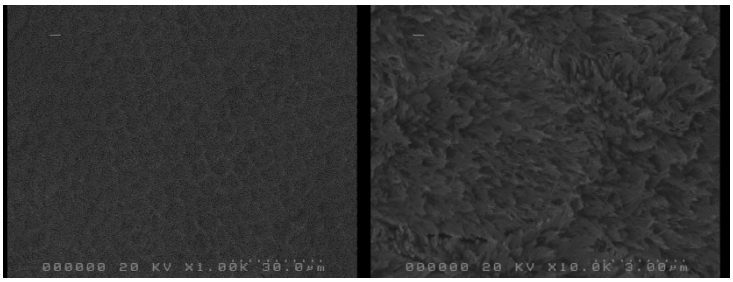


Figure 2-11: Knoop microhardness measuring SMHc (KHN) when samples were immersed for 120s and 300s in the experimental solution, lines and (***) and(o) represent significant difference (P<0.001). Significant difference pattern in 120s is exactly as same as in all samples immersed for 300.

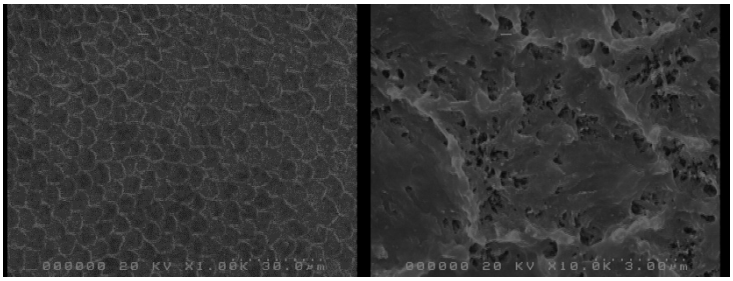
2.4.3 SEM

SEM images using two magnifications (1.00k and 10.00k) revealed the pattern of prism dissolution on samples immersed for 300s in the experimental solutions. At 1.00k magnification, all experimental samples show changes in enamel crystalline structure with variable thickness.

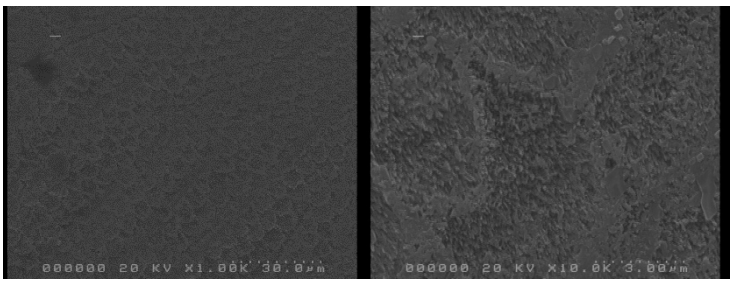
At 10.00k magnification, samples immersed in CA showed minor dissolution of prism cores with an intact prism periphery revealing a honey-comb structure and with the presence of AEP the prism core shows even less dissolution {Figure 2-12a, 2-12b}. When samples were immersed in HCl, deeper dissolution of the prism core can be seen with intact prism periphery and this became more defined when samples where immersed in saliva and formed an AEP prior to the acid exposure {Figure 2-12c, 2-12d}. Whereas immersion in AGJ shows more variation in the dissolution of the prism core with minimal dissolution of the prism periphery and with the effect of AEP the prism periphery were diminished {Figure 2-12e, 2-12f}.



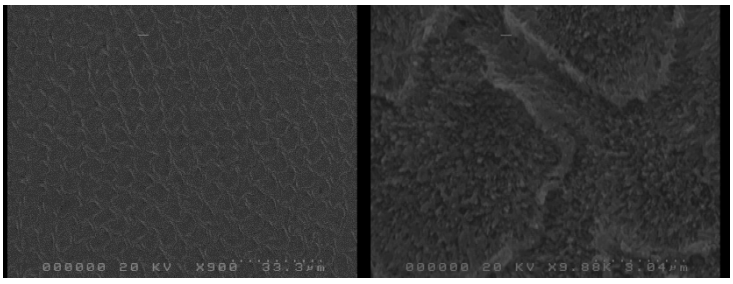
2-12a: Citric acid



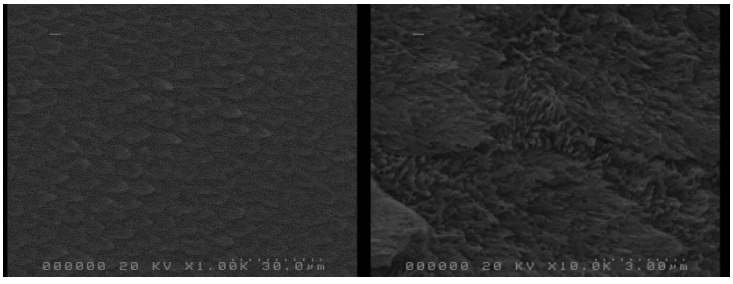
2-12b:Citric acid with AEP



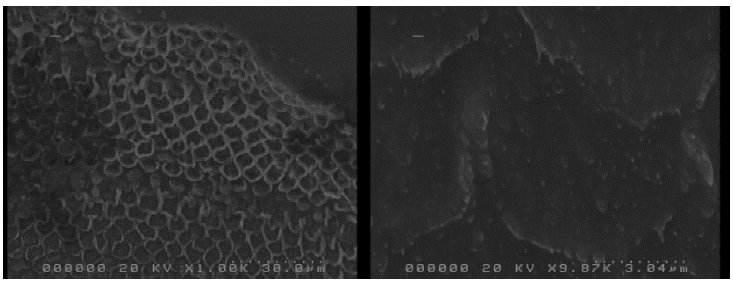
2-12c: HCl



2-12d: HCl with AEP



2-12e: AGJ



2-12f: AGJ with AEP

Figure 2-12: Qualitative measures using SEM at x1.00k and x10.00k magnifications for all samples immersed for 300s in the corresponding experimental solution.

2.4.4 ICP-MS

The mean values (mg/L) for Ca and P concentration are presented in {Figure 2-13}. The calcium and phosphorous concentrations in the AGJ solution without AEP increased from baseline at 300 seconds and the difference were statistically significant (Ca 788.15 mg/L to Ca 15041.9 mg/L; p=0.021) and (P 212.8 mg/L to P 7191.3 mg/L; p=0.021) respectively. The mean of calcium and phosphorous concentrations increased with increase of immersion time but lacked statistical significance. Furthermore, the calcium and phosphorous concentrations in AGJ after immersion in 120 seconds with AEP were higher than baseline levels (Ca 788.15 mg/L to Ca 7181.9 mg/L) and (P 212.8 mg/L to P 3579.7 mg/L; p=0.021) respectively. but the differences were not statistically significant.

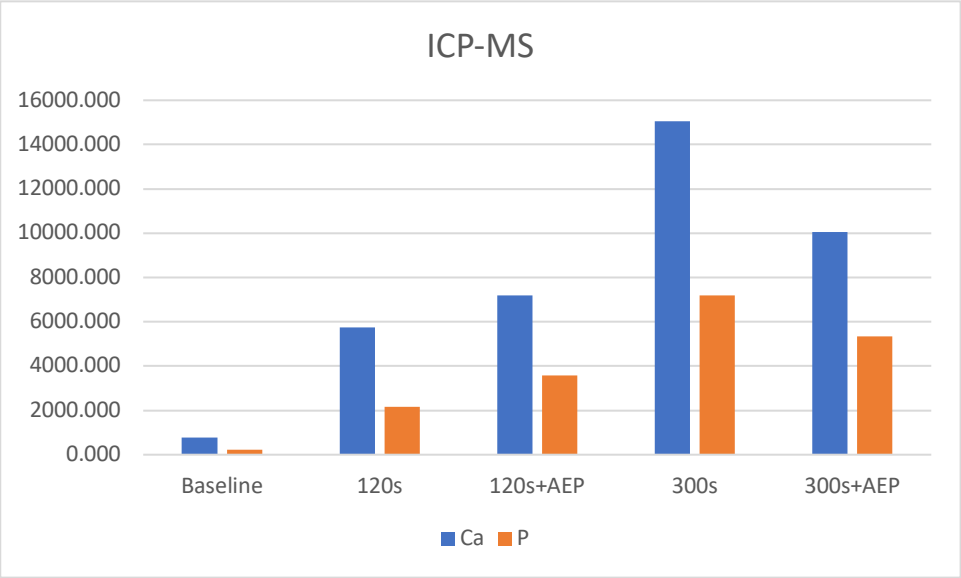


Figure 2-13: Ca 44 and P 31 concentration (ug/L) for non-AEP and AEP groups at 120s and 300s. The baseline represents the Ca and P concentration within the AGJ solution.

2.5 Discussion

This in-vitro study investigated the effect of acids simulating dietary (extrinsic) and gastric (intrinsic) acids on human polished enamel samples. The study assessed the impact of Hydrochloric acid and artificial gastric juice on polished human enamel samples at different time points and the effect of AEP on protection against acids immersion in citric acid (CA), hydrochloric acid (HCl) and artificial gastric juice (AGJ) at different time points increased surface microhardness change causing softer enamel surface and increased step height formation causing loss of enamel surface, which has also been observed in other studies [Austin et al., 2016b; Eisenburger et al., 2001; Hove et al., 2007]. The presence of AEP provided protection against dietary acid (citric acid) but not against gastric acids (HCl and AGJ). This indicates that AEP has a role as a physical barrier in dietary erosion but is not as effective in prevention of softening caused by gastric juice. Therefore, the null hypotheses were partially rejected.

The experimental design should simulate the real clinical situation as far as possible, therefore the solutions used simulated acids found in common dietary intakes and artificial gastric juice as well as HCl. Citric acid is the most frequent acid found in foods and beverages at (0.3%, pH 3.2) [Austin et al., 2016; Lussi et al., 2000; Mylonas et al., 2018]. HCl is the main acid in the gastric refluxate found at (HCl pH 2.2, AGJ pH 1.1) [Amoras et al., 2012; Hove et al., 2011; Mann et al., 2014; Schlueter et al., 2010]. In patients with GORD, It is well known that pepsin reaches the oral cavity during vomiting or regurgitation and was present in the artificial gastric juice used in this study.

The protocol followed previously published protocols by our group [Mistry et al., 2015; Mutahar et al., 2017a; Mylonas et al., 2018]. Human extracted teeth were disinfected using sodium hypochlorite (NaOCl) as it has been reported that the use of NaOCl for chemically cleaning natural human teeth does not affect the enamel surface nor the physiochemical properties [van 't Spijker et al., 2007]. In addition, Lippert et al. [2004b] demonstrated that the application of strong 14% of NaOCl did not affect the physical properties of unexposed enamel.

Only the buccal surfaces of molars were used in the current study, as it was demonstrated by Mistry et al. [2015] that the mean step height of buccal and lingual surfaces of molars (6.52 (0.78), 7.74(1.82)) respectively, or premolars (7.35(1.03), 8.166(1.63)) respectively, did not result in a significant difference when measured using a white light profilometry. A significant difference was reported when comparing molars and premolars using microhardness test ($p=0.001$). Tucker et al. [1998] reported that buccal surface was less susceptible to erosion compared to lingual surface, due to the difference in the wear extension of the fluoride rich layer. Therefore, to avoid the variability in mineral content and for standardisation, only buccal surface was included in the current study. Furthermore, enamel samples were polished and checked for flatness of the surface prior to use, to ensure erosion measurements were accurate and maximum sensitivity of profilometry measurement was obtained. Following polishing and embedding, samples were immersed in DIW and ultrasonicated. The ultrasonication effect on ETW in in-vitro models has was previously been studied. It was demonstrated that when samples were immersed in DIW for 4 hours it did not result in demineralisation of enamel. Whereas when enamel samples were exposed to citric acid for periods

between 30 minutes and 4 hours, ultrasonication resulted in an increase of enamel surface loss measured using profilometry [Jaeggi and Lussi, 2014]. However, in the present study, ultrasonication was used after polishing samples and prior to the erosive experimental procedure. It was used to ensure surface cleaning and removal of any debris on the polished enamel surface.

One of the influencing factors of ETW rate is the manner of immersion of the erosive agent. One factor that has been shown to have an influence is the flow rate of the acid, Shellis et al. [2005] showed that with the increase of acid flow rate, the erosion depth increases. Moreover, it has been demonstrated that with an increasing speed of agitation, the experimental solution could physically remove the dissolved tissue resulting in an increase dissolution rate [Attin and Wegehaupt, 2014]. Furthermore, previous studies have used various models including no stirring [Schlueter and Luka, 2018], minimal agitation [Bartlett et al., 1999], and other studies used various velocities of agitation [Bartlett, 2003]. However, in the present in-vitro model, experimental solutions were stirred at 62 rpm representing the clinical oral conditions. The stirrer was calibrated to ensure controlled and reproducible flow rate of the solution [Bartlett, 2003]. Another factor influencing ETW is the exposure time of an erosive challenge. Austin et al. [2016] reported that changes on human enamel samples could be detected as early as 30 seconds of acidic exposure. Mullan et al. [2017] detected significant increase in surface roughness after polished enamel samples exposure to orange juice for 5 minutes. Therefore, in the present study, citric acid and HCl were applied for 30, 60,120 and 300 seconds simulating early erosion models [Austin et al., 2016a; Field et al., 2017; Mylonas et al., 2018]. However, acid reflux in healthy individuals cleared by saliva

and oesophageal peristalsis within 1-2 minutes but at longer period in patients with GORD [Orr, 2003]. Therefore, artificial gastric juice in the present study was applied for 120 and 300 seconds to mimic the true clinical situation.

For the purpose of quantitative assessments, non-contact surface profilometry was used to quantify the level of step height in relation to the non-treated area, as it is considered a gold standard measurement used in in vitro and in situ studies [Paepegaey et al., 2013b]. The laser used in this experiment was red light NCSP of 0.01 µm resolution and laser spot sizes of 2 µm, which provide the ability to analyse deep pits of the enamel. The disadvantage of such small spot size is that it could possibly produce increased measurement reading.

Moreover, surface microhardness was used to quantify the hardness changes within the surface. In this experiment, a Knoop indenter was used which penetrates the surface by 1.5 µm providing higher sensitivity compared to Vickers indenters to changes on the erosive lesion within the superficial layer. Rakhmatullina et al. [2013] reported that the relationship is not linear between softening of enamel surface and tooth surface loss. This is similar to our findings, our data showed that there was a non-AEP group (citric acid and HCl at 30s and 60s) with statistically significantly higher surface microhardness change, but a lower step height formed. Also, in our study, it was shown that the presence of AEP in the AEP groups resulted in increased surface microhardness change, but no changes reported for the step height. However, the produced softer enamel surface may be more susceptible to loss in mechanical wear [Lussi et al., 2014].

SEM was used in this study for a qualitative assessment and to obtain visual images of enamel dissolution produced by different acids. It can be visualised that the ETW occurred as a layer-by-layer dissolution of the hydroxyapatite crystals, resulting in tissue loss and softening of the enamel surface [Lussi et al., 2012b]. The enamel crystals were exposed to different acids for 300s showing how HCl rapidly destroys the hydroxyapatite crystalline structure, and the presence of AEP enhances this effect, and a similar finding was seen in a higher dissolution rate when samples were exposed to AGJ. This observation is important for understanding the fast progression of ETW in patients with GORD when compared to dietary ETW.

Numerous factors have been identified to affect the erosive potential of acids contacting human enamel tissue, these are either chemical factors or biological. Chemical factors include the type of acid, pH level, titratable acidity and chelating properties [Lussi et al., 2012b]. Thereby, results of this study may be influenced by the different pH level used and the different type of acids. However, little is known about the different effects of in-vitro dietary acid (citric acid) compared to intrinsic acid (HCl and AGJ with pepsin) mimicking the real clinical situation.

The protective effect of saliva and AEP against erosive attacks in the oral cavity is still a matter of debate. Some studies have shown that saliva and AEP are the biological protective factors against ETW [Van't Spijker et al., 2009], whereas others have demonstrated that saliva or AEP offered limited or no protection [Bartlett and O'Toole, 2019]. In this in-vitro study, AEP was formed from pooled human saliva which was frozen immediately after collection and thawed prior to the time of use. Pooled saliva was

chosen over an individual donor as it was observed that protective effect of in-vitro formed AEP varies between individuals [Bartlett et al., 2019]. Therefore, on the grounds of avoiding bias and overcoming salivary variabilities, it was decided to use pooled human saliva to form an in-vitro AEP. However, the frozen saliva was carefully handled and mixed vigorously after thawing to ensure protein precipitation were re-suspended [West et al., 2013a].

In the current study, in-vitro AEP was formed after immersing samples for 24 hours in natural pooled human saliva. As it was reported by Mutahar et al. [2017a] that the highest protection against acid erosion was when the AEP was formed for 24 hours compared to 30 minutes and 60 minutes. However, studies showed that AEP formation continues in maturation at longer immersion times ranging between 24 hours and several days [Amaechi et al., 1999; Hannig and Balz, 1999; Hannig et al., 2004b].

Furthermore, the results from AEP groups showed an increase in surface microhardness after immersion in saliva for 24 hours. Similarly, Mutahar et al. [2017] reported an increased surface microhardness change in samples with 24 hours formed in-vitro AEP compared to those without the presence of AEP. The softer enamel surface reported after the AEP is formed could be due to many reasons. It could be due to the mineral layer formed, Ganss et al. [2001] reported that the protective effect of AEP could be through forming a mineral layer on the enamel surface, under an acidic attack, this layer would be dissolved protecting the underlying tissue from dissolution. Moreover, it could be due to the prolonged immersion time in saliva (24 hours), which could affect the uptake rate of salivary protein binding [Johansson, 2002; Shellis et al., 2013]. In addition, it could be due to a formed organic layer on the enamel surface, which would

create a more porous enamel surface hence the greater microhardness change and the softer the surface measurement [Young and Tenuta, 2011]. For all the mentioned reasons, the baseline measurement of the AEP groups was considered as the post saliva immersion microhardness measurement.

Our profilometry data showed that immersion of the polished enamel samples in saliva for 24 hours prior to the acid exposure resulted in significant reduction in the loss of enamel when samples were immersed in CA. This finding confirms previous findings [Amaechi and Higham, 2001; Buzalaf et al., 2012a; Cheaib and Lussi, 2011]. The protective effect of natural saliva was investigated by Mutahar et al. [2017] comparing it to artificial saliva and DIW. They immersed enamel samples for 24 h prior to an erosion cycle of 10 minutes citric acid exposure for five times. They observed significant lower step height in the natural saliva group compared to artificial saliva and DIW groups. However, the lacking maturation of the AEP that occurs in-vivo might be an explanation for the surprising results, which makes it difficult for comparison.

Interestingly, when samples were immersed in HCl and AGJ, they resulted in an increased enamel loss and more softening than those immersed in CA. This finding could be explained by the different chemical erosion process between the used acids. The ETW process partly depends on the hydrogen ions present in weak (CA) and strong acids (HCl); these ions attack the hydroxyapatite crystals and dissolve it by binding to either carbonate or phosphate ions resulting in a chelation process as seen in CA attack. Whereas when the attack is by a strong acid like HCl, the acid dissociates completely in water and directly dissolves the crystalline structure [Shellis et al., 2014], which explains

the loss and softening findings of the present study specially in conditions where the acid attack is prolonged or repeated as seen in GORD patients.

Digestive enzymes like pepsin could reach the oral cavity in patients suffering from GORD especially during vomiting, and affect the oral tissues by degrading the organic matrix. However, unlike acids, few studies referenced the association between pespin and ETW. Thus, the presence of pepsin in artificial gastric juice of this in-vitro study is believed to enhance the ETW process. Shlueter et al. [2010] investigated the effect of pepsin and trypsin enzymes on dentines, and reported 45% increase in mineral loss when these two enzymes were combined which led to increased degradation of the organic matrix. However, there are many rationales that might explain the significant loss of the enamel surface when exposed to artificial gastric juice with pepsin. It could be due to the enamel microstructure, enamel is a non-collagenous highly mineralised tissue [Hughes et al., 2000], and dentin as a collagen-rich calcified tissue that supports the brittle outer enamel surface [Grenby, 1996]. Enamel is composed of an organic prism sheaths, that run between enamel rods of about 100-400 um thickness, that extend from the dentine-enamel junction [Shellis et al., 2011]. Under the effect of the used artificial gastric juice, we speculated that pepsin could cause an aggressive dissolution of the organic matrix, which dominate at the inner region of enamel, and in combination to HCl in the solution the enamel distruction is even widely affected. In addition, ETW of human enamel have been proven as depth dependant. In the current study, enamel samples were polished exposing deeper enamel layer. This inner enamel region has been reported as less acid resistant compared to outer surfaces of enamel.

Another hypothesis of the significant enamel surface loss with the presence of AEP, could be that pepsin is a larger molecule compared to HCl [Cheng et al., 2009c; Hjortsjo et al., 2010], it has the ability to physically displace and remove the AEP from enamel surface [Grobler et al., 1990; Milosevic, 1997], which clears the surface for the HCl to easily penetrate resulting in distruction and dissolution of enamel. Although our study investigated the effect of the pepsin enzyme present in artificial gastric juice on human enamel surface at a concentration of (0.002g/ ml) to reach an optimum pH level of 1.1 [Johansson, 2002; Lussi et al., 2012a], to the authors knowledge there are no studies investigating the effect of pepsin enzyme on enamel surfaces which makes direct comparison difficult.

CA is a weak organic acid that dissociates progressively as pH rises, whereas HCl is a strong inorganic acid that fully dissociates at any pH level. Moreover, the HCl component becomes diluted by saliva increasing the availability of hydrogen ions resulting in a more erosive effect on the enamel structure, which supports the finding by Hannig and Balz. [1999] that AEP layers are permeable to protons. Though it cannot be ruled out that the presence of AEP caused dilution and barrier effect providing protection against erosion in CA, these effects do not appear consistent with how it performs when the attack is by HCl and AGJ. This could be explained by an interference to the protein binding by chemical alterations to the enamel surface, due to the HCl ingressing deeper in the enamel surface and dissolving the hydroxyapatite structure [Lussi et al., 2012b]. This agrees with Hannig et al. [2004b] and Hara et al. [2006a] reporting that AEP does not fully inhibit enamel erosion. However, surfaces with AEP may have been more prone to softening when exposed to the experimental solutions.

It could be hypothesised that the increased tooth tissue loss in the presence of AEP would be due to excess release of ions into the experimental solution; hence calcium and phosphate ion release were measured using ICP-MS in the samples immersed in AGJ. As saliva contains Ca and P, the formed AEP could contain these ions as well, this method cannot separate the ionic release from the pellicle itself; hence the baseline measured in the study represent the DIW used to wash the enamel samples after immersion in saliva for 24 hours. Increase in the calcium and phosphorous level was found when compared to baseline and when between the AEP non-AEP groups. This could be an explanation of the results seen in NCSP where the presence of AEP caused an increase is tooth tissue loss (higher step height) when samples were immersed in HCl and AGJ.

There are some limitations to the present study, including the use of polishing protocol which removes around 400um from enamel samples resulting in faster progression of erosion than natural unpolished enamel samples [Hemingway et al., 2006]. Moreover, natural unpolished samples display more mineralised surface which would reflect the true clinical situation and interactions. In addition, although it was demonstrated that the use of frozen or fresh human saliva did not result in a significant difference [Hara and Zero, 2008], the salivary protective effect could be altered due to the process of collection, storage and thawing [Jensdottir et al., 2005b]. Furthermore, although in vitro studies have the advantage of standardisation and in this study, efforts were made to mimic the clinical situation as far as possible, there are still differences to biomechanics within the oral cavity, which cannot necessarily be extrapolated to in-vivo conditions, making direct comparison between studies difficult.

However, the novel findings of this study provide some insight into the variation of ETW seen in GORD patients and dietary ETW patients. It demonstrates the protective effect of AEP against softening and loss of enamel when human teeth are exposed to extrinsic acids whereas it had a negative influence on enamel softening and tissue loss when human teeth were exposed to intrinsic acids.

2.6 Conclusion

Intrinsic acids caused more tooth tissue loss and softer enamel when compared to extrinsic acids. Presence of AEP resulted in softening of tooth enamel samples for all experimental solutions. Interestingly, presence of AEP reduced tissue loss in the groups representing dietary acid but resulted in increased tissue loss for groups representing intrinsic acids. This could be an explanation for the fast progression of ETW in patients suffering from GORD but needs further investigation in future studies

3 Chapter 3: Predictive Factors for Erosive Tooth Wear (ETW) In Patients with Gastro-oesophageal Reflux Disease (GORD) Symptoms: A Prospective Cross-Sectional Case Control Study

3.1 Introduction

Upper endoscopy is the first step for diagnosis of structural abnormalities of the oesophagus in patients suffering from gastro-oesophageal symptoms (such as dysphagia). If no structural abnormalities are detected, oesophageal manometry is used to assess oesophageal motor function. Manometry also determines the position of the upper and lower oesophageal sphincters and is followed by intralumenal impedance and pH monitoring for the diagnosis of Gastro-Oesophageal Reflux Disease (GORD).

High resolution manometry (HRM) and intraluminal 24hr-pH-impedance monitoring (pH-MII) have been established as gold standard diagnostic tools for pathological Gastro-Oesophageal Reflux (GOR). HRM is superior to conventional manometry and provides pressure topography plotting of the oesophageal pressure and functional contractility of the sphincters. It is used in the diagnosis of oesophageal motility disorders (achalasia, ineffective oesophageal motility, absent peristalsis, obstruction, absent of contractility). Intraluminal 24hr-pH-impedance monitoring is the latest reproducible technique used in clinical practice for its ability to provide detailed parameters including the nature of the reflux (gas, liquid, mixed) irrespective of the pH,

the frequency of individual reflux events and the symptoms association to reflux. The association between reflux episodes and symptoms during the test is carried out using "symptoms correlation analysis" which uses symptom index (SI) and symptom association probability index (SAP). The combination of both indices exhibits clear association of symptoms to gastro-oesophageal reflux disease (GORD).

The Reflux Symptom Questionnaire (RESQ) is a validated self-reported questionnaire used as a practical tool based on frequency and severity of symptoms. It is used to evaluate patients with GORD symptoms especially those who partially respond to proton pump inhibitors [Dent et al., 2010].

Previous studies have investigated GORD diagnosis as a risk factor for developing erosive tooth wear. However, it is not clear which parameters contribute most or indeed whether a combination of parameters increase the risk of erosive tooth wear. To the author's knowledge, there is no study to date that has used intraluminal 24-hr-pH-Impedance monitoring and HRM parameters to investigate the association of GORD parameters and erosive tooth wear in adults.

3.2 Aim, objectives and hypotheses

Aim

1. To investigate the association between GORD symptoms and ETW.
2. To investigate the severity and frequency of GORD symptoms in patients with and without ETW.

Objectives

1. To identify predictive factors associated with presence of ETW in patients with GORD symptoms using 24h-pH-impedance monitoring test.
2. To identify the association between oesophageal motility diagnosis and the presence of ETW using high-resolution manometry (HRM).
3. To identify the frequency and severity of GORD symptoms 7 days prior to the monitoring test using RESQ questionnaire.

Null Hypothesis

1. There is no association between GORD symptoms and ETW.
2. There is no association between oesophageal motility and ETW.
3. There is no association between frequency and intensity of GORD symptoms before the test and ETW.

3.3 Materials and methods

The study was a single-centre, prospective, case control study conducted at Guy's hospital, London, UK. Ethical approval was granted by National Research Ethics Service (NRES) in North East-York Research Ethic Committee (REC Ref 18/NE/0099).

3.2.1 Participants

Consecutive patients with symptoms suggestive of gastro-oesophageal reflux referred to the Oesophageal laboratory & breath test clinics at Guy's hospital by general practitioners for the assessment of GORD symptoms were approached between April 2018 and November 2019.

Inclusion criteria were:

1. Aged 18 to 95 years
2. Have a minimum of 20 natural occluding teeth present
3. Give written informed consent
4. Be in good general health other than GORD symptoms

Exclusion criteria were:

1. Pregnant or breast feeding
2. Presence of severe periodontal disease or active caries on more than one tooth.
3. Unable to speak or understand English
4. Wearing an appliance

5. Restoration of the occlusal or incisal surfaces of upper anterior teeth and first molars.
6. No signs or symptoms of GORD

3.3.2 Power calculation

The power calculation estimated the need for 282 participants to identify a 10%-difference in the prevalence of any GORD parameter between patients with and without ETW, assuming a recruitment ratio of one-to-one (141 GORD patients with ETW and 141 without ETW. The prevalence for the parameter of 15% and 5% in GORD patients with and without ETW respectively, 80% statistical power and 5% significance level. Sample size was increased to 300 (150 in each group) to compensate for potential exclusions due of missing values (up to 6%). A sample of 300 participants will also give 82% statistical power to detect a standarised mean difference of 0.33 units in a continuous GORD parameter (i.e., duration, etc.) between GORD patients with and without ETW, assuming a recruitment ratio of one-to-one (150 GORD patients with ETW and 150 without ETW), a common standard deviation of 1 unit in both groups and 5% significance level.

3.2.2 The study protocols

Patients attended on two consecutive days for HRM and pH-impedance tests. On the first day the HRM and placement of the intraluminal pH- Impedance were carried out by the gastrointestinal (GI) physiologist. Patients returned the next day for removal of the pH-impedance catheter and downloading of data from the data logger. For the purposes of the study all patients were approached on the first day and given a patient information sheet (PIS in appendix 7.7). Adequate time was offered during their appointment to consider the study. Those who agreed to take part in the study signed an informed consent form (ICF appendix 7.8). A single trained examiner (RS) carried out all oral clinical examinations in a clinical bed in an upright position under ideal lighting.

A general oral examination was carried out to select patients who met the inclusion/exclusion criteria. Those patients were subsequently entered into the study. A BEWE examination was carried out and each sextant was given a score. They were then asked to fill in the questionnaire (RESQ) while waiting. Patients subsequently attended their HRM appointment followed by the intraluminal pH-impedance testing. Figure 3-5 illustrates a flow diagram of the study.

Those who did not tolerate the insertion of the catheter-based test were referred by the GI physiologist for a wireless catheter-free test (BRAVO) (detailed in chapter 1 section 1.8.4).

3.2.3 Data collection

Data were collected as follows:

1. BEWE score
2. Self-administered questionnaire RESQ
3. Clinical information obtained from:

   a- High resolution manometry (HRM)

   b- 24h-pH-impedance monitoring (pH-MII)

- BEWE score:

The presence and severity of erosive tooth wear was measured using the Basic Erosive Wear Examination (BEWE) index. The examination was carried out by the same clinician (RS), who was trained and calibrated.

3.2.3.1 Training and inter-examiner reliability

The clinical investigator (RS) was trained by a gold standard examiner (RM) and calibrated, each examiner recorded the scores separately and blinded to the other examiner scores. Training was done on study casts first followed by training on patients. First, casts were examined, all surfaces (n=56) (cervical, buccal/labial, occlusal/ incisal and lingual/palatal) were graded separately of each tooth except: third molars, teeth with >50% of restoration, carious and traumatised. A score was assigned to each sextant according to the highest present wear score, and a cumulative BEWE score calculated by adding the scores of each sextant. Scores ranged between 0 to 18, according to the original BEWE criteria [Bartlett et al., 2008]. The criteria were: 0= no loss of surface

characteristics, 1= initial loss of surface texture, 2= loss of hard tissue <50% of surface area and 3= loss of hard tissue >50% of surface area).

Second, training was done on patients attending the dental care units at Guy's hospital. Patients were approached on the day of their appointment and asked to participate for the training practice. Consent was obtained verbally from ten patients to undertake the examination and were examined on a dental chair at a reclined position under an ideal light source. All examined teeth were dried using air compressor and graded following the above-described process.

Kappa scores for categorical variables were analysed for inter-examiner test reliability, weighed Kappa scores for ordinal variables were analysed as well. Inter-examiner agreement percentage for BEWE score was assessed and reported. The strength of agreement for Kappa score categorised according to Masson et al [2003] into : poor <0.20, fair 0.21 to 0.40, moderate 0.41 to 0.60, good 0.61 to 0.80, very good 0.81 to 1.00.

When assessing study casts, the inter-examiner Kappa score between (RS) and (RM) Kappa was 0.44, the weighted Kappa 0.47 was and the percentage agreement was very good 85.4%. When assessing patients, the kappa was 0.75 , the weighted kappa was 0.75 and the percentage agreement was very good scoring 92.6%.

In the current study, all assessments were carried out under ideal lighting, without magnification. The cervical, buccal/labial, occlusal/ incisal and lingual/palatal surfaces of each tooth were examined in the same manner for all participants. Those with a cumulative score of 12 or more with at least 1 sextant scoring 3 were included in the case-group and those with a cumulative score of less than 12 were included in a control-group [O'Toole et al., 2017].

- Questionnaire:

Participating patients were asked to fill out a questionnaire (RESQ), which is a validated, self-reported questionnaire to assess frequency and intensity of gastro-oesophageal reflux symptoms in the 7 days prior to the tests. A 6-point Likert response format was used for both frequency (0 to 5) and intensity (0 to 5). Refer to Appendix 7.10 for the questionnaire form. The licence for the use of RESQ was obtained from AstraZeneca AB, Möndel, Sweden (Appendix 7.11). Patients were asked to complete the questionnaire in a paper form prior to their appointment, which took around 5 minutes of their time.

- High resolution manometry (HRM)

Patients were asked to discontinue any acid inhibitory drugs and medications that control gastrointestinal motility for 7 days prior to the test. They were also asked to fast for 12 hours prior to the procedure. HRM procedure was performed by a GI Physiologist, providing a diagnosis of the oesophageal motor function using The ManoScan™ ESO high resolution manometry system (Sierra Scientific Instruments, USA). The system specifically quantifies oesophageal contractions and LOS to identify any abnormal outflow resistance. It can be performed in 10 minutes, delivering useful information about the oesophageal pressure profile of patients and complete physiological mapping throughout the oesophagus, as well as determining the location of the LOS.

The manometric findings were then interpreted using the Chicago classification by the GI Physiologist, which is considered a standard approach for categorising oesophageal motility disorders.

The following data were used in this study: normal oesophageal motility, achalasia, absent of contractility, fragmented peristalsis, oesophageal junction (OGJ) outflow obstruction, ineffective oesophageal motility (IOM) and hiatus hernia. A topographical illustration of a high-resolution manometry obtained from the recruited patients can be shown in figures 3-1 and 3-2.

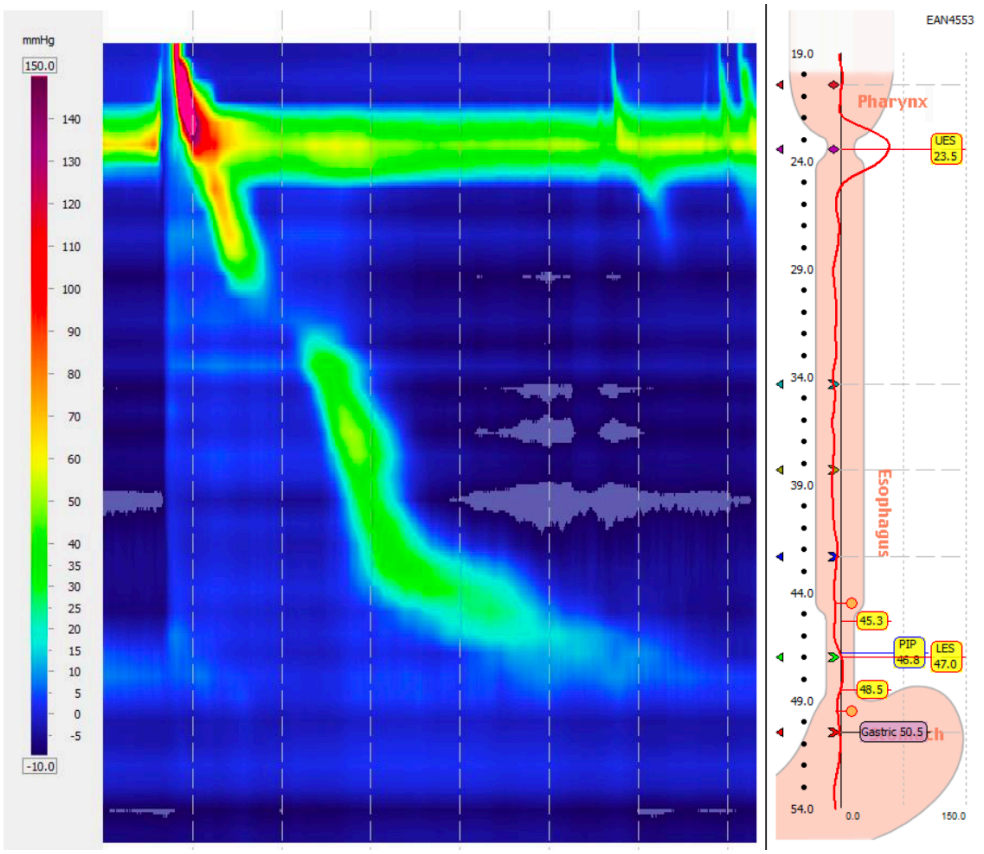


Figure 3-1: illustration of HRM finding, An example of a patients classified by the Chicago classification as: ineffective oesophageal motility (minor disorder). UOS and LOS showing complete relaxation on swallow, the oesophageal body showed weak peristalsis and no hiatus hernia.

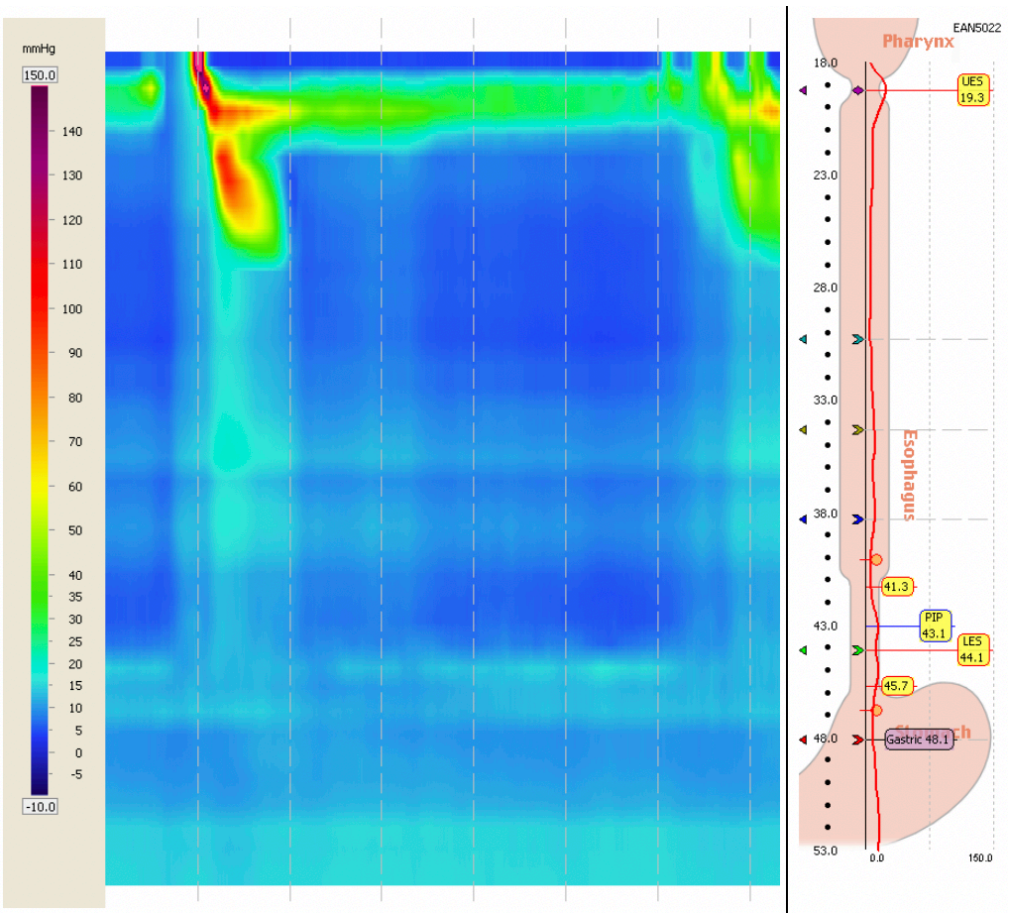


Figure 3-2: illustration of HRM finding, an example of Chicago classification as: absent of contractility (major disorder), UOS and LOS were hypotensive with complete relaxation on swallow, the oesophageal body produced no peristaltic contractions and dysphagia was reported during solid swallow.

- Intraluminal 24h-pH-Impedance monitoring (pH-MII)

Patients underwent intraluminal pH-MII monitoring test after the HRM. The Oesophageal pH-MII monitoring was performed to measure pH levels and impedance values through a 2.13 mm multichannel impedance-pH catheter (ZAI-BG-44, Sandhill Scientific, Inc.; Highland ranch, CO, USA).

The catheter was inserted trans-nasally under topical anaesthesia. The catheter includes two pH channels (gastric sensors and oesophageal sensor), one of which was placed 5cm above LOS and the other placed 15 cm below the proximal pH sensor {Figure 3-3}. The catheter also includes 6 intraluminal impedance channels which were placed throughout the oesophagus at 2, 4, 6, 8, 10, 14, 16, 18 cm from tip of the catheter with 2 cm space in between, (Details in section 1.8.4).

Patients were instructed to carry the data collection device on a belt and press the symptom button on the datalogger whenever they experienced a symptom as illustrated in {Figure 3-4}. They were encouraged to keep a diary of their daily meals and record body position (upright, supine) during the 24 hours of monitoring period. Data were collected at the end of the observation period from the worn device (patients instruction sheet in appendix 7.13).

The pH-MII parameters were analysed by the reporting physician using BioView analysis software (Sandhill Scientific Inc. 2014, Colorado, US). The auto-scan screening for reflux is set to creating pH <4 threshold (excluding meal periods).

The following pH-MII parameters from the collected data were analysed in this study using specified cut-off points:

- Percent of acid exposure time (AET) defined as the total time of oesophageal pH<4 divided by total monitoring time, according to the Lyon consensus [Gyawali et al., 2018]: Normal <4, inconclusive 4-6, abnormal >6.
- Percent of acid exposure in upright position (AE_UP): normal if =<6.3%.
- Percentage of acid exposure in supine position (AE_SUP): normal if =<1.2%.
- The total number of reflux episodes categorised as: normal< 40, inconclusive 40-80, conclusive >80.
- DeMeester score: a global measure for analysing oesophageal acid exposure. It is a composite score including 6 parameters, these parameters and the normal threshold are detailed in Table 3-1. Considered normal if the composite score is < 14.7.
- Reflux episode activity impedance analysis: number of reflux episodes detected by impedance and categorised as acid or non-acid by pH in both distal and proximal (proximal migration level at 15 cm from the LOS oesophagus) oesophagus, only liquid and mixed reflux episodes were reported.
- Symptom-reflux association analysis: statistical parameter to define the relationship between the reflux episode and symptomatic event, the analysis is done using:

    1. Symptom index (SI), an index that quantifies the symptoms episodes that are related to reflux. It is a percentage calculated as the number of reflux episodes related to symptoms divided by total number of symptoms episodes X 100 [Vaezi, 2012].

    2. Symptom associated probability (SAP), an index that quantifies the probability that the observed result is not brought by chance. It is a percentage

calculated by a complex formula, which is time consuming if done manually. Hence usually the analysis is done automatically through an incorporated analysis within the monitoring software.

Symptom-reflux association analysis in this study was considered positive if SI≥ 50% and/or SAP≥ 95% and negative when SI< 50% and SAP< 95% [Desjardin et al., 2016].

- GORD diagnosis: the diagnosis performed by the GI Physiologist according to patients' endoscopic, manometric, and pH-impedance metric findings. The diagnosis of pathological GORD includes abnormal values of: DeMeester score, percent of acid exposure in supine position, percent of acid exposure in upright position and percent of total acid exposure time.

• Parameters	Threshold value
% of total time the pH<4	< 4.5
% upright time pH<4	< 8.4
% supine time pH<4	< 3.5
number of reflux episodes	< 46.9
number of reflux episodes over 5 minutes	< 3.5
longest reflux episode	< 19.8

Table 3-1: Parameters included within Demeester score

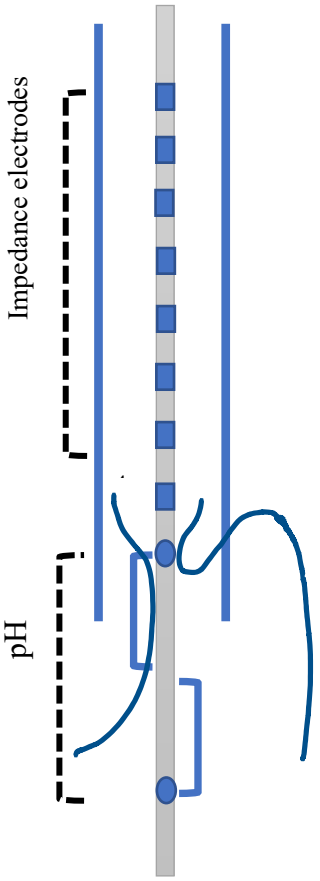


Figure 3-3: schematic demonstration of combined pH and impedance catheter, 2 sensors place 5 cm above and below the LOS, 8 impedance electrodes with 2 cm intervals.

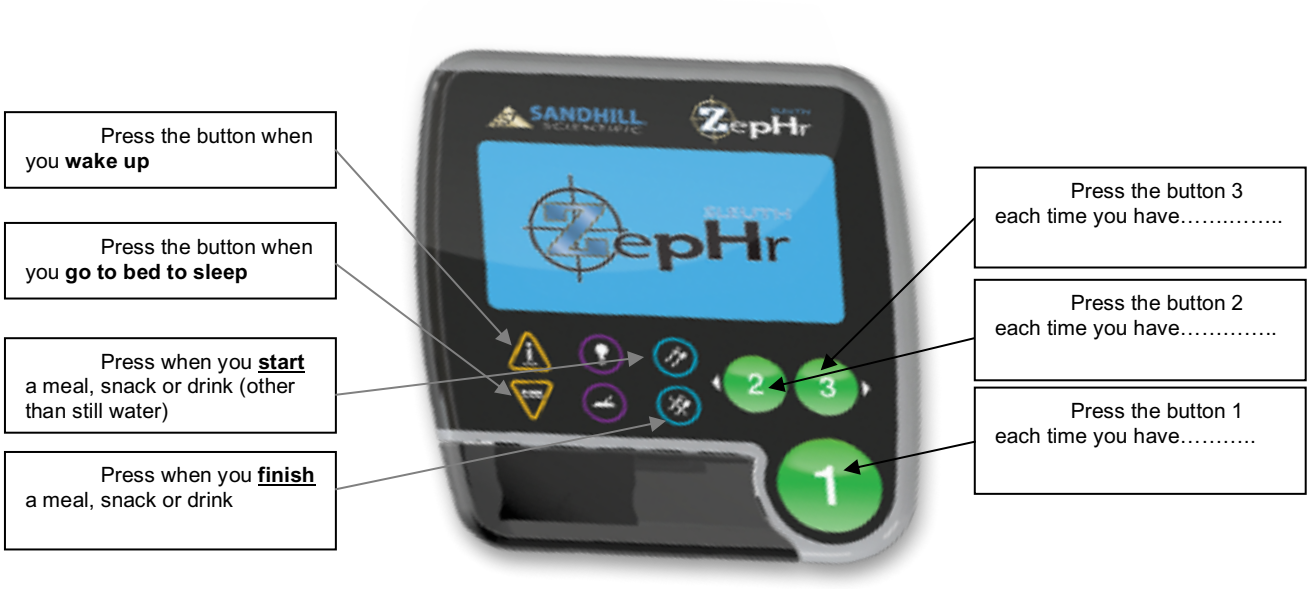


Figure 3-4: the data collecting device worn by patients on a belt, the instructions of how to use the device to keep records of their symptoms, meals and body position

3.2.4 RESQ data analysis method

All data were analysed using STATA software (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC). The frequency and intensity of symptoms were analysed using the RESQ questionnaire through a modified scoring system, 13-items were aggregated to 6 frequency and 6 intensity domain scores as follows:

- Heartburn (5 items): burning feeling behind the breastbone, pain behind the breastbone, burning feeling in the centre of upper stomach, pain in the centre of upper stomach, heartburn.
- Regurgitation (4 items): acid taste in your mouth, bitter taste in your mouth, unpleasant movement of material upwards from the stomach, stomach content reaching the throat of mouth.
- Burping (1 item).
- Cough (1 item).
- Hoarseness (1 item).
- Difficulty swallowing (1 item).

Items were coded from 0 to 5 for both frequency and intensity, frequency data were re-coded before calculating the domain score: (0=0) (1=1) (2=2) (3=3.5) (4=5.5) (5=7), giving the minimum/ maximum score for frequency 0-7 and intensity 0-5. Missing values were computed as a mean score of the non-missing items within a domain, according to the “half-scale” method (appendix 7.12).

In order to facilitate analysis, the scoring system for both frequency and intensity were subcategorised into mild, moderate and severe as detailed in tables 3-2, 3-3:

Frequency:

Score level	Definition	Subcategory
0	Non on any day of the week	0
1	Present on 1-2 day of the week	1
2	Present on 2 days of the week	1
3-4	Present on 3-4 days of the week	2
5-6	Present on 5-6 days of the week	3
Daily	Present every day of the week	4

Table 3-2: RESQ frequency scoring system used in the study

Intensity:

Score level	Definition	Subcategory
0	Did not have the symptom	0
1	Very mild	Mild
2	Mild	Mild
3	Moderate	Moderate
4	Moderately severe	Severe
5	Severe	Severe

Table 3-3: RESQ intensity scoring system used in the study

A receiver operating characteristic (ROC) curve analysis was used to assess the cut-off point for ETW for each of the above symptoms. The cut-off was determined using the value that maximised both sensitivity and specificity. Firstly, the sum of the scores for the frequency and intensity of all the symptoms were plotted. Subsequently the frequency and intensity of the symptoms that were statistically significant ($P<0.05$) between cases and controls were plotted.

For the purpose of analysis, collected data were divided into: (1) motility results and (2) impedance results. Whereas data about symptoms were divided into: (1) symptoms reported 7 days before the test and (2) symptoms during the test.

3.3 Statistical analysis

Comparison between case and control groups was firstly analysed using single variable logistic regression with the presence of ETW as the dependant variable. The variables considered were GORD diagnosed, AET, DeMeester, motility disorder, and symptoms reported in the impedance (heartburn, regurgitation), symptoms reported in RESQ (frequency of heartburn, regurgitation, difficulty swallowing, coughing, hoarseness and (intensity of: heartburn, regurgitation, coughing). Further analysis was done according to the GORD diagnosis as a dependant variable.

Results are reported as n (%), Odds Ratio and 95% Confidence Interval for categorical data, median (Interquartile Range), Odds Ratio and 95% Confidence Interval for non-normally distributed continuous data and mean (SD), Odds Ratio and 95% Confidence Interval for normally distributed continuous data.

A backwards stepwise multivariable logistic regression was then conducted to identify which variables were significantly and independently related to ETW. Variables with a p-value <0.2 were considered for inclusion in the multivariable analysis. Age and gender were included in the model regardless of significance.

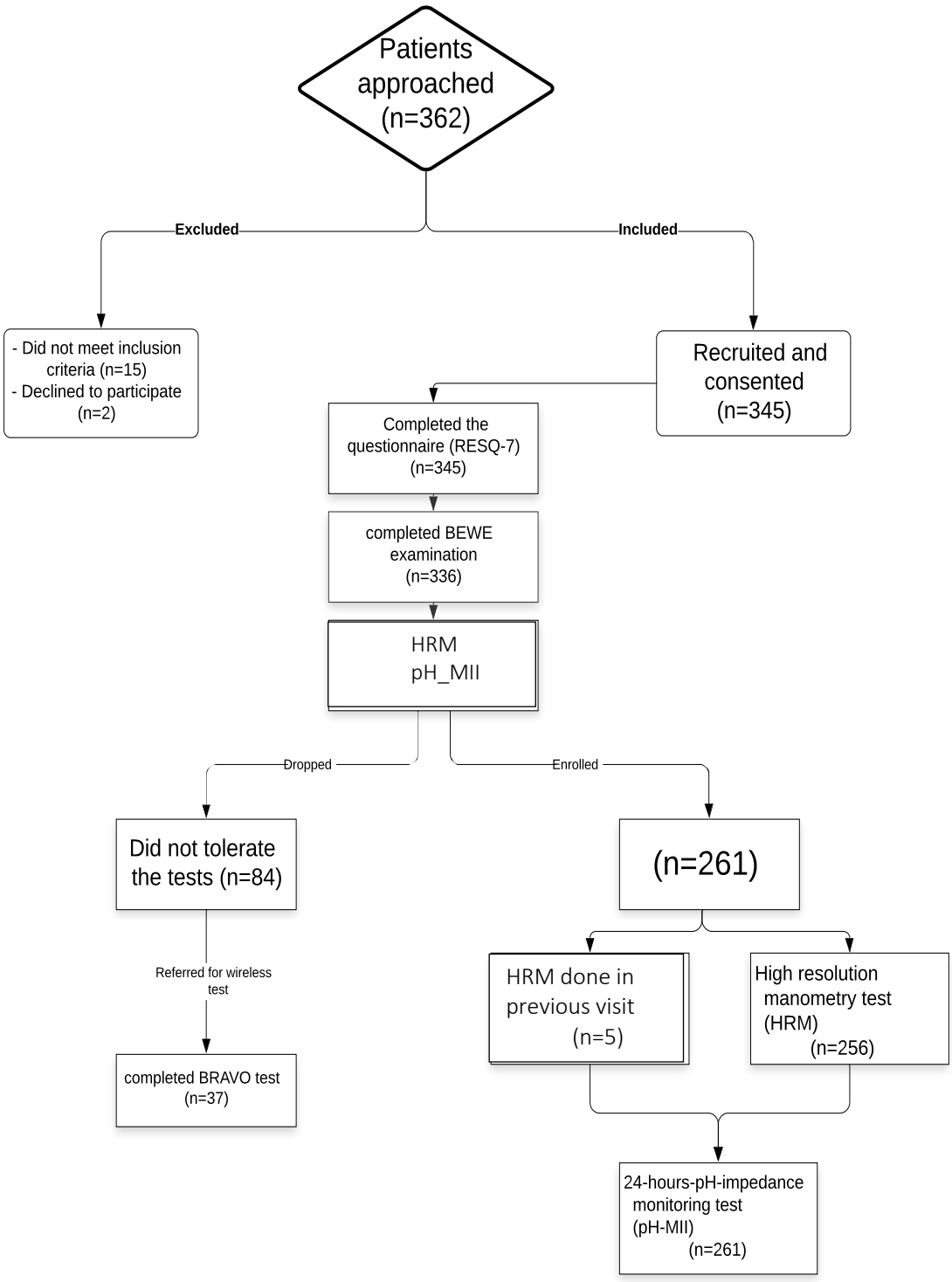


Figure 3-5: Flow chart of the protocol followed in the clinical study

Results

A total of 362 consecutive patients were approached, 15 patients did not meet one or more of the inclusion criteria and 2 did not wish to participate. A total of 345 patients completed the questionnaire (RESQ). From this group, 9 patients did not have a BEWE examination due to appointment logistics. A total of 84 patients were not able to tolerate the catheter-based tests (HRM or pH-MII), out of which 37 were referred for the wireless monitoring procedure (BRAVO). In total therefore, 261 patients completed the intraluminal 24hr-pH-impedance monitoring test. Five of the patients were having the test for the second time they were not scheduled for HRM test, giving a total of (n=256) who underwent the HRM. Using the criteria 150 patients were allocated to the case and 111 to the controls.

Two hundred and sixty-one patients were included in the study analyses {Figure 3-5}. Table 3-4 shows the demographic and clinical characteristics of cases and controls. A total of 111 male and 150 female patients participated in the study. There was no statistically significant difference for ETW between male and female (p=0.7). The mean (SD) age was 48.9 (15), two-sample t-test shows that age was significantly different between cases (53.3 (13.)) and controls (42.9 (14.9)) ($p<0.0001$). Therefore, it was decided to divide the age into those below and above 50 years old following a paper published by [Rauber et al., 2020]. The chi-square test {Table 3-5} shows a significant association between presence of ETW and age (p=0.001), and the odd of developing ETW is 2.13 for those above 50 years old (OR 2.13, 95% CI: 1.38-3.28; $p<0.001$).

The mean (SD) of BEWE scores were 15.3 (2.0) for cases and 8.7 (2.9) for control, t-test shows that BEWE score was significantly higher in cases compared to control ($p<0.0001$) with a difference of 6.83.

	Case		Control		P value
	N	%	N	%	
N (%) of cases and controls	150	57.47%	111	42.53%	<0.0001
Gender					
Male	65	58.56%	46	41.44%	
female	85	56.67%	65	58.56%	0.76
Age groups					
18-25	2	13.33%	13	86.67%	
26-35	13	32.50%	27	67.50%	
36-45	27	56.25%	21	43.75%	
46-55	40	57.97%	29	42.03%	<0.0001
56-66	33	73.33%	12	26.67%	
66+	35	79.55%	9	20.45%	
Age in years					
N	150		111		
Mean ± SD	53.3± 13.8		42.9± 14.9		<0.0001
95% CI	51.1-55.5		40.1-45.7		
BEWE					
Mean ± SD	15.4 ± 2.5		8.6 ±3.2		<0.001
Range	12-18		0-11		

Table 3-4: Demographic and clinical characteristics of participants

Parameter	Case	Control			
	N %	N %	OR	95% CI	P value
Age					<0.001
<50 years old	55(36.6%)	56 (50.5%)	1.00	-	
>50 years old	95(63.3%)	55 (49.5%)	2.13	1.38-3.28	

Table 3-5: Age and erosive tooth wear

3.3.1 Results for motility test

Table 3-6 details HRM parameters in both case and control groups. Out of the 261 recruited patients (n=5) already had the HRM test previously and the data could not be retrieved. A total of 256 patients completed the HRM test and analysed, the majority of patients were diagnosed with ineffective oesophageal motility (IOM) 62.5% (n=160). When comparing cases and controls, a higher proportion of patients had erosive tooth wear although no significant difference was found between the two groups.

Parameter	Case	Control			
	N %	N %	OR	95% CI	P value
Hernia					
Yes	22 (8.53%)	9 (3.49%)	1.61	0.86-4.44	0.09
No	126 (48.84%)	101 (39.15%)	1.00	-	
Oesophageal motility (n=256):					
Normal	35 (13.6%)	18 (7%)	1.16	0.36-3.72	0.7
Ineffective IOM	88 (34.37%)	72 (28.1%)	0.73	0.25-2.11	
Achalasia	4 (1.56%)	4 (1.56%)	0.6	0.1-3.33	
Fragmented peristalsis	3 (1.17%)	2 (0.78%)	0.9	0.11-7.0	
OGJ obstruction	10 (3.9%)	6 (2.3%)	1.0	-	
Absent contractility	7 (2.73%)	7 (2.73%)	0.6	0.13-2.57	

Table 3-6: findings from motility test using HRM

3.3.2 Results for ambulatory 24hr-pH-impedance monitoring test

Table 3-7 displays findings of the intraluminal 24hr-pH-MII tracing analysis.

GORD diagnosis: Out of 261 patients, 136 were diagnosed with pathological GORD of which 77 were female and 59 males. No statistical significance observed with regards to gender (p=0.77) or between age and GORD diagnosis was observed (p=0.15). Those who had GORD had a statistically significantly higher BEWE score, the mean (SD) of BEWE score in GORD 13.0 (4.1) patients and no-GORD patients 12.0 (4.0); p=0.042). Logistic regression showed that erosive tooth wear was almost twice as likely in those diagnosed with pathological GORD (OR=1.98, 95% CI: 1.2 -3.2, P=0.007) compared to those who do not have GORD {Table 3-8}.

Acid exposure time (AET%): for cases, the mean (SD) of normal <4, inconclusive 4-6, abnormal >6 acid exposure times were (1.2(1.0); 4.8(0.8); 18.3(28.2)) respectively. Whereas within controls, mean (SD) of those with normal, inconclusive and abnormal acid exposure times were (1.07(0.9); 5.0(1.1); 10.9(5.4)) respectively. A strong association was observed between erosive tooth wear and patients diagnosed with inconclusive AET. Logistic regression showed that ETW was almost 4 times more in those with inconclusive AET (OR 4.0, 95% CI: 1.88-8.8) compared to those with normal AET. If the previous AET% criteria were applied (i.e.: <4.2% normal, >4.2% considered abnormal), those with ETW were almost twice more likely in those with abnormal AET% (OR 2.3, 95% CI: 1.54-3.54, p=0.0005) than those with normal AET.

Acid exposure according to the body position: in an upright position, 69 patients demonstrated abnormal percentage of acid exposure in an upright position (AE_UP) (26.4%) in comparison with 192 patients with normal percentage of AE_UP (73.5%).

Logistic regression showed that ETW was more in those with abnormal percentage of AE_UP% (OR 1.00, 95% CI: 0.2- 0.68; p=0.002) than those with normal percentage.

In a supine position, 106 patients demonstrated abnormal percentage of acid exposure in a supine position AE-SUP (40.6%) in comparison with 155 patients with normal percentage of AE-SUP (59.3%). No significant association between ETW and AE_SUP% (p=0.2).

DeMeester score: ninety-eight patients had abnormal DeMeester score (37.5%) with a mean (SD) score of 42.2 (33.3) in case group in comparison to 38.9 (23.2) in the control group. Whereas patients with normal DeMeester score had a mean (SD) of 5.8 (4.3) in case group in comparison to 3.9 (3.2) in the control group. Logistic regression showed that ETW was more in those with abnormal DeMeester score (OR 1.00, 95% CI: 0.35-1.00; p=0.05) compared to those with normal DeMeester score.

Post-hoc Kruskal-Wallis analysis corresponding results for DeMeester score: the median (IQR) BEWE scores were significantly different in patients with normal or abnormal Demeester score (12 (9-15) and 14 (11-16); p=0.04), respectively. For percentage of acid exposure time: the median (IQR) BEWE scores were significantly different in patients with normal AET%, inconclusive AET% and abnormal AET% were (12 (8.5-15), 15 (12-18), 13 (9.75-15.2); p=0.0002) respectively. Also for total reflux episode: median (IQR) BEWE scores were not significantly different in patients with normal reflux episodes (<73) and abnormal reflux episodes (>73) (14 (10.75-16) and 13 (10-16); p=0.30) respectively.

Parameter	Case			Control			OR	95% CI	P value
	N	%	Mean ± SD	N	%	Mean ± SD			
GORD diagnosis									
YES	89	59.33		47	42.3		1.98	1.2-3.2	0.007
No	61	40.67		64	57.66		1.00	-	
Total Acid exposure Time (AET%)									
Normal<4%	66	44	1.2±1.0	75	67.5	1.07±0.9		1.88-8.8	0.0002
inconclusive 4-6%	36	24	4.8±0.8	10	9.0	5.0±1.1	4.0		
Abnormal >6%	48	32	18.3±28.2	26	23.4	10.9±5.4	2.0	1.17-3.7	
Upright acid exposure (AE_UP%)									
Normal < 6.3	99	66	2.2±1.8	93	83.7	1.89±1.75	0.37	0.20-0.68	0.002
Abnormal >6.3	51	34	13.1±7.9	18	16.7	11.4±3.7	1.00	-	
Supine acid exposure (AE_SUP%)									
Normal < 1.2	85	56.67	0.12±0.24	70	63.0	0.13±0.26	0.76	0.49-1.26	0.2
Abnormal > 1.2	65	43.33	14.7±18.0	41	36.9	10.6±11.4	1.00	-	
DeMeester score									
Normal < 14.7	83	56.4	5.8±4.3	74	68.5	3.9±3.2	0.59	0.35-1.00	0.05
Abnormal > 14.7	64	43.5	42.2±33.3	34	31.4	38.9±23.2	1.00	-	

Total reflux episodes									
Normal <40	68	29.31	23.6±10.5	54	23.28	22.1±10.4	1.00	0.79-2.48	0.2
Inconclusive 40-80	55	23.71	55.8±11.13	31	13.36	53.4±10.7	1.40		
Conclusive >80	11	4.74	110.4±27.2	13	5.60	107.4±47.2	0.67	0.27-1.61	

Table 3-7: Intraluminal 24hr-pH-impedance results in case and control groups.

Parameter	N	Mean±SD	95% CI	diff	P value
GORD					
no	125	12.00±4.0	11.2-12.7	1.03	0.042
yes	136	13.03±4.1	12.3-13.7		

Table 3-8: Two-sample t-test of patients GORD diagnosis and BEWE score

Table 3-9 demonstrates the difference in general reflux profile between acid and non-acid reflux (NAR) in proximal and distal oesophagus collected from all participating patients irrespective of their ETW group. In Table 3-10, in cases controls, the number of episodes of acid and non-acid reflux were higher in the distal oesophagus in both positions (upright and supine) compared to the proximal oesophagus. Also, comparing the number of episodes of acid non-acid reflux between cases and controls, no statistically significant differences, although there was a trend of a higher number of episodes in the case group.

Spearman correlation analysis

There was a significant correlation between the BEWE score and percentage of acid exposure time (AET%) (rho=0.15, p=0.014) and the total number of reflux episodes (rho=0.85, p<0.0001). There were no statistically significant differences for other parameters.

	N of patient	mean	SD	Min	Max
number of Proximal acid episode	249	29.0	26.39	0	185
number of Proximal NAR episode	247	15.19	13.31	0	76
number of Distal acid episode	217	11.31	16.34	0	92
number of Distal NAR episode	214	4.42	7.1	0	41

Table 3-9: Table showing mean (SD) number of acid and non-acid reflux episodes in proximal and distal oesophagus.

	Case			Control			
	N	Median	IQR	N	Median	IQR	p value
Total Proximal acid	128	5	1-13	86	3	0.75-13.25	0.28
Total Proximal NAR	126	2	0-5.25	85	2	0-5.5	0.90
Total Distal acid	142	24.5	12-42	104	21	8.25-43.5	o.23
Total Distal NAR	142	10.5	6-19	102	11	6-20	0.51

Table 3-10: Table comparing the mean (SD) of number of acid and non-acid reflux episodes in proximal and distal oesophagus between cases and controls.

3.3.3 Results for symptoms 7 days prior to the test

The self-reported questionnaire (RESQ) was using a modified scoring system (section 3.3.5), aggregating the 13 items into six parameters. Table 3-11 shows summary of mean (SD) of symptoms reported by patients during the preceding 7 days prior to the test date. There were, 261 patients recruited for the motility, PH-MII and ETW data, but 9 failed to fill the questionnaire due to appointment time restriction. There were a total of 252 patients' data analysed in this section. Using the criteria 107 patients were allocated to the case and 145 to the controls.

Table 3-12 illustrates the mean (SD) of symptoms frequency and intensity reported by case and control groups. Both frequency and intensity of symptoms scores were higher amongst cases in comparison to controls as illustrated in Figure 3-6. However, the statistically significantly higher (mean (SD)) symptoms in cases compared to controls were: frequency and intensity of heartburn (p=0.0006, p=0.004), frequency of difficulty swallowing (p=0.05), frequency and intensity of coughing (p=0.0001, p=0.0006), and frequency of hoarseness (p=0.01).

3.3.3.1 *Symptoms frequency*

Patient responses to each symptom frequency domain are presented in Table 3-12. In general, all symptoms were more frequent in cases compared to controls. Heartburn was reported by (n= 142; 56.34%) of patients as a daily symptom during the preceding 7 days prior to the test. The odds of developing ETW increased with the increase of frequency of heartburn, the results showed that ETW is 1.21 times more likely

in those who reported having heartburn daily compared to those who did not have any heartburn symptom reported (OR 1.21, 95% CI: 0.37-3.83; p=0.04).

Similarly, regurgitation and coughing were reported by (n=82; 56.55%) (n=60; 41.38%) of patients as a daily symptom during the preceding 7 days prior to the test. The odds of ETW increased with the increase of frequency of regurgitation/ coughing, the results showed that ETW is 2.72 more likely in those who reported having regurgitation daily compared to those who did not have any regurgitation symptom reported (OR 2.72, 95% CI: 0.33-1.38; p=0.05). And ETW is 3.77 more likely in those who reported coughing daily compared to those who did not have any coughing symptom reported (OR 3.77, 95% CI: 1.95-7.28; p=0.002). Difficulty in swallowing was reported by (n=46; 31.72%) of patients as a daily symptom. But the highest odds of ETW was those who reported to have difficulty swallowing 3-4 days during the preceding 7 days prior to the test date (OR 3.99, 95% CI: 1.39-11.43; p=0.02).

3.3.3.2 *Symptoms intensity*

Patients' responses to each symptom intensity domain are presented in Table 3-13. All symptoms were more severe/intense among cases in comparison to the controls.

Intensity of heartburn was reported by (n=143; 56.74%) as severe intensity during the preceding 7 days prior to the test date. The odds of ETW increased with the increase of intensity of heartburn. Results showed that ETW is 1.50 more likely in those who reported sever heartburn compared to those with less intensity (OR 1.50, 95% CI: 0.64-3.48; p=0.01).

Intensity of coughing was reported by (n=67) as mild intensity during the preceding 7 days prior to the test date. ETW is 1.37 more likely in those with mild coughing intensity compared to those who did not report any intensity (OR 1.37, 95% CI: 0.71-2.64; p=0.001).

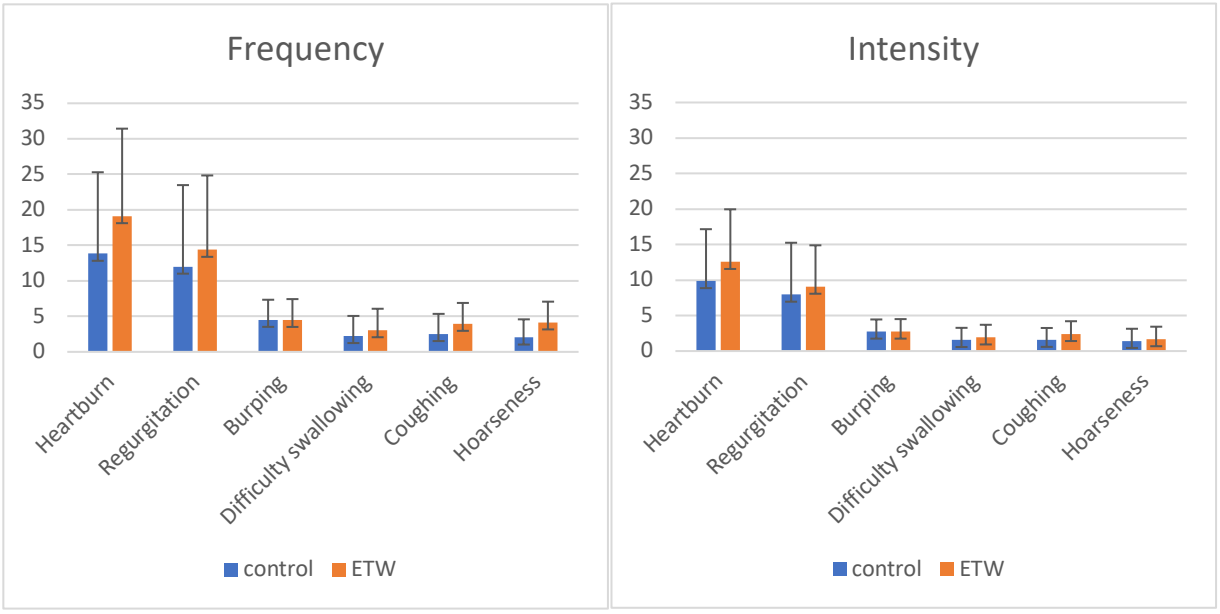


Figure 3-6:Symptoms frequency and intensity for cases and controls

Parameter	Control		Case		P value
	Mean	SD	Mean	SD	
Heartburn					
Frequency	13.82	11.47	19.12	12.31	0.0006*
Intensity	9.86	7.3	12.56	7.40	0.004*
Regurgitation					
Frequency	12.01	11.47	14.38	10.46	0.07
Intensity	7.95	7.30	9.07	5.82	0.14
Burping					
Frequency	4.52	2.82	4.51	2.92	0.85
Intensity	2.75	1.70	2.75	1.75	0.91
Difficulty swallowing					
Frequency	2.24	2.82	3.06	3.01	0.05*
Intensity	1.57	1.70	1.93	1.77	0.25
Coughing					
Frequency	2.52	2.83	3.96	2.93	0.0001*
Intensity	1.59	1.66	2.41	1.79	0.0006*
Hoarseness					
Frequency	2.03	2.55	4.16	2.91	0.01*
Intensity	1.44	1.70	1.68	1.75	0.21

Table 3-11: summary of mean (SD) of reported score of frequency and intensity of symptoms

Parameter	Control N (%)	Case N (%)	OR	95% CI	P value
Heartburn					
Did not have	11(10.48%)	16(12.40%)	1.00	-	
1-2 days	21(20.00%)	12 (8.28%)	0.39	0.13-1.11	
3-4 days	15(14.02%)	13 (8.97%)	0.59	0.20-1.73	0.04*
5-6 days	8 (7.48%)	14 (9.66%)	1.20	0.37-3.83	
Daily	52(48.60%)	90(62.07%)	1.21	0.51-2.75	
Regurgitation					
Did not have	19(17.76%)	13(8.97%)	1.00	-	
1-2 days	19(17.76%)	16(11.03%)	1.23	0.46-3.24	
3-4 days	15(14.02%)	22(15.17%)	2.14	0.81-5.61	0.05*
5-6 days	10 (9.35%)	12(8.28%)	1.75	0.58-5.24	
Daily	44(41.12%)	82(56.55%)	2.72	0.33-1.38	
Burping					
Did not have	18(16.82%)	26(17.93%)	1.00	-	
1-2 days	15(14.02%)	24(16.55%)	1.1	0.45-2.67	
3-4 days	12(11.21%)	9 (6.21%)	0.52	0.18-1.48	0.6
5-6 days	10 (9.35%)	11 (7.59%)	0.76	0.26-2.16	
Daily	52(48.60%)	75(51.72%)	0.99	0.46-2.00	
Difficulty swallowing					
Did not have	54(50.47%)	54(37.24%)	1.00	-	0.02*

1-2 days	20(18.69%)	20(13.79%)	1.00	0.48-2.06	
3-4 days	5 (4.67%)	20(13.79%)	3.99	1.39-11.43	
5-6 days	2 (1.87%)	5 (3.45%)	2.50	0.46-13.44	
Daily	26(24.30%)	46(31.72%)	1.76	0.96-3.25	
Coughing					
Did not have	47(43.93%)	34(23.45%)	1.00	-	
1-2 days	18(16.82%)	24(16.55%)	1.84	0.83-3.91	
3-4 days	11(10.28%)	17(11.72%)	2.13	0.88-5.13	0.002*
5-6 days	8 (8.41%)	10(6.90%)	1.53	0.56-4.18	
Daily	22(20.56%)	60(41.38%)	3.77	1.95-7.28	
Hoarseness					
Did not have	51 (47.66%)	56(38.62%)	1.00	-	
1-2 days	21 (19.63%)	20(13.79%)	0.69	0.43-1.94	
3-4 days	15 (14.02%)	25(17.24%)	0.27	0.62-2.95	0.15
5-6 days	4 (3.74%)	6 (4.14%)	0.64	0.40-5.75	
Daily	16 (14.95%)	38(26.21%)	0.03	1.13-4.66	

Table 3-12: RESQ results of reported symptoms frequency, (*) indicate significant difference of P<0.05

Parameter	Control N (%)	Case N (%)	OR	95% CI	P value
Heartburn					
Did not have	12(11.32%)	14(9.72%)	1.00	-	
Mild	25(23.58%)	13(9.03%)	0.44	0.16-1.23	0.01*
Moderate	17(16.04%)	26(18.06%)	1.31	0.49-3.50	
Severe	52(49.06%)	91(63.19%)	1.50	0.64-3.48	
Regurgitation					
Did not have	19(17.92%)	15(10.42%)	1.00	-	
Mild	20(18.87%)	24(16.67%)	1.52	0.61-3.73	0.2
Moderate	21(19.81%)	28(19.44%)	1.68	0.69-4.08	
Severe	43(43.40%)	77(53.47%)	2.21	0.98-4.57	
Burping					
Did not have	17(16.04%)	23(15.97%)	1.00	-	
Mild	27(25.47%)	39(27.08%)	0.87	0.48-2.36	0.4
Moderate	26(24.53%)	24(16.67%)	0.37	0.29-1.57	
Severe	36(33.96%)	58(40.28%)	0.64	0.56-2.52	
Difficulty swallowing					
Did not have	52(49.06%)	51(35.42%)	1.00	-	
Mild	17(16.04%)	36(25.00%)	2.15	1.07-4.32	0.06
Moderate	19(17.92%)	21(14.58%)	1.12	0.54-2.34	

Severe	18(16.98%)	36(25.00%)	2.03	1.02-4.04	
Coughing					0.001*
Did not have	44(41.51%)	35(24.31%)	1.00	-	
Mild	32(30.19%)	35(24.31%)	1.37	0.71-2.64	
Moderate	14(13.21%)	28(19.44%)	1.00	1.15-5.48	
Sever	16(15.09%)	46 (31.94)	1.33	1.75-7.43	
Hoarseness					0.5
Did not have	53 (50%)	59(40.97%)	1.00	-	
Mild	24(22.64%)	36(25.00%)	1.34	0.71-2.54	
Moderate	12(11.32%)	23(15.97%)	1.72	0.78-3.79	
Sever	17(16.04%)	26(18.06%)	1.37	0.67-2.80	

Table 3-13: RESQ results of reported symptoms intensity, (*) indicate significant difference of P<0.05

3.3.3.3 Frequency and intensity cut-off points

ROC curves were plotted to determine the value of cut-off points that maximise both sensitivity and specificity observed. ROC curves were constructed according to a prespecified method for scoring RESQ items (section 3.3.5).

For all symptoms frequency {Table 3-14} {Figure 3-7}, using an optimum cut-off of 40.5 gave a sensitivity of 61.38% and a specificity of 60.75%, with 61.11% correctly classified. For all symptoms intensity {Table 3-15} {Figure 3-8}, using an optimum cut-off of 22 gave a sensitivity of 70.14% and a specificity of 48.11%, with 60.96% correctly classified. In total, the optimum cut-off for frequency and intensity of all symptoms of 63 gave a sensitivity of 61.26% and a specificity of 60.56%, with 60.96% correctly classified.

Parameter	Cut-off point at	Specificity	Sensitivity
Heartburn	18.00	69.16%	52.41%
Regurgitation	11.00	50.47%	62.07%
Difficulty swallowing	2.00	58.88%	55.17%
Coughing	3.50	62.62%	60.00%
Hoarseness	2.00	57.01%	55.17%

Table 3-14: Frequency cut-off values determined using the value that maximised both sensitivity and specificity collected from all participating patients.

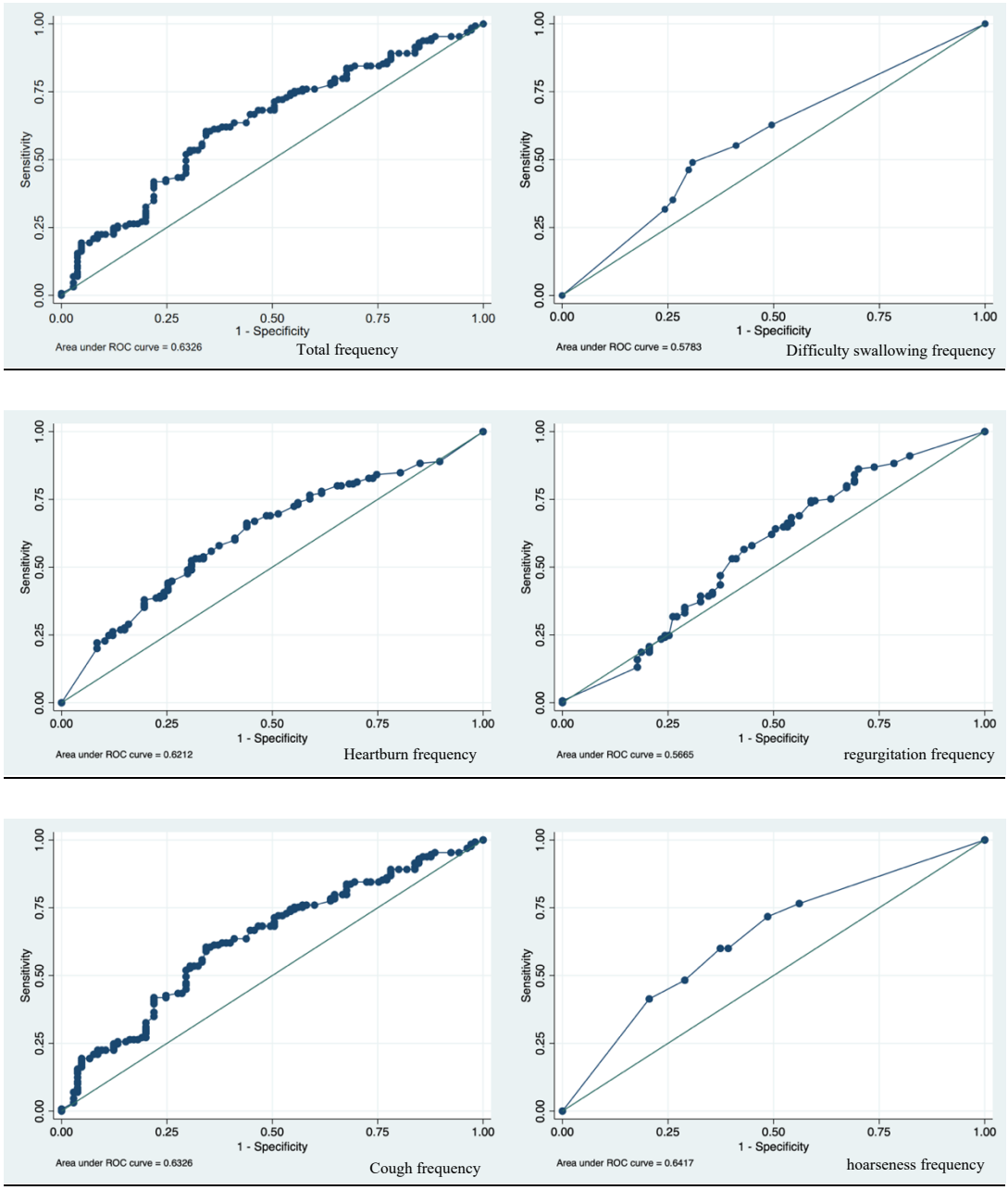


Figure 3-7: Receiver operating characteristic (ROC) curve showing the trade-off between sensitivity and specificity for the prespecified scoring method of RESQ responses of all frequency items.

Parameter	Cut-off point at	Specificity	Sensitivity
Heartburn	10.00	50.94%	65.28%
Regurgitation	9.00	53.77%	52.08%
Difficulty swallowing	1.69	57.55%	55.56%
Coughing	2.03	71.70%	51.39%
Hoarseness	1.00	56.60%	49.31%

Table 3-15: Intensity cut-off values determined using the value that maximised both sensitivity and specificity collected from all participating patients.

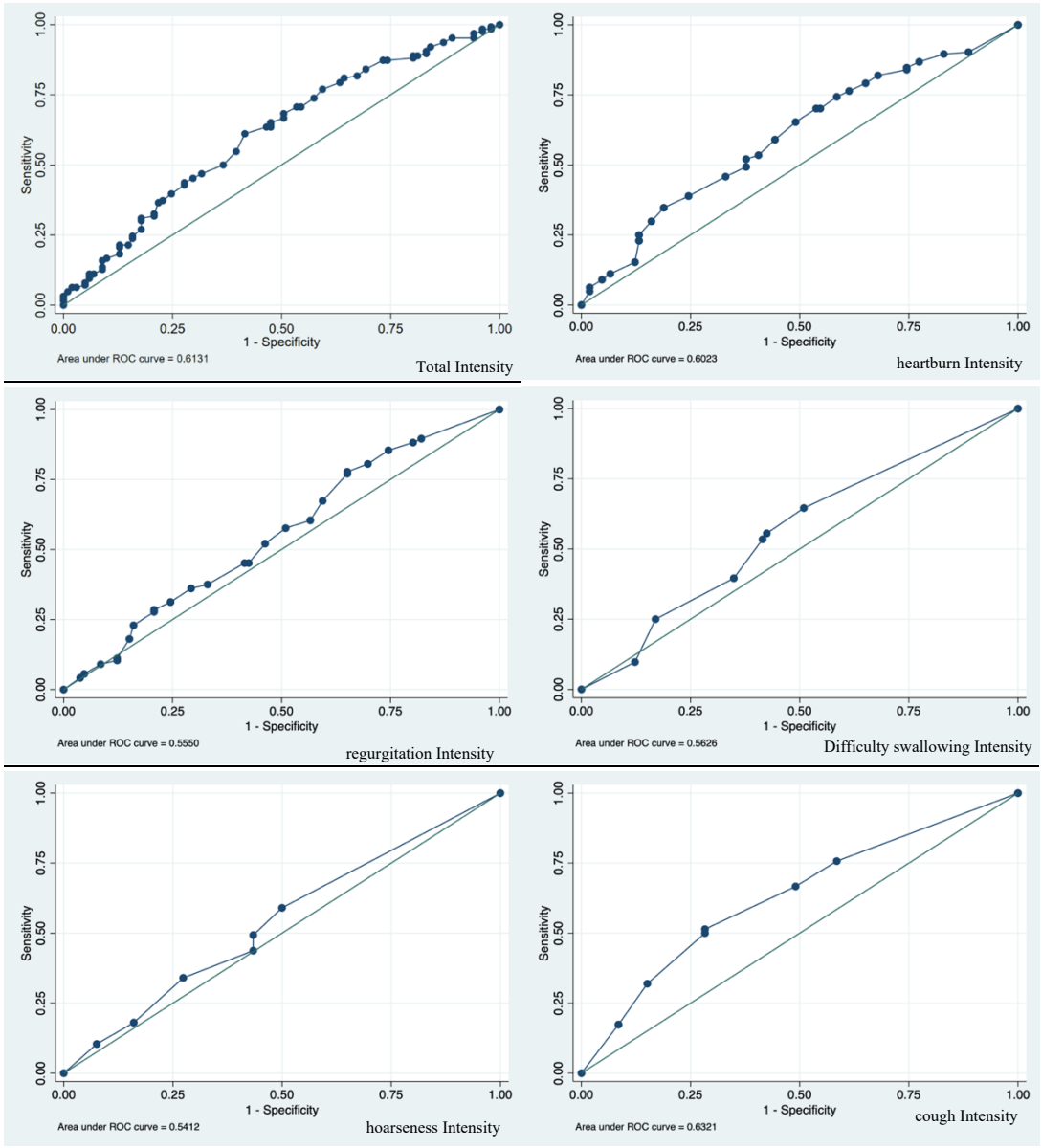


Figure 3-8: Receiver operating characteristic (ROC) curve showing the trade-off between sensitivity and specificity for the prespecified scoring method of RESQ responses of all intensity items.

3.3.4 Results for symptoms during the test

From the 261 patients recruited: heartburn 144/261 (55.5%), regurgitation 147/261 (56.3%), belching 109/261 (42.1%), epigastric pain 89/261 (34.5%). Chestpain was reported by 73/261 (28.3%) of patients, cough by 34/261 (13.4%), acid taste 4/261 and hoarseness by 4/261 (1.9%) was reported. A total of 8675 episodes of symptoms were detected {Figure 3-9}, a box plot illustration of all symptoms detected in Figure 3-10. Table 3-16 summarise the mean (SD) and maximum number of each symptom captured during the 24 hours monitoring period.

Symptom index (SI) and symptom association probability (SAP) analyses were applied according to the criteria mentioned in section 3.2.3. 95/144 (36.3%) of patients with heartburn had either positive SI or SAP. Results reported that ETW is 2.40 more likely in those who had heartburn and showed positive reflux association compared to those who did not have heartburn during the monitoring test (OR 2.40, 95% CI: 1.37-4.22; p=0.003). Whereas results showed that ETW is 2.33 more likely in those who reported heartburn during the test regardless of reflux symptom association compared to those who did not report it (OR 2.33, 95% CI: 1.41-3.85; p= 0.001).

In addition, 95/147 (36.3%) of patients with regurgitation had either positive SI or SAP. ETW reported to be 1.72 more likely in those who had regurgitation and showed positive reflux association compared to those who did not have regurgitation during the monitoring test, but no significant difference reported (OR 1.72, 95% CI: 0.98-2.97; P=0.08). In addition, ETW was 1.75 more likely in those who reported regurgitation during the test regardless of reflux symptom association compared to those who did not report it (OR 1.75, 95% CI: 1.06-2.87; p=0.027).

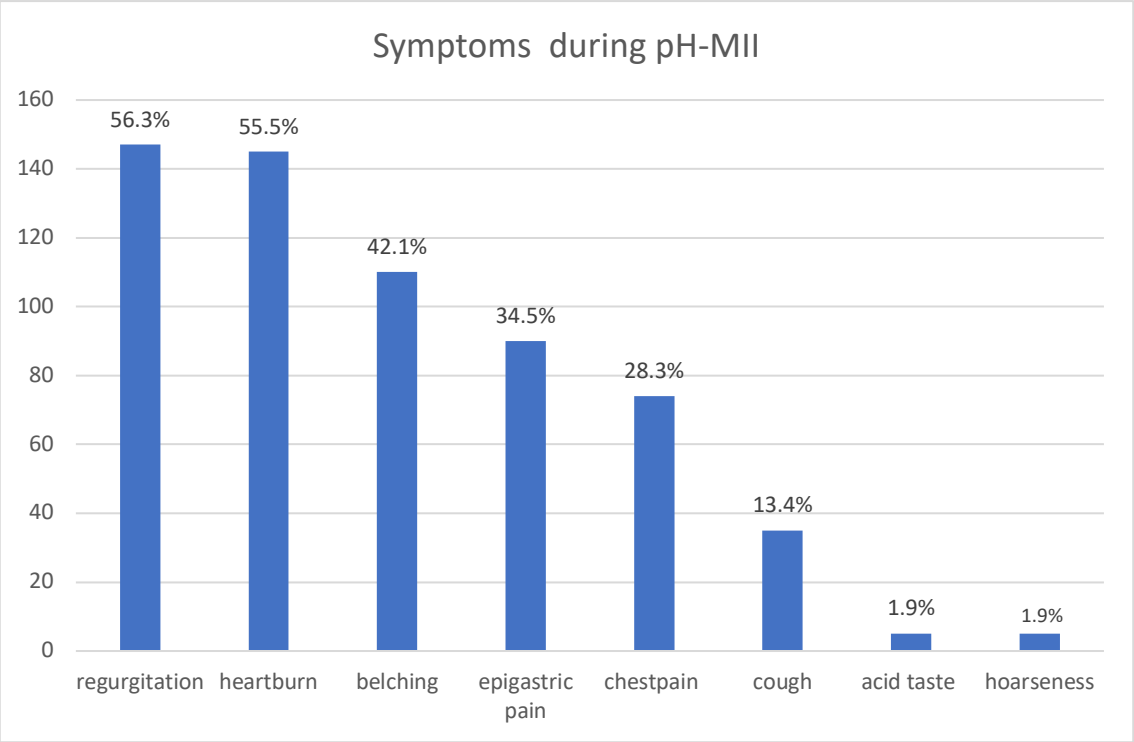


Figure 3-9: Summary of reported symptoms during the pH_MII monitoring period, number and percentage of patients.

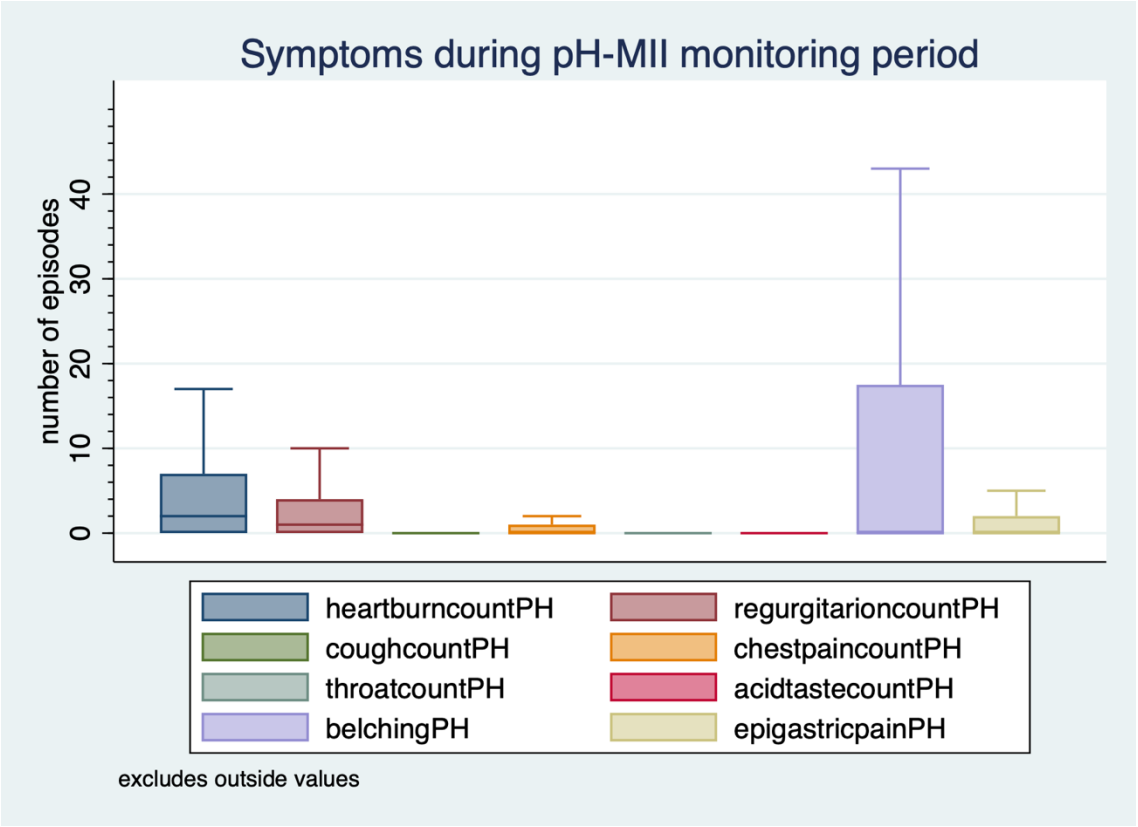


Figure 3-10: Box plot of each symptom reported by patients during the 24 hours pH-MII monitoring test, illustrating the median, interquartile and range of each symptom.

Parameter	Control N (%)	Case N (%)	OR	95% CI	P value
Heartburn	Total episodes = 1768, Mean = 4.7				
+ve association	31 (27.93%)	64 (42.67%)	2.40	1.37-4.22	0.003*
no association	17 (15.32%)	32 (21.33%)	2.19	1.09-4.38	
no symptom reported	63 (56.76%)	54 (36.00%)	1.00	-	
Regurgitation	Total episodes = 1225, Mean = 6.8				
+ve association	35 (31.53%)	60 (40.00%)	1.82	0.98-2.97	0.08

no association no symptom reported	17 (15.32%) 59 (53.15%)	31 (20.67%) 59 (39.33%)	1.72 1.00	0.91-3.64 -	
Cough	Total episodes = 793, Mean = 1.0				
+ve association no association no symptom reported	7 (6.31%) 4 (3.60%) 100 (90.09%)	15 (10.00%) 4 (2.67%) 131(87.33%)	1.63 0.76 1.00	0.64-4.16 0.18-3.12 -	0.5
Hoarseness					
+ve association no association no symptom reported	0 (0.0%) 1 (0.90%) 110 (99.10%)	2 (1.33%) 1 (0.67%) 147 (98.0%)	1 0.74 1.00	- 0.04-12.0 -	0.8
Chestpain	Total episodes = 525, Mean = 2.0				
+ve association no association no symptom reported	16 (14.41%) 13 (11.71%) 82 (73.87%)	16 (10.67%) 28 (18.67%) 106(70.67%)	0.77 1.66 1.00	0.36-1.63 0.81-3.41 -	0.2
Acid in throat	Total episodes =134, Mean = 0.5				
+ve association no association no symptom reported	1 (0.90%) 4 (3.60%) 106 (95.50%)	3 (2.00%) 3 (2.00%) 144 (96.0%)	2.20 0.55 1.00	0.22-21.5 0.12-2.51 -	0.48

Epigastric pain	Total episodes =924, Mean = 3.56				
+ve association	13 (11.71%)	25 (16.67%)	1.57	0.75-3.28	0.4
no association	20 (18.02%)	30 (20.00%)	1.23	0.64-2.33	
no symptom reported	78 (70.27%)	95 (63.33%)	1.00	-	
Belching	Total episodes =3281, Mean = 12.61				
+ve association	37 (33.33%)	50 (33.33%)	0.93	0.54-1.58	0.47
no association	12 (10.81%)	10 (6.67%)	0.57	0.23-1.41	
no symptom reported	62 (55.86%)	90 (60.00%)	1.00	-	

Table 3-16: Symptoms association analysis shown comparing both cases and controls, 95% confidence interval and p value reported.

3.3.5 Multivariable analysis

All variables were chosen manually based on the study hypothesis for the multivariable analysis, variables were adjusted for were age and gender. The initial model started with those with statistical significance of $p<0.2$ in the single variable analysis {Table 3-17}, which included 18 potential parameters:

From HRM: motility disorder, hernia

From pH-MII test: GORD diagnosis, percentage of acid exposure time, total number of reflux episodes, DeMeester score.

From symptoms during the test: heartburn, regurgitation, chestpain

From symptoms before the test: frequency of total heartburn, intensity of total heartburn, frequency of total regurgitation, intensity of total regurgitation, frequency of difficulty swallowing, intensity of difficulty swallowing, frequency of cough, intensity of cough, frequency of hoarseness.

Based on the p values, variables with the highest p value were subsequently withdrawn from the initial model. Tables 3-18 reports the final model for the strongest associations observed for the development of ETW, reporting only variables with p value less than 0.05 independently after adjusting for age and gender. Although DeMeester score was a borderline significance in the univariable analysis, it was highly significant when it was included in the multivariable analysis ($p=0.04$).

The predictors for ETW were age (age 66+) (OR 32.86, 95% CI: 5.30-203.5; $p<0.0001$), abnormal DeMeester score (OR 3.92, 95% CI: 0.88-17.46; $p=0.04$), inconclusive percentage of acid exposure time (OR 12.65, 95% CI: 3.07-52.14; $p<0.001$),

abnormal percentage of acid exposure time (OR 6.49, 95% CI:1.33-31.55; p=0.02) and daily regurgitation reported by patients (OR 2.90, 95% CI:01.15-7.31; p=0.02).

Furthermore, the analyses were repeated for patients above and below 50 years old. Table 3-19 represents the initial model analysed and Table 3-20 represents the final model.

The predictors for ETW were age (+50) (OR 2.90, 95% CI: 1.83-6.00; p<0.0001), abnormal DeMeester score (OR 4.04, 95% CI: 0.95-17.15; p=0.05), inconclusive percentage of acid exposure time (OR 12.18, 95% CI: 3.10-47.84; p<0.0001), abnormal percentage of acid exposure time (OR 8.5, 95% CI:1.81-39.89; p=0.007) and daily regurgitation reported by patients (OR 2.90, 95% CI:0.89-6.39; p=0.01).

variable	OR	95% CI	P value
Age group			
18-25	1.00	-	
26-35	1.11	0.08-15.08	0.933
36-45	7.94	0.59-106.03	0.117
46-55	11.23	1.00-125.09	0.049
56-66	16.73	1.36-205.17	0.028
66+	71.13	4.62-1094.73	0.002
Gender			
male	1.00	-	
female	3.02	1.08-8.38	0.03
motility disorder	0.21	0.07-0.65	0.007
Hernia	1.35	0.26-6.86	0.99
GORD diagnosis	1.90	0.43-8.36	0.28
AET %			
Normal	1.00	-	
inconclusive	34.50	2.82-420.72	0.00
abnormal	13.42	0.84-212.58	0.02
Total reflux episodes			
Normal <40	1.00	-	
Inconclusive 40-80	1.10	0.36-3.34	0.85
Conclusive >80	0.13	0.02-0.87	0.03
DeMeester score	10.76	0.72-160.45	0.08
Heartburn associated with reflux			

negative	1.00	-	
not reported	1.09	0.23-5.11	0.91
positive	2.38	0.50-11.33	0.27
Regurgitation associated with reflux			
negative	1.00	-	
not reported	0.50	0.12-2.00	0.33
positive	0.70	0.15-3.13	0.64
chestpain associated with reflux			
negative	1.00	-	
not reported	0.30	0.08-1.1o	0.07
positive	0.22	0.03-1.39	0.22
Frequency of total heartburn			
Did not have	1.00		
1-2 days	0.35	0.01-8.07	0.51
3-4 days	0.09	0.003-2.49	0.15
5-6 days	3.55	0.09-154.26	0.50
Daily	0.40	0.01-13.31	0.59
Intensity of heartburn			
Did not have	1.00	-	
Mild	0.35	0.01-8.74	0.52
Moderate	3.38	0.10-104.94	0.48
Sever	2.41	0.06-83.12	0.62
Frequency of total regurgitation			

Did not have	1.00	-	
1-2 days	18.77	1.06-331.61	0.04
3-4 days	113.45	4.21-3055.7	0.00
5-6 days	5.67	0.20-156.85	0.30
Daily	59.01	2.27-1529.52	0.01
Intensity of regurgitation			
Did not have	1.00	-	
Mild	0.06	0.003-1.34	0.07
Moderate	0.06	0.00-1.26	0.07
Sever	0.01	0.00-0.42	0.01
Frequency of difficulty swallowing			
Did not have	1.00	-	
1-2 days	2.52	0.21-29.83	0.46
3-4 days	16.54	0.82-332.0	0.06
5-6 days	8.76	0.24-316.87	0.23
Daily	6.48	0.35-119.9	0.20
Intensity of difficulty swallowing			
Did not have	1.00	-	
Mild	1.00	0.09-10.79	0.99
Moderate	0.13	0.00-2.53	0.18
Sever	0.17	0.00-3.67	0.28
Frequency of coughing			
Did not have	1.00	-	

1-2 days	10.42	0.59-181.3	0.10
3-4 days	16.40	0.81-328.6	0.06
5-6 days	13.70	0.53-348.4	0.11
Daily	22.93	1.31-400.4	0.03
Intensity of coughing			
Did not have	1.00	-	
Mild	0.1	0.00-2.04	0.13
Moderate	0.11	0.00-2.31	0.15
Sever	0.43	0.02-8.45	0.57
frequency of hoarseness			
Did not have	1.00	-	
1-2 days	0.32	0.07-1.49	0.14
3-4 days	0.73	0.15-3.53	0.69
5-6 days	0.07	0.00-1.19	0.06
Daily	0.16	0.03-0.84	0.03

Table 3-17: Initial model composed in the multivariable analysis, including those with $p<0.02$ in the single variable analysis

variable	OR	95% CI	P value
Age group			
18-25	1.00	-	
26-35	2.98	0.50-17.48	0.22
36-45	12.66	2.22-72.20	0.004
46-55	10.01	1.84-54.34	0.008
56-66	23.13	3.96-135.06	0.000
66+	32.86	5.30-203.5	0.000
Gender			
male	1.00	-	
female	1.37	0.74-2.53	0.3
AET %			
Normal	1.00	-	
inconclusive	12.65	3.07-52.14	0.00
abnormal	6.49	1.33-31.55	0.02
DeMeester score	3.92	0.88-17.46	0.04
Frequency of total regurgitation			
Did not have	1.00	-	
1-2 days	1.07	0.34-3.31	0.90
3-4 days	1.96	0.64-5.92	0.23
5-6 days	1.35	0.35-5.17	0.65
Daily	2.90	1.15-7.31	0.02

Table 3-18: Final model of multivariable analysis with the strongest association of parameters for developing ETW

variable	OR	95% CI	P value
Age group			
<50 years old	1.00	-	<0.0001
>50 years old	6.75	2.44-18.63	
Gender			
male	1.00	-	
female	3.02	1.13-8.10	0.02
motility disorder	0.21	0.07-0.60	0.004
Hernia	1.35	0.28-6.46	0.71
GORD diagnosis	1.06	0.26-4.25	0.92
AET %			
Normal	1.00	-	
inconclusive	30.02	3.36-267.91	0.002
abnormal	13.72	1.17-159.73	0.03
Total reflux episodes			
Normal <40	1.00	-	
Inconclusive 40-80	1.22	0.42-3.49	0.71
Conclusive >80	0.21	0.03-1.25	0.08
DeMeester score	6.94	0.66-72.92	0.10
Heartburn associated with reflux			
negative	1.00	-	
not reported	0.89	0.20-3.88	0.87
positive	2.38	0.42-7.41	0.43
Regurgitation associated with reflux			

negative	1.00	-	
not reported	0.76	0.20-2.84	0.69
positive	1.09	0.26-4.44	0.90
chestpain associated with reflux			
negative	1.00	-	
not reported	0.25	0.07-0.90	0.03
positive	0.24	0.04-1.42	0.11
Frequency of total heartburn			
Did not have	1.00		
1-2 days	0.35	0.01-7.12	0.49
3-4 days	0.08	0.003-1.93	0.12
5-6 days	1.73	0.05-56.01	0.75
Daily	0.29	0.01-8.72	0.48
Intensity of heartburn			
Did not have	1.00	-	
Mild	0.55	0.02-11.22	0.70
Moderate	3.77	0.14-95.73	0.42
Sever	3.24	0.11-90.02	0.48
Frequency of total regurgitation			
Did not have	1.00	-	
1-2 days	11.81	0.71-196.03	0.08
3-4 days	61.48	2.64-1430.1	0.01
5-6 days	3.15	0.11-86.81	0.49

Daily	26.24	1.14-0.87	0.04
Intensity of regurgitation			
Did not have	1.00	-	
Mild	0.18	0.01-3.13	0.24
Moderate	0.11	0.00-1.99	0.13
Sever	0.04	0.00-0.87	0.04
Frequency of difficulty swallowing			
Did not have	1.00	-	
1-2 days	1.58	0.14-17.50	0.70
3-4 days	12.78	0.72-225.31	0.08
5-6 days	10.39	0.33-454.12	0.17
Daily	12.27	0.27-73.40	0.28
Intensity of difficulty swallowing			
Did not have	1.00	-	
Mild	1.48	0.14-15.32	0.73
Moderate	0.26	0.01-4.27	0.34
Sever	0.26	0.01-4.71	0.36
Frequency of coughing			
Did not have	1.00	-	
1-2 days	8.13	0.61-107.2	0.11
3-4 days	8.35	0.55-125.5	0.12
5-6 days	10.39	0.5-210.2	0.12
Daily	12.27	0.94-158.8	0.05

Intensity of coughing			
Did not have	1.00	-	
Mild	0.18	0.13-2.60	0.21
Moderate	0.25	0.01-3.44	0.30
Sever	0.74	0.01-10.66	0.89
frequency of hoarseness			
Did not have	1.00	-	
1-2 days	0.36	0.08-1.62	0.18
3-4 days	0.58	0.13-2.65	0.49
5-6 days	0.11	0.00-1.39	0.08
Daily	0.21	0.04-0.97	0.04

Table 3-19: Initial model of multivariable analysis with the strongest association of parameters for developing ETW using age group above and below 50

variable	OR	95% CI	P value
Age group			
<50 years old	1.00	-	
>50 years old	3.32	1.83-6.00	<0.0001
Gender			
male	1.00	-	
female	1.59	0.86-2.95	0.12
AET %			
Normal	1.00	-	
inconclusive	12.18	3.10-47.84	<0.0001
abnormal	8.5	1.81-39.89	0.007
DeMeester score	4.04	0.95-17.15	0.05
Frequency of total regurgitation			
Did not have	1.00	-	
1-2 days	1.57	0.53-5.19	0.41
3-4 days	2.28	0.89-9.00	0.13
5-6 days	1.46	0.33-4.94	0.55
Daily	2.90	0.89-6.39	0.01

Table 3-20: Final model composed in the multivariable analysis using age group above and below 50, including those with p<0.02 in the single variable analysis

3.4 Discussion

This cross-sectional case control study investigated predictive factors for erosive tooth wear in patients with symptoms suggestive of gastro-oesophageal reflux disease (GORD), using subjective (self-reported questionnaire RESQ) and objective measures via the latest available diagnostic techniques (high resolution manometry (HRM), pH-impedance monitoring test (pH-MII).

The results of the self-reported questionnaire showed that patients with a higher frequency of heartburn, regurgitation, difficulty swallowing, coughing and hoarseness were at a higher risk of erosive tooth wear. In addition, this study using pH-MII has confirmed the association between GORD diagnosed patients and erosive tooth wear. Patients in this study with abnormal acid exposure time, abnormal acid in an upright position, abnormal DeMeester score and positive association of heartburn to reflux were at higher risk of erosive tooth wear than those with normal scores.

The most important and predominant oral manifestation of gastro-oesophageal reflux disease is erosive tooth wear. Erosive tooth wear initially affects the enamel surface but can progress to dentine and eventually may result in total destruction of the tooth. The severity of erosive tooth wear is affected by various factors such as the source and origin of the acid, time of contact of the acid with the teeth and nature of the acid as well as protective factors such as salivary flow rate and buffering capacity [Ganss et al., 2012]. Understanding the aetiology of erosive tooth wear and interpreting clinical findings are critical for assessing the association between erosive tooth wear and reflux symptoms.

Erosive tooth wear was characterised in this study using basic erosive tooth wear examination index (BEWE), which is a validated index with high sensitivity and specificity

compared to other indices [Dixon et al., 2012; Margaritis et al., 2011]. BEWE is one of the most widely used indices for erosive tooth wear, it has been used in 96 peer-reviewed publications since it was introduced in 2008 [Bartlett et al., 2019].

Gastro-oesophageal reflux disease (GORD) is a symptom driven disease, however symptoms of GORD are not always associated with diagnosis or changes in the oesophageal lining [Sifrim and Zerbib, 2012]. A recent study reported that erosive tooth wear was not correlated to histopathologically of diagnosed oesophagitis, hence patients with non-erosive reflux disease could also develop erosive tooth wear [Milani et al., 2016]. Therefore, the primary aim of this study was to evaluate the association between erosive tooth wear and symptoms of GORD, however it also investigated the association of erosive tooth wear with diagnosis of GORD. The case-group was classified as those who had a cumulative score of 12 or more with at least one score of grade 3 in one of the sextants, control-group as those with a cumulative score of less than 12 following a previously published study investigating the presence of erosive tooth wear in patients with dietary acid intake [O'Toole et al., 2017].

In this study high resolution manometry (HRM) was used which provides a more accurate and precise evaluation of transient lower oesophageal relaxations (TLER), lower oesophageal sphincter pressure (LOS) and oesophago-gastric junction (OGJ) morphology and function. It is considered the gold standard test for diagnosing oesophageal motor dysfunction. This study showed that when comparing cases to controls, there was no statistical difference between patients with normal oesophageal motility and patients with oesophageal hypomotility. This is in contrast to another study by Moazzez et al. [2005] which demonstrated a correlation between poor oesophageal motility (achalasia) and erosive tooth wear. However, the oesophageal motility disorders in our study

included: achalasia, absence of contractility, fragmented peristalsis, oesophageal junction (OGJ) outflow obstruction and ineffective oesophageal motility (IOM).

There may be several possible reasons for this finding:

First, the acid clearance mechanism determines the duration of exposure to the irritant. A normal oesophagus clears the refluxates by peristalsis in two steps, oesophageal primary peristalsis which clears most of the oesophageal content followed by secondary peristalsis which occurs by swallowing saliva and this is followed by salivary buffering, which neutralises any remaining acids [Alfaro et al., 2008; Helm et al., 1984]. Although cases in our study reported most of our patients (79.2%) had oesophageal hypomotility (55.1%) had erosive tooth wear but no statistical difference was reported. This suggests that for these patients, oesophageal hypomotility was less important than other parameters included in this study for developing erosive tooth wear. Second, salivary variables such as saliva content, ion concentration, presence of acquired enamel pellicle and buffering capacity are also principal factors influencing presence of acids within the oral cavity [Filipi et al., 2011], therefore it is studied in depth in the next chapter of this thesis. Third, the magnitude and frequency of exposure to the acid also a factor in developing erosive tooth wear in these patients, however Triadafilopoulos et et al. [2016]reported similar ambulatory pH findings in patients with normal and ineffective oesophageal motility, which could affect the results of our study. Fourth, age could be considered as a predisposing factor for the lack of association between oesophageal hypomotility and erosive tooth wear, the mean age of the present study was 48-year-old. It was reported previously that with age the risk of developing ineffective peristalsis increases [Achem et al., 2003]. In addition, Gutschow et al. [2011] reported that with age the peristaltic contractions decrease, and it is correlated to oesophageal abnormalities.

In the present study, a new reproducible technique which monitors oesophageal reflux for 24 hours including a combined technique (pH and impedance channels) was used. The use of this multiple intraluminal impedance technology made it possible to detect gastro-oesophageal reflux irrespective of pH, and therefore define the type of refluxate (liquid, gas, mixed), as well as identifying antegrade and retrograde bolus movement. It aids to clarify the diagnosis of GORD with higher accuracy, by eliminating the artefacts and errors which are overlooked by the conventional method. Furthermore, the pH probe provides chemical characterisation of the refluxate [Sifrim et al., 2005].

Using pH-MII, our study confirms the association between GORD diagnosed patients and erosive tooth wear. Patients who were diagnosed with pathological GORD were 1.98 times more likely to have erosive tooth wear when compared to those who were not diagnosed with GORD. This is in supports the review by Jordão et al. [2020] including 27 studies in a meta-analysis. They assessed subjective and objective GORD measurements and the severity of erosive tooth wear using dental indices. Based on the meta-analysis of 15 studies using objective tools for GORD diagnosis, it was concluded that patients diagnosed with GORD objectively were 4 times more likely to develop erosive tooth wear, whereas patients diagnosed with GORD subjectively had 2.7 times more likely to develop erosive tooth wear.

In addition, our results reported significantly higher BEWE scores in patients diagnosed with GORD compared to those who were not diagnosed with GORD using pH-MII. This is in agreement with a study carried out by Oginnini et al. [2005a]. They reported that GORD patients had higher tooth wear scores compared to controls using tooth wear index (TWI), although contradictory results have been reported [Di Fede et al., 2008; Jensdottir et al., 2004].

However, our data showed no association between GORD diagnosed patients and gender (n=136) (p=0.77), which confirms the findings of the meta-analysis by Eusebi et al. [2018]. In this study there was no association between gender and GORD symptoms in studies conducted in North America and Europe. However, in South America and the Middle East GORD was reported more in women compared to men.

From the pH-MII analysis, the percentage of acid exposure time (AET%) is the most reliable and reproducible parameter [Wiener et al., 1988], it can also predict post medical and surgical therapy response [Patel et al., 2015]. Our results reported an interesting strong association between patients with inconclusive acid exposure time ranging between 4-6% and the presence of erosive tooth wear when the Lyon consensus criteria was applied. It was recommended to consider AET% less than 4% as definitely normal, and more than 6% as definitely abnormal, with the range between 4% to 6% as inconclusive, hence it was used in this study analysis. However, when the previous criteria was applied (<4.2% normal, >4.2% considered abnormal) it showed a strong association between abnormal percentage of acid exposure time (>4.2%) and erosive tooth wear. In addition, there was a significant correlation between BEWE score and the percentage of acid exposure time (rho=0.15, p=0.014) and total number of reflux episodes (rho=0.85, p<0.0001). This finding shows that the characteristics of reflux episodes do not only determine the presence of the reflux but also perceive the nature of the episode. Acid exposure time is considered the main factor in the occurrence of typical and atypical symptoms.

There was a significant association between the percentage of acid exposure in an upright position and erosive tooth wear (p=0.002), whereas percentage of acid exposure in supine position was not significant (p=0.2). This could be explained by the

fact that frequency of reflux episodes in an upright position was significantly more than in a supine position [Bredenoord et al., 2006]. This could be caused by the absence of oesophageal peristalsis and the decrease in salivary secretion [Orr et al., 1984].

As for the nature of reflux (acid vs non-acid), our findings showed no significant difference between acid and non-acid reflux in the distal and proximal oesophagus. However, when applying Spearman correlation between number of acid reflux episodes and the total number of reflux episodes, the coefficient was (rho=0.85) with a high statistical significance ($p<0.0001$). The result confirms in part previously published work [Sifrim et al., 2001], that significantly more acidic reflux episodes reported with the increase of total reflux events.

The term “non-acid reflux” has been described in various ways. However, an international consensus agreed to define it as “reflux episodes that decrease oesophageal pH across 4, or reflux that occurs when oesophageal pH is already below 4” [Vakil et al., 2006a]. The term “weakly acidic” is to be used for the refluxate pH ranging between 4 to 7. However, in the present study the term “non-acidic” was used for reflux events with a pH of >4 as per the analysis criteria followed in the clinical practice. Though Sifrim et al [2001] reported that a third of reflux episodes in patients under acid suppressive therapy are weakly acidic. Therefore, performing the analysis including weakly acidic reflux episodes could determine whether or not these episodes could cause erosive tooth wear.

Although each individual parameter accounted for in the Demeester score did not result in statistical significance, the accumulative score of those parameters: total time pH less than 4, upright and supine times the pH less than 4, total number of reflux episodes, number of reflux episodes over 5 minutes and longest reflux episodes, resulted

in a difference. Our results show that an abnormal Demeester score was statistically significantly associated with the presence of erosive tooth wear (p=0.05) in univariate analysis, with a stronger association found in multivariable analysis (OR=3.92; p=0.04).

Patients may present with a range of troublesome typical and atypical symptoms of GORD, although some clinical studies have shown that classic reflux symptoms were neither sensitive nor specific to GORD diagnosis [Colas-Atger et al., 2002; Ott et al., 1997; Tefera et al., 1997]. Symptoms have been classified by the Montreal consensus to oesophageal and extraoesophageal symptoms with established or proposed association [Vakil et al., 2006a]. In the present study symptoms were assessed subjectively through patient's perspective given the history of symptoms a week prior to the test, and by clinical perspective/findings during the 24 hours monitoring.

The frequency and intensity of each symptom was quantified using a self-reported questionnaire, RESQ. This is a validated and reliable questionnaire consisting of the most relevant symptoms. As GORD is a symptom-related disease it was suggested by the Montreal consensus to evaluate the occurrence, frequency, and intensity of the symptoms [Vakil et al., 2006a], RESQ is a suitable tool in clinical trials specially when symptoms are fluctuating, recording patients' symptoms daily to capture patients symptoms experience. Our findings demonstrated that both frequency and intensity scores of all symptoms were higher in patients with erosive tooth wear in comparison to the control group. Moreover, daily occurrence of any of the symptoms increased the risk of developing erosive tooth wear ranging between 1.2 to 3.77 times, supporting previous studies that demonstrated an association between symptom frequency and presence of erosive tooth wear [Bartlett et al., 2011; Moazzez and Bartlett, 2014]. Two typical GORD oesophageal symptoms are heartburn and regurgitation and tend to usually be present

in combination. Daily heartburn and regurgitation were found statistically higher in patients with erosive tooth wear compared to the controls. These findings are in agreement with previous studies. Oginni et al [2005a] assessed the prevalence of erosive tooth wear and gastro-oesophageal reflux symptoms. They reported a strong association between the severity of erosive tooth wear (using tooth wear index) and frequency of regurgitation. Picos et al.[2020] assessed the association between erosive tooth wear using the BEWE index and the presence of heartburn in GORD patients using Gerd Q questionnaire, they observed an increase in frequency and severity of erosive tooth wear in patients with heartburn. Moreover, recently Rauber et al. [2020] reported a significant association between erosive tooth wear and regurgitation but not with heartburn.

Extraoesophageal symptoms such as chronic cough and difficulty swallowing have been studied extensively in the literature; however, little is known about the association between these symptoms and erosive tooth wear. In the present study, the intensity of coughing and difficulty swallowing scores in RESQ were statistically higher in patients with erosive tooth wear. Two mechanisms may be involved in the association between extraoesophageal symptoms and erosive tooth wear: 1) reflux contents reaching the high proximal extension, resulting in irritation of the pharynx and/or larynx or micro-aspiration of the refluxate into the airway. This theory is supported by Ing et al. [1994], they found that acid infusion into higher levels of the oesophagus increased cough frequency. 2) reflux contents reaching the distal oesophagus causing the activation of oesophageo-respiratory pathway [Pauwels, 2015]. Which is in agreement with Javorkova et al., [2008], they reported an increase in cough reflux frequency in patients with GORD.

Symptoms were assessed objectively as well using pH-MII for 24 hours monitoring. The relationship between symptoms and the occurrence of reflux events

during the pH-MII for 24 hours monitoring was evaluated by the most frequently used indicators, symptom reflux association analysis (SI) and symptoms association probability (SAP) indices. The SI has been recognised as the best evidence to provide relevant clinical association between symptoms and reflux events, especially when both SI and SAP are positive [Roman et al., 2017a]. Once the gastric refluxate passes the lower oesophageal sphincter (LOS) causing symptoms it may or may not reach the oral cavity, which may explain that there was no significant correlation found between the severity of erosive tooth wear and any of the reflux monitoring parameters within this study. Symptomatic reflux episodes have been investigated using the combined intraluminal impedance and pH monitoring, the most reported symptoms in 261 patients were regurgitation (56.3%) and heartburn (55.5%), patients who had positive association of heartburn or regurgitation were more likely to develop erosive tooth wear. The mechanism of these two symptoms have been investigated previously and found that they are evoked when the pH drop is large and when the refluxate reaches higher levels in the proximal oesophagus [Bredenoord et al., 2006].

This study demonstrated no statistical significance between groups (control and case) in respect to gender (p=0.7), which was adjusted for in the statistical analysis of the study. The lack of association between gender and erosive tooth wear has been observed in epidemiological studies [Auad and Moynihan, 2007; Bartlett et al., 2013; Peres et al., 2005] , with others observing an increased association between gender and erosive tooth wear [Alvarez Loureiro et al., 2015; Huew et al., 2012; Okunseri et al., 2015].

Moreover, there was a highly significant difference in age between case and control groups, patients with erosive tooth wear had a 10.39 year higher mean age compared with those without erosive tooth wear. The systematic review by Eusibi et al. [2018]

concluded that the prevalence of GORD was significantly higher in patients older than 50, this is in agreement with our study where patients older than 50 were twice more likely to develop erosive tooth wear than those below 50. The higher frequency of erosive tooth wear in this age group may be due to either an increase in prevalence of GORD [Nirwan et al., 2020], or due to prolonged use of proton pump inhibitors which may in turn have an effect on salivary function and therefore indirectly result in erosive tooth wear [Richter, 2000]. By contrast, a recent study assessed the association between age and erosive tooth wear reported that within the study population, those below 50 years were at a higher risk of developing erosive tooth wear [Rauber et al., 2020].

There are some limitations to our study, particularly the use of symptoms-association analysis applying SI and SAP indices, the limitation of these indices is mainly the variability of symptom occurrence during the 24 hours monitoring period. It must also be borne in mind that the sample size of our case-control study is not too large, thus larger sample size would increase the power of the study. However, several aspects of this study deserve comment. This is, to our knowledge, the first study that demonstrates an assessment and analysis of symptoms in patients suggestive of GORD and its relation to erosive tooth wear from both perspectives (subjective and objective). Our study provides new evidence on the association between erosive tooth wear and pH-MII findings and questionnaire responses.

Using logistic regression analysis in a multivariable model, which is an appropriate model for cross-sectional studies with a categorical outcome (presence of erosive tooth wear or not), the predictive factors for developing erosive tooth wear in patients with GORD suggestive symptoms are abnormal percentage of acid exposure time, abnormal DeMeester score, and daily frequency of regurgitation.

3.5 Conclusion

The results of this study indicated the predictive factors for developing erosive tooth wear in patients with GORD symptoms are: age (+50), abnormal DeMeester score, inconclusive and abnormal percentage of acid exposure time and daily regurgitation reported by patients.

4 Chapter 4: Protein Components in Human Acquired Enamel Pellicle (AEP) on Eroded and Un-Eroded tooth surfaces from Patients with GORD Symptoms: An in-vivo study

4.1 Introduction

The association between GOR symptoms and ETW was investigated in Chapter 3. ETW was twice more likely in patients diagnosed with pathological GORD than those who were not diagnosed with GORD, the association between the two conditions has also previously been reported in the Montreal consensus in statement no.48 [Vakil et al., 2007b]. One factor that could contribute to this association amongst others, could be altered salivary parameters; either an altered salivary volume as reported in patients suffering from reflux, or due to drop in salivary pH level as regurgitated content has a low pH ranging between 1 and 3 [Milosevic et al., 1997]. Patients with non-erosive oesophageal reflux and oesophageal reflux disease have different proteomic profiles of the oesophageal mucosa [Calabrese et al., 2011]. Patients with erosive oesophageal reflux do not have the same protective ability against acid and pepsin attacks, which has been explained by the reduced mucosal integrity, keratinised oesophagus, cell migration and cell proliferation [Calabrese et al., 2009].

Saliva is one of the major biological factors protecting both the gastrointestinal tract and the oral system. In the oral system an organic bacteria free layer is formed on enamel surfaces as a result of salivary protein adsorption forming an “acquired enamel pellicle (AEP)” and is composed mainly from proteins and glycoproteins [Siqueira et al., 2012; Siqueira et al., 2007c]. The protective effects of AEP have been studied extensively. AEP

acts as a semi-permeable membrane, a barrier between tooth surfaces and the surrounding environment, a modulator for the demineralisation/remineralisation process, a modulator to proteins adherence and has a lubrication property which enhances speech and mastication efficiency [Buzalaf et al., 2012a; Hahn Berg et al., 2004; Hannig and Balz, 1999; Hannig and Hannig, 2014; Hannig and Joiner, 2006b; Vukosavljevic et al., 2014]. However, previously reported in this thesis (Chapter 2) the in-vitro AEP did not protect against intrinsic acids whereas it protected the enamel surface against extrinsic acid attack.

In-vitro studies have investigated the protein components of AEP and demonstrated the function of different protein components against erosive tooth wear [Carlen et al., 1998; Jensen et al., 1992; Leinonen et al., 1999a; Li et al., 2004; Siqueira et al., 2007a; Yao et al., 2001]. However, the unique nature of in-vivo formed AEP has differences to in-vitro formed AEP for many reasons; there are dissimilarities in the salivary flow rate dynamics, AEP thickness, mineral components and enzymatic activities [Hannig and Hannig, 2009; Yao et al., 2001].

Previous studies by our group have assessed the protective effect of AEP in-vivo. Moazzez et al. [2014] compared the protective effect of an hour formed AEP against an in-vitro erosive challenge on enamel blocks worn by healthy individuals and individuals with ETW and to no-AEP enamel blocks. The study reported a significant difference in surface roughness (SA) between the three groups. The study suggested that AEP was protective against acidic challenges in healthy individuals compared to those with ETW. Moreover, Mutahar et al. [2017c] investigated the total protein concentration in in-vivo formed AEP between eroded and un-eroded enamel surfaces from patients presented with ETW due to dietary acids. The authors observed reduced total protein concentration

in AEP harvested from eroded surfaces compared to un-eroded surfaces within the same patient. However, the impact of intrinsic acids and enzymes have been proven to be more destructive to dental tissue compared to dietary acids [Moazzez and Bartlett, 2014; Schlueter et al., 2010]. This could result in different effect of gastric acid on protein concentration levels in patients suffering from ETW. Therefore, this study was designed to investigate the total protein concentration in AEP from eroded surfaces compared to un-eroded surfaces within the same patient suffering from GORD symptoms.

4.2 Aims, objectives and null hypotheses

Aims

1. To compare the total protein concentration in in-vivo AEP from teeth with ETW and without ETW in patients suffering from GORD symptoms.

Objectives

1. To compare the total protein concentration in an in-vivo film and AEP from tooth surfaces with ETW and without ETW in the same patient suffering from GORD symptoms using bicinchoninic acid assay (BCA).
2. To compare the total protein concentration in an in-vivo film and AEP from tooth surfaces with ETW and without ETW between patients with symptoms of GORD and diagnosed with GORD and those who had symptoms but no GORD diagnosis using bicinchoninic acid assay (BCA).

Null hypothesis

1. There is no difference in total protein concentration of in-vivo AEP between teeth with ETW and without ETW in the same patient suffering from GORD symptoms.
2. There is no difference in total protein concentration of in-vivo AEP between teeth with ETW and without ETW in patients with and without GORD diagnosis.

4.3 Materials and methods

This study was approved by the National Research Ethics Service (NRES) in North East-York Research Ethic Committee (REC Ref 18/NE/0099). The protocol for this in-vivo study has been published previously on patients presenting with ETW as a results of dietary acids [Mutahar et al., 2017c].

4.3.1 Participants

Consecutive patients with symptoms suggestive of GORD presenting to the Oesophageal laboratory & breath test clinics at Guy's hospital London for an intralumenal 24hr-pH-impedance monitoring test were approached between April 2018 and November 2019 and invited to take part in the clinical study described in chapter 3. A smaller group of patients within this cohort were also asked to participate in this current study and were given a new patient information sheet (PIS appendix 7.7). Those who consented and agreed to take place in the present study were recruited for this in-vivo study (ICF appendix 7.8). Patients who did not have an eroded and un-eroded surface on the same side were excluded. The inclusion and exclusion criteria were:

Inclusion criteria:

1. Aged 18 to 95 years
2. Have a minimum of 20 natural occluding teeth present
3. Give written informed consent
4. Be in good general health other than GORD symptoms

Exclusion criteria:

1. Pregnant or breast feeding
2. Presence of severe periodontal disease or active caries on more than one tooth.

3. Unable to speak or understand English

4. Wearing an appliance

5. Restoration of the occlusal or incisal surfaces of upper anterior teeth and first molars.

6. No signs or symptoms of GORD

7. Patients who did not have an eroded and un-eroded surface on the same side

4.3.2 Sample collection

A single trained and calibrated investigator (RSH) assessed patients and performed an oral examination. Examination was done under an ideal source of light using a headlamp and patient in a reclined position. Basic erosive tooth wear (BEWE), using the same procedures explained in section 3.3, examination was performed on all surface areas (buccal, lingual/palatal and occlusal) and examined without magnification. Those with an accumulative score of 12 and more with at least one score of 3 and had an eroded and un-eroded tooth on the same side, were invited to take part in the study.

Participants were fasting for at least 12 hours prior to their assigned appointment for the intraluminal 24hr-pH-impedance monitoring test. One eroded and one un-eroded surface on the lower right molars were identified and the same on the lower left molars (ideally 6 and 7) in the same patient. Patients were divided into two groups based on the intraluminal 24hr-pH-impedance diagnosis to GORD and No-GORD.

4.3.3 In-vivo film and AEP harvest and recovery

The salivary film and AEP collection, harvest and recovery were carried out according to previously published protocols [Moazzez et al., 2014; Mutahar et al., 2017c; Siqueira et al., 2007d; Svendsen et al., 2008]. Salivary film and AEP were collected by same investigator (RS), a total of 4 salivary film and 4 AEP were collected resulting in 8 samples from each patient.

The labelling system for collected samples is illustrated in Table 4-1.

	Definition	Labels
Participant Number	10	10
ETW status	Eroded Un-eroded	E UE
Type of sample	Film Pellicle	F P
Side of the mouth	Right Left	1 2

Table 4-1: Sequence of the labelling system used for collecting salivary film and AEP (an example of participant number 10)

Salivary film was collected first to ensure the that the identified tooth was clear of salivary film prior to the AEP collection, and to separately analyse the total protein content of film and AEP. Films were collected by applying a dry sialopaper strip using a sterilized blunt ended tweezer for 5 seconds on the occlusal surface of the tooth to ensure the removal of salivary film prior to the AEP, it was then placed individually in a microtube with the tail of the sialopaper strip secured by closing the lid.

AEP was then harvested by soaking 5 mm of the sialopaper strips in sodium dodecyl sulphate (SDS) buffer (0.5% w/v) (Novex, Thermo Fisher Scientific Inc, UK). The solution was prepared by adding 0.5 g of SDS powder using electronic analytical scale (Fisher Scientific, Loughborough, UK) to 100 ml of deionised water, the solution was stirred using a magnetic stirrer until the SDS particles were dissolved completely in the deionised water. The SDS solution was made fresh every morning.

The 5 mm of the sialostrips soaked in the SDS solution were placed against the tooth surface to collect AEP by rubbing against the occlusal surface for 15 seconds (approximately 3x3 surface area). AEP samples were placed individually in microtubes and both AEP and film were placed on an ice pack and transported to a freezer where they were frozen at -80ºC until analysis.

The total number of samples from each patient were (n=8), upon analysis, samples were pooled together by combining the following: two eroded films, two eroded AEP, two un-eroded film and two un-eroded AEP, given a total of (n=4) samples per patient for the analysis.

The harvest process of film and AEP was done by a recruited analyst, placing the sialopaper strips carrying either the film or AEP in a 0.5ml Eppendorf tube, these tubes were perforated from the bottom and placed in another 1.5ml tube for the centrifuge.

The protein recovery was performed by adding: 15 uL of 0.5% SDS, (1:4) 5 uL of lithium dodecyl sulfate (LDS) buffer and (1:10) 1.8 uL of dithiothreitol (DTT) reducing agent. These were applied directly to the sialostrips. The 1.5 ul tubes were then placed in the centrifuge for 11 minutes at 800 rpm, they were then heated in 100C for 5 minutes. The recovered films and AEP were placed in a -20C freezer afterwards until the analysis.

4.3.4 Testing

Bicinchoninic acid assay (BCA)

Total protein concentration analysis was carried out by a recruited analyst, using a dye-based absorbance measurement method. The technique is composed of bicinchoninic acid assay kit (BCA) (Pierce Chemical, Rockford, III., USA) and 96- well plates. The 96 well is composed of 12 columns (numbered) and 8 rows labelled in letters from A to H.

The BCA measures the reduced amount of cuprous ion generated by chelating copper to proteins in the sample. The cuprous ions then react with BCA forming a purple water-soluble complex. The kit is composed of a standard and working reagent, purified bovine serum albumin standard (BSA) in single use 1 mL ampule for consistent curve standard generation. BSA was used as a protein standard in a concentration of (2mg/ mL) (Pierce Chemical, Rockford, Ill., USA).

Reference or blank samples was prepared by pipetting 200 uL of BSA standard solution to each well of the first two columns of the wells in the first two plates. 100 ul of deionised water was pipetted from the top column all the way to the 7^{th} row of the first two columns. 1 uL of each film and AEP diluted in deionised water at 1:10 in duplicate to total volume of 100 uL. Samples were placed into the 96-microtitter plates.

The working BCA reagent was prepared according to the manufacturer instructions, by combining reagents A and B in a ratio of 50:1. 25 uL of each standard, pipetted into a microtiter plate (96-wells, Fisher Scientific, Leicestershire, UK). A total of 200 uL of the working reagent was pipetted into the wells with the blank solution, BSA standard

solution and the film/AEP samples. The components were mixed for 30 seconds and incubated at 37 C for 30 minutes and left to cool for 5 minutes.

A spectrophotometer was used to measure the absorbance of all samples at wavelength of 562 nm (BioRad laboratories Ltd, Hemel Hempstead, UK). The absorbance is the density of the solutions or the intensity of transmitted light of all samples placed in the plate reader. BSA standard curves generated by plotting the line of best fit of BSA and blank samples. Plotting absorbents versus concentration in mg/mL, the formula used to measure protein concentration is:

$$\text{conentration} = \frac{absorbance - b}{m} X \frac{1}{dilution}$$

4.3.5 Power calculation

Based on previous studies comparing means of protein levels [Carpenter et al., 2014; Piangprach et al., 2009] and based on paired t-test and an effect size of 0.6 and 80% statistical power, 24 participants were required to identify a 5% difference and 5% significance level.

4.3.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.3 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com". Data were analysed for normality using Shapiro-Wilk test, those normally distributed were reported as mean and standard deviation (SD). Data were analysed using one-way ANOVA test and Tukey's multiple comparison test. ($p<0.05$) was set as the level of significance. Data were then analysed using GORD diagnosis as a dependant variable into GORD and No-GORD.

4.4 Results

Forty-five patients were approached and accepted to participate, six were excluded because they either did not have any un-eroded surfaces or did not have both un-eroded and eroded on the same side, leaving 39 patients from which 312 samples were collected and analysed, of which 298 samples were viable to analyse. The age range of participating patients was (21 to 80) with a mean (SD) age of 49.9(16.1), gender distribution was similar (female=21, male=18).

For the purpose of the analysis, samples from all recruited patients with symptoms were grouped into four: eroded film (n= 38), eroded AEP (n= 39), un-eroded film (n= 33), and un-eroded AEP (n= 39) as some of the samples were not viable and we were not able to recover the collected film/AEP, could be due to the small size of collected sample.

Table 42 shows the mean (SD) total protein concentration of in-vivo film (F) and AEP (P) from eroded (E) and un-eroded (U) surfaces. For film, mean (SD) of total protein concentration from eroded surfaces were 2.33 (0.94) mg/mL, un-eroded surfaces were 2.62 (1.59) mg/mL. For AEP, mean (SD) of total protein concentration from eroded surfaces were 2.74 (0.97) mg/mL, un-eroded surfaces were 2.80 (1.32) mg/mL. No statistically significant difference observed for film and AEP between surfaces ($p>0.05$).

Sample	Total protein concentration (mg/mL) mean (SD)
EF (n= 38)	2.33 (0.94)
UF (n= 33)	2.62 (1.59)
EP (n= 39)	2.74 (0.97)
UP (n= 39)	2.80 (1.32)

Table 4-2: Total protein concentration (mg/mL) from patients with symptoms of GORD

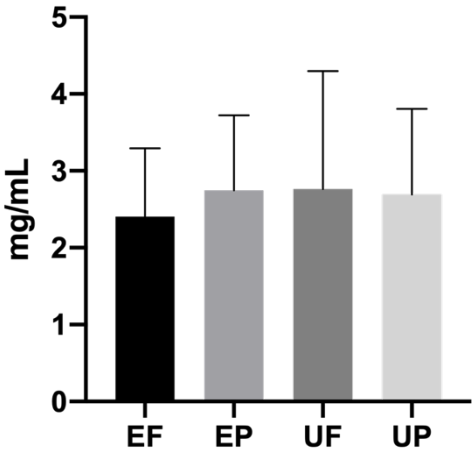


Figure 4-1: Total protein concentration (mg/mL) from patients with symptoms of GORD

Out of the total 39 patients recruited to the study with GORD symptoms, (n=20) of the participating patients were diagnosed with GORD (GORD group) and (n=19) were not diagnosed with GORD even though they had symptoms. The mean (SD) of BEWE score of those with GORD was 15.3 (0.74) and for NO-GORD 12.8 (0.83) but lacked statistical significance.

Film from NO-GORD patients: the mean (SD) of total protein concentration from eroded film (NG-EF) was 2.63 (0.84) mg/mL, un-eroded film (NG-UF) was 2.97 (2.0) mg/mL.

Film from GORD patients: the mean (SD) of total protein concentration collected from eroded film (GEF) was 1.97 (0.92) mg/mL and un-eroded film (GUF) was 2.22 (0.81) mg/mL. Although total protein concentration in films from both eroded and un-eroded surfaces were lower in GORD patients compared to NO-GORD patients, there was no statistically significant difference.

AEP from NO-GORD patients: the mean (SD) of total protein concentration from eroded AEP (NG-EP) was 3.27 (1.01) mg/mL, un-eroded AEP (NG-UP) was 3.33 (1.57) mg/mL.

AEP from GORD patients: the mean (SD) of total protein concentration collected from eroded AEP (GEP) was 2.17 (0.49) mg/mL and un-eroded AEP (GUP) was 2.24 (0.66) mg/mL.

There were no statistically significant differences between total protein concentration in AEP between eroded and uneroded surfaces for the GORD and the NO GORD groups. However, when comparing the AEP total protein concentration between the eroded and uneroded surfaces between the GORD and NO GORD groups statistically significant differences were found. The total protein concentration form eroded surfaces in the GORD group was statistically significantly lower than that on eroded surfaces in the No GORD group ($p=0.007$). Likewise, the total protein concentration form uneroded surfaces in the GORD group was statistically significantly lower than that on uneroded surfaces in the No GORD group ($p=0.008$).

Film samples	total protein concentration (mg/mL)
NG-EF	2.63 (0.84)
NG-UF	2.97 (2.0)
GEF	1.97 (0.92)
GUF	2.22 (0.81)

Table 4-3: Film total protein concentration (mg/mL) from GORD/ NO-GORD patients eroded and un-eroded surfaces

AEP samples	total protein concentration (mg/mL)
NG-EP	3.27 (1.01)
NG-UP	3.33 (1.57)
GEP	2.17 (0.49)
GUP	2.24 (0.66)

Table 4-4: AEP total protein concentration (mg/mL) from GORD/ NO-GORD patients eroded and un-eroded surfaces

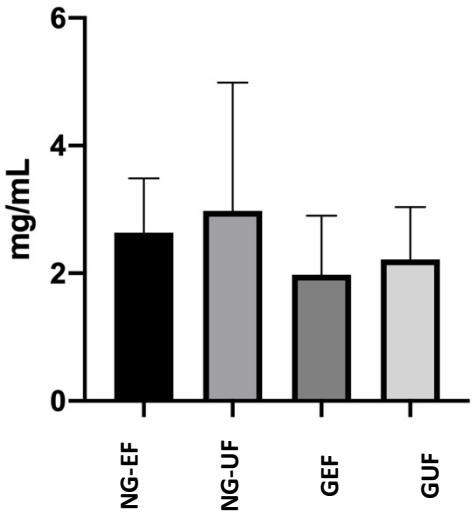


Figure 4-2: Film total protein concentration (mg/mL) from GORD / NO-GORD patients

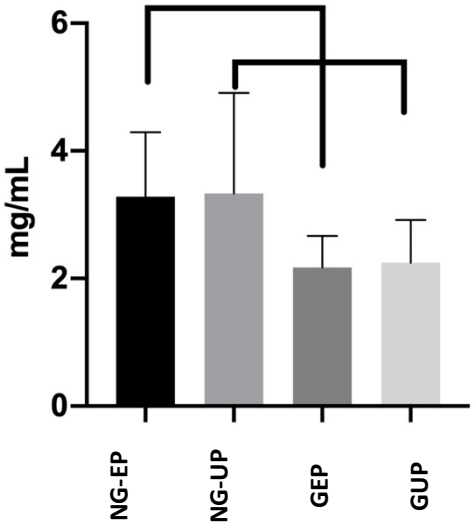


Figure 4-3: AEP total protein concentration (mg/mL) from GORD/ NO-GORD patients

4.5 Discussion

To the authors knowledge, this is the first study comparing total protein concentration of salivary film and AEP on eroded and uneroded surfaces within the same patient with GORD symptoms. There were no statistical significant differences between the total protein concentration on eroded and un-eroded surfaces collected from the same patient with GORD symptoms. However, when AEP was compared on eroded and uneroded surfaces between patient diagnosed with a GORD diagnosis and those with a No GORD diagnosis total protein concentrations were statistically lower in the GORD group for both eroded and uneroded surfaces. Therefore, the first null hypothesis was accepted and the second one was rejected.

In the current study, the site of film/ AEP collection protocol, the harvest and recovery method followed a recently published methodology by our group [Mutahar et al. 2017]. The film/AEP were collected from lower molars. This was based on the fact that previous work has shown AEP to be site-specific in regard to ETW [Amaechi et al., 1999a]. Amaechi et al. [1999] found the thickest AEP on mandibular posterior teeth, whereas the thinnest AEP was found on maxillary anterior teeth, it was concluded that the thickness of AEP may affect the presence of erosive tooth wear. Therefore, this study analysed AEP within the same patients mandibular molars only.

The amount of AEP collected from the individual surfaces is small and therefore samples from more than one tooth within the same patient were collected and pooled for the purpose of analysis.

The acquired enamel pellicle was formed for 12 hours prior to collection from patients, as patients were asked to fast for 12 hours prior to attending for their procedure. Hannig [1999a] examined the acquired enamel pellicle formation period

between 1 hour and 24 hours in an in-vitro study, the study reported the thickest pellicle was found in a 24 hours formed AEP with a dense globular layer of 1000 to 1300 nm. It was concluded that the longer the formation time the thicker the AEP. To the authors knowledge there are no previous in-vivo studies investigating AEP from natural human enamel surface after a 12-hours formation period.

Our results showed a significant lower total protein concentration in patients diagnosed with GORD compared to those with no-GORD (p=0.007). In addition, the mean BEWE scores of GORD patients (15.3) were higher than those with no-GORD (12.8), which denotes higher severity of ETW, although it lacked significance. According to the he inclusion/exclusion criterial of the participants had a total BEWE score of 12 or more and all had symptoms of GORD. These findings could therefore be due to salivary variables. Studies have linked the presence of GORD to changes in the salivary mechanisms [Filipi et al., 2011; Holbrook et al., 2009; Moazzez et al., 2004a]. The reduced salivary flow rate and reduced salivary clearance reported in patients with GORD, could result in gastric acid remaining longer in the oral cavity compared to individuals with normal flow rate [Jarvinen et al., 1991]. On the other hand, some studies reported an increased flow rate in patients with GORD during reflux episodes [Skoczylas et al., 2014]. However, the reduced salivary factors qualitatively and quantitatively have been associated with GORD pathogenesis [Menezes and Herbella, 2017; Moazzez et al., 2004b].

Acquired enamel pellicle (AEP), varies in thickness within the oral cavity, this in turn has a role in determining the site and severity of erosive tooth wear. Martini et al. [2019] investigated the proteomic profile of AEP from GORD patients with and without erosive tooth wear, they identified exclusive proteins in the AEP from GORD with erosive tooth wear patients, which are membrane proteins. They concluded that those proteins could

change the AEP structure reducing its protective effect and increasing incidence of erosive tooth wear.

Moreover, Hara et al [2003] and Hara et al [2006] showed that the proteins found in salivary film provided limited protection when formed on dentine (as ETW in most of GORD patients is severe and results in dentine exposure), whereas better protection against an erosive challenge was reported when the salivary film was formed on enamel surface. The results of our study agree with a previous study by Carpenter et al [2014]. They compared the total protein concentration between thirty patients with and without erosive tooth wear. Their study reported lower total protein concentration in patients with erosive tooth wear compared to healthy patients. However, Carpenter et a; [2014] compared the total protein concentration between healthy individuals and patient diagnosed with dietary erosive tooth wear, whereas in our study the comparison was done between patients diagnosed with and without GORD, but all patients had ETW.

The film/AEP total protein concentration collected from patients with GORD symptoms did not result in a significant difference when comparing eroded and un-eroded surfaces from the same patient. In contrast, Mutahar et al. [2017] reported significantly lower total protein concentration on eroded surfaces compared to un-eroded surfaces from the same patient diagnosed with dietary erosive tooth wear. A plausible explanation could be that patients in our study all had moderate/severe erosive tooth wear as an inclusion criterion (BEWE 12 or more with at least one 3), whereas the study by Mutahar et al. [2017] included patients with a BEWE score of 8 or more. Hence the protein delivery by the salivary film could be slower compared to those with less severe erosive tooth wear. This is supported by an in-situ study showing no significant

difference in the AEP composition on enamel splints and hydroxyapatite between patients with and without erosive tooth wear [Carpenter et al., 2014]. In addition, the AEP maturation time differs in the study by Mutahar et al.[2017c] , AEP was collected after an hour of fasting, whereas in our study the AEP collection was done after 12 hours of fasting. Therefore, the protein absorption varies in these patients as it depends on many factors such as the pH level, the surface charge and the surface area. Moreover, many factors could influence the results including the acid origin pH level, exposure time and tooth surface roughness. Mutahar et al. [2017] investigated the total protein concentration in patients with dietary erosive tooth wear whereas our study included patients with intrinsic acid origin. The pH level varies in dietary products ranging between 2.6 to 3.8 [Cheng et al., 2009c; Hjortsjo et al., 2010], whereas the pH of gastric acid ranges between 0.9 to 1.5 [Bartlett and Coward, 2001b], which is more destructive to the dental tissues. The chemical process varies between organic and inorganic acids. Most of dietary acids are weak, organic acids causing erosion through a chelation process, whereas gastric (intrinsic) acids are strong, inorganic acids causing erosion through full dissociation in water providing hydrogen ions that dissolve minerals on the tooth surface. This is in agreement with an in-vitro study by O'Toole et al [2020] investigating the interaction between enamel, AEP and extrinsic or intrinsic acids. After 300 s of citric acid (extrinsic) exposure, the total protein concentration decreased significantly. Whereas after 300 s of exposure to HCl (intrinsic), the total protein concentration did not change significantly. This indicates that the changes to the AEP protein composition is dependent on the type of acid. Similarly, earlier in chapter 2 of this thesis, the in-vitro study reported the difference in the chemical and physical interactions between intrinsic (HCl, artificial gastric juice) and dietary (citric acid) with the enamel surface. It was found that AEP

protected against citric acid whereas it did not offer any protection when human enamel samples were exposed to HCl or artificial gastric juice.

Like many in-vivo experiments, there are limitations to this study. First, the difficulty to standardise the size of surface area for the film/AEP collection, as larger areas could result in larger amount of proteins. However, the use of sialostrips does standardise the amount of proteins collected from film/AEP collected. In addition, the collected samples were pooled following previous protocol [Lee et al., 2013; Delecrode et al., 2015b; de Souza- E-Silva et al., 2017; Ventura et al., 2017] in order to increase the amount of collected protein and reduce individual variability.

The findings of this study contribute further to the understanding of the role of AEP during an acidic challenge. They also aid in explaining the differences between in-vitro and in-vivo data.

4.6 Conclusions

The total protein concentration of in-vivo acquired enamel pellicle was lower in patients diagnosed with GORD compared to patients with GORD symptoms but no diagnosis of GORD. The total protein concentration did not differ when comparing eroded and un-eroded surfaces within the same patient with GORD symptoms.

5 Chapter 5: General discussion

This thesis has investigated the predictive factors of ETW in patients with gastro-oesophageal reflux (GOR) symptoms and disease in a series of in-vitro, clinical and in-vivo studies. These assessments were carried out by exploring the relevant parameters including the effect of in-vitro gastric juice on enamel samples, the severity and frequency of GOR symptoms and oesophageal parameters with erosive tooth wear; and in-vivo protein components of acquired enamel pellicle in patients with GOR symptoms.

This thesis adds to previous literature by establishing several novel findings. The laboratory study reported increased enamel step height (tissue loss) when human enamel samples were exposed to intrinsic acids (HCl, artificial gastric juice) compared with dietary acid (citric acid) and water. The presence of acquired enamel pellicle resulted in increased tissue loss with intrinsic acids. The clinical studies indicated that predictors of erosive tooth wear in patients undergoing intralumenal 24hr-pH-impedance monitoring are those with abnormal DeMeester score, inconclusive or abnormal percentage of acid exposure time and those reporting daily regurgitation. In addition, within the study population, erosive tooth wear was more likely in those older than 50 years old with gastro-oesophageal reflux symptoms compared to younger patients. In vivo, it was demonstrated that total protein concentration from eroded and un-eroded surfaces in patients diagnosed with pathological GORD were lower than those not diagnosed with GORD. However, no statistical significance was observed between total protein concentration from eroded and un-eroded surfaces within the same patient with GOR symptoms.

The applied methodology in the laboratory study including the acid immersion times, the immersion method, acquired enamel pellicle formation time, qualitative and quantitative techniques for assessment of erosive tooth wear followed previously published protocol by our group [Mistry et al., 2015a; Mutahar et al., 2017a; Mylonas et al., 2018]. The findings of our in-vitro study were followed by further investigation on the effect of gastric refluxate clinically in patients suffering from reflux symptoms.

Previous studies have evaluated the association between gastro-oesophageal reflux symptoms or disease and erosive tooth wear. In a recent study, Rauber et al. [2020] reported that predictors of erosive tooth wear were heartburn and regurgitation, these symptoms were evaluated using digestive upper endoscopy and a self-assessment questionnaire considering only these two symptoms. However, in our study every individual symptom was evaluated by quantifying patient's self-assessment and clinically by the use of the latest diagnostic tools monitoring the oesophagus for 24 hours. Moreover, in our study symptoms were assessed from the patients' perspective using a standardised validated self-assessed questionnaire (RESQ), for both frequency and severity of each symptom and calculated an accumulative score of all symptoms. Questionnaires have been reported as a valid tool of diagnosing gastro-oesophageal reflux disease using a composite score of various symptoms questionnaires [Wang et al., 2004].

Symptoms were also assessed clinically through symptoms-association analysis. This was done by means of oesophageal testing using the latest technique 24-hours pH-impedance monitoring test. With this technique, we were able to detect the movement of a bolus (solid, liquid, air) within the oesophagus in both antegrade and retrograde directions irrespective of the pH. Our findings showed that GOR patients with erosive

tooth wear reported statistically increased heartburn and regurgitation compared to those without erosive tooth wear, there were no association between acid non-acid reflux and erosive tooth wear. Likewise, using this technique Vela et al.[2001] reported that acid and non-acid reflux were responsible for the generation of heartburn and regurgitation in patients off PPI. However, other studies have demonstrated an association between reflux symptoms and acid exposure in the oesophagus [Colas-Atger et al., 2002]. The 24hr-pH-impedance has some limitations, most notably the period of 24 hours may not be long enough as symptoms frequency varies daily. It was shown that a prolonged ambulatory monitoring period of 48 hours double the SI and SAP compared to 24 hours monitoring period in patients with atypical symptoms [Clouse et al., 2003].

Although age was shown to be one of the predictive factors of erosive tooth wear in patients with GOR, one could argue that tooth wear could be an age-related condition rather than a predictive factor. Physiological tooth wear was defined by the erosive tooth wear consensus [Schlueter et al.,2020] as “some degree of tooth wear expected over a lifetime. The rate of progression varies between individuals and not all tooth wear needs treatment”. In addition, pathological tooth wear was defined as “tooth wear beyond the physiological; level relative to the individual’s age and interferes with the self-perception of well-being”. Those included in the clinical study as an erosive tooth wear group were considered according to the mentioned criteria as those with BEWE score of 12 and more with at least 1 score of 3. In the [Schlueter et al.,2020] consensus, it was 100% agreed on describing BEWE score 3 as “severe wear”. Therefore, for the given reasons, age in this study within the given study group is considered as one of the predictive factor.

Previous studies have investigated the proteomic profile of the acquired enamel pellicle from patients diagnosed with GORD with erosive tooth wear and others without erosive tooth wear [Martini et al., 2019]. Also, others have compared the protein components of eroded and un-eroded surfaces within the same patient presented with erosive tooth wear but from an extrinsic dietary acidic origin [Mutahar et al., 2017c]. However, our study is the first to compare the total protein concentration of an acquired enamel pellicle formed in-vivo from eroded and un-eroded surfaces in patients diagnosed with pathological GORD presented with erosive tooth wear.

Erosive tooth wear is not necessarily correlated to histopathological oesophagitis, patients with non-erosive reflux disease also suffer from erosive tooth wear [Friesen et al., 2017]. Along with the findings of this thesis, it is highly recommended that dental screening for the presence of erosive tooth wear should be carried out on patients with GOR symptoms not only in those with histopathological diagnosis.

Overall, given the worldwide distribution of the association between gastro-oesophageal reflux and erosive tooth wear, clinicians should be made aware of the predisposing factors for developing erosive tooth wear in this group of patients and health care providers should be aware of the oesophageal and extraoesophageal symptoms and signs of gastro-oesophageal reflux disease.

6 Chapter 6: Clinical implications and future work suggestions

Findings of this thesis imply that erosive tooth wear could be predicted in patients with gastro-oesophageal reflux symptoms through a self-assessed questionnaire and through key parameters analysed from the impedance-pH-monitoring test. It suggests that referral for dental examination should be considered to patients above 50 years old with GOR symptoms, patients with abnormal/inconclusive acid exposure time, abnormal DeMeester score and those who complain of frequent regurgitation. It also suggests that enhancing the protective ability of AEP in patients within this study population would result in prevention of erosive tooth wear progression.

There are several findings of this thesis that deserve further investigation:

Findings observed provided an interesting information regarding the response of AEP to the intrinsic acid stimuli. The results indicate that AEP does not protect against intrinsic acids in-vitro, which could further be evaluated in-situ and in-vivo. The relationship between HCl/artificial gastric juice, AEP and erosive tooth wear has not been fully answered. This could be repeated including chemical analyses and total protein concentration in-vitro.

The findings of the clinical study in chapter 3 specify certain parameters as predictors of erosive tooth wear, these could be further investigated on larger number of participants. Further research could also include other parameters that were not investigated in this study such as the nature of reflux (gas, liquid, mixed) and the duration of refluxate in distal oesophagus. Although it was observed that the use of a self-assessed questionnaire (RESQ) would specify symptoms associated to the presence of erosive

tooth wear, this needs to be validated and confirmed with higher sensitivity and specificity for the detection of symptoms association.

The in-vivo study only included the measurement of total protein concentration of film and AEP from eroded and un-eroded surfaces, it would be beneficial to measure individual proteins within the AEP from eroded and un-eroded surfaces from the same patient suffering from GOR symptoms. It would also be interesting to test the activity of certain enzymes (pepsin, trypsin) in those patients, which are relevant for erosive tooth wear progression. Using the qualitative and quantitative proteomic approaches to investigate the dynamic process of the AEP would be noteworthy.

7 Chapter 7: Appendices

Volunteer information sheet (Version 2) 15/07/2015

Title of project: Protection of erosive tooth wear (donation of extracted tooth)

REC ref: 12/LO/1836

Investigator: Professor David Bartlett

7.1 PIS for teeth collection

You will be given a copy of the information sheet and a signed consent form to keep.

Part 1

Invitation paragraph

You are being invited to donate your tooth for a research study. Before you decide it is important for you to understand why the research is being done and what it will involve:

Part 1 tells you the purpose of the potential studies and what will happen if you decide to participate.

Part 2 gives you more detailed information about the conduct of the potential studies.

Please take time to read the following information carefully. Ask us if there is anything that is not clear. Talk to others about the research if you wish and the following organization could give you independent advice:

Guy's and St Thomas' Hospital NHS Foundation Trust Patient Advice and Liaison Service Telephone 020 7188 8801 or 020 7188 8803 email: pals@gstt.nhs.uk

Post: Patient information team, Knowledge and information centre, St Thomas' Hospital London, Westminster Bridge Road, SE1 7EH

What is the purpose of the study?

Tooth wear is a condition where the teeth wear away faster than normal and is caused by acid erosion (from acidic foods and drinks and stomach acid), tooth grinding and over brushing. Tooth wear is a common condition that can affect anyone, and it appears to be happening more and more nowadays. Severe tooth wear can cause teeth to become very sensitive, as well as causing cosmetic and chewing problems due to shortened teeth and even in severe cases can cause tooth loss. Certain toothpastes and mouth rinses have the potential to prevent and treat tooth wear. However the scientific evidence for this is lacking and the studies we plan to carry out may provide important information regarding the disease process, progression of the disease and possible prevention of the disease.

Why have I been chosen?

You are suitable for this study because you are a healthy individual who needs a tooth removed.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I decide to take part?

At your first visit, when you are consulted about the tooth extraction, you will be invited to join the study by a clinician. At your second visit we will confirm that you still want to donate your tooth and then you will have your tooth removed in the normal way. After your tooth is extracted it will be transferred to the Biomaterials laboratory at King's College Hospital Dental Institute (Department of Biomaterials, 17th Floor, Guy's Tower, Guy's Hospital, London Bridge SE1 9RT). Once the tooth is extracted your participation in the study is over.

What do I have to do?

You will just have to attend your set appointments as normal.

What is the drug, device or procedure being tested?

Various methods of studying the surface changes of the extracted teeth and the effects of dietary acids, fluorides and other protective agents are being investigated in this study on the extracted teeth.

What are the alternatives for diagnosis or treatment?

The research does not involve any volunteer treatment and you will receive your routine standard treatment as usual.

What are the side effects of any treatment received when taking part?

There are no risks associated with this study, other than the usual risks of a tooth extraction which will be explained to you by the clinical team who are carrying out the treatment.

What are the other possible disadvantages or risks of taking part?

There are no risks associated with this study, other than the usual risks of a tooth extraction which will be explained to you by the clinical team who are carrying out the treatment.

What are the possible benefits of taking part?

We do not expect that you will receive any benefit from taking part in this study.

What happens when the research study stops?

We aim to publish the results in medical journals.

What if there is a problem? And contact details:

No problems can be foreseen however the contact number for complaints or concerns is for:

Professor David Bartlett 0207 188 5390 or email david.bartlett@kcl.ac.uk

Will my taking part in the study be kept confidential?

We will not be collecting any information about you and your confidentiality is safeguarded during and after the study. Our procedures for handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998.

Contact for further information:

Professor David Bartlett 0207 188 5390 or email david.bartlett@kcl.ac.uk

This completes Part 1 of the Information Sheet. If the information sheet in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2

What if relevant new information becomes available?

We are a leading establishment in this area of research and if any new information relevant to this study becomes available the researchers will discuss this with you. You are free to withdraw from the study at any time.

What will happen if I don't want to carry on with the study?

You can withdraw from study. Just advise the clinician treating you that you do not want to donate your tooth and your tooth will be disposed of once extracted, or you can keep it to take home.

What if there is a problem?

If you have any concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer their questions.

Professor David Bartlett 0207 188 5390 or email david.bartlett@kcl.ac.uk

If you remain unhappy and wish to complain formally, you can do this through the NHS complaints procedure. If you are harmed by taking part in this research project there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay privately for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way that you have been approached or treated during the course of this study, the normal NHS complaints mechanisms should be available to you.

Details of how to complain can be obtained from the Volunteer Advice and Liaison Service (PALS)

Guy's and St Thomas' Hospital NHS Foundation Trust Patient Advice and Liaison Service

Telephone 020 7188 8801 or 020 7188 8803 email: pals@gstt.nhs.uk

Post: Patient information team, Knowledge and information centre, St Thomas' Hospital London, Westminster Bridge Road, SE1 7EH

Will my taking part in this study be kept confidential?

We will not be collecting any information about you and your confidentiality is safeguarded during and after the study. Our procedures for handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998.

What will happen to any samples that I give?

After your tooth has been removed, it will be anonymised (i.e. there will be no way of linking the tooth to your personal data or medical records) and then transported to the Biomaterials laboratory at King's College Hospital Dental Institute (Department of Biomaterials, 17th Floor, Guy's Tower, Guy's Hospital, London Bridge SE1 9RT). The tooth will be used in a laboratory study or clinical study investigating erosive tooth wear. The study may be laboratory experiment which involves simulating erosive wear on the enamel blocks from the donated teeth in the laboratory, as well as exposure to topical protection or it may be a clinical study where participants may wear mouthgaurds containing sterilised blocks containing the enamel from the donated teeth. In both cases, measurements of the amount of wear on the tooth surface are taken.

What will happen to the results of the research study?

The results of the study will be published in medical journals. Participants will not be identified in any report or publication.

Who has reviewed the study?

This study was given a favourable ethical opinion REC ref: 12/LO/1836

Will any genetic tests be done?

No.

Thank you for considering taking part and for taking time to read this sheet – please ask any questions if you need to.

7.2 ICF for teeth collection

Consent Form (Version 2: 22/07/2015)

Title of project: Protection of erosive tooth wear (donation of extracted tooth)

REC ref: REC ref: 12/LO/1836

Investigator: Professor David Bartlett

Please complete this form after you have read the Information Sheet and/or listened to an explanation about the research

Patient Identification: Date

Thank you for considering taking part in this research. The person organising the research and/or a member of the clinical team who is trained for this purpose must explain the project before you agree to take part.

If you have any questions arising from the Information Sheet or explanation given to you, please ask the researcher before you decide whether or not to join in. You will be given a copy of this Consent Form to keep and refer to at any time.

I confirm that I have read and understand the information sheet dated (version 1) for the above study.

I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

I agree to take part in the above study.

Name of Patient.....

Signature.....Date.....

.....

Name of Person taking consent.....

Signature.....Date.....

.....

7.3 PIS for saliva collection

Participant Information Sheet

Healthy Volunteers Group

Study Title: Role of Saliva/pellicle in dental erosion and dental caries

REC ref: Northampton REC, 14/EM/0183

Investigator: Dr Rebecca Moazzez

Invitation paragraph

You are being invited to donate saliva for a research study. You should only participate if you want to. Choosing not to take part will not disadvantage you in any way. It is up to you to decide whether to take part or not. If you decide to take part you are still free to withdraw from the study at any time and without giving a reason. You can withdraw your data at any point up until the conclusion of your final clinic visit. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. Before you decide it is important for you to understand why the research is being done and what it will involve.

Part 1 tells you the purpose of the study and what will happen if you decide to participate.

Part 2 gives you more detailed information about the conduct of the study.

Part 1: Purpose of the study and what will happen

What is the purpose of the study?

The goal of this study is to collect saliva from healthy individuals, individual with dental erosion (wear of teeth by acids) and individual with dental caries (tooth decay).

Dental erosion is a condition where the teeth wear away faster than normal and is caused by acids (from acidic foods and drinks and stomach acid). Dental erosion is a common condition that can affect anyone and it appears to be happening more and more nowadays. Severe dental erosion can cause teeth to become very sensitive, as well as causing cosmetic and chewing problems due to shortened teeth and even in severe cases can cause tooth loss.

Dental caries (tooth decay) results when foods and drinks high in sugary carbohydrates, bacteria in plaque (a sticky film that forms on the teeth when they are not brushed) use these carbohydrates to produce acid. Acid in plaque begins to break down the tooth's surface and result in decay. Left untreated it can result in pain and death of the nerve inside the tooth and tooth loss.

A number of research studies have shown a relationship between the properties of saliva and salivary pellicle (a thin film formed from saliva on the tooth surface immediately after brushing) and dental erosion and dental decay. Some proteins in saliva and pellicle may offer a protective role against these two conditions developing. However the scientific evidence is lacking about the role of these proteins in the hardening and loss

of enamel and dentin through these conditions. This study will help us in our understanding of the role of saliva and pellicle in preventing dental erosion and decay.

Why have I been chosen?

You are suitable for this study because you do not have any signs of dental caries (tooth decay) or dental erosion (Abnormal wear of teeth by acids).

Do I have to take part?

You do not have to take part. It is up to you decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I decide to take part?

At your first visit, you will be invited to join the study by a clinician and given this patient information sheet. At your second visit we will confirm that you still want to donate saliva. After your saliva is collected it will be anonymised and transferred to the Biomaterials laboratory at King's College Hospital Dental Institute and used in a Laboratory study. After the completion of the study the sample will be discarded.

What do I have to do?

You will just have to attend your set appointments as normal.

Once your consent is taken, you will be given a general oral exam and we will ask you some questions regarding your medical history to ensure that you meet our study criteria. You will then be asked to provide a sample of unstimulated saliva by dribbling any saliva collected in your mouth into a tube. Following this you will be asked to chew on a tasteless piece of paraffin wax for 5 minutes and dribble any saliva collected in your mouth into another tube (Stimulated saliva).

Next, a saliva sample will be collected from the sides of your cheeks inside your mouth from one of the salivary glands (parotid gland). This will be collected by placing a sterile suction cup on the inside of your mouth on the surface of your cheeks. The whole process will take up to 30 minutes. The saliva secretion will be stimulated by placing 2 drops of citric acid 2% solution on the back of your tongue every 30 seconds.

What are the side effects of any treatment received when taking part?

There is no treatment and no side effects.

What are the other possible disadvantages or risks of taking part?

There are no risks associated with this study.

What are the possible benefits of taking part?

We do not expect that you will receive any benefit from taking part in this study.

Will any genetic tests be carried out?

No

What happens when the research study stops?

We aim to publish the results in medical journals. Our procedures for handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998. Any samples collected for the study will be discarded.

What will happen if I don't want to carry on with the study?

You can withdraw from participation at any time. Just advise the clinical researcher or the chief investigator that you do not want to continue taking part and any collected saliva, if any, will be discarded.

This completes Part 1 of the Information Sheet. If the information sheet in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2: Study Conduct

What if relevant new information becomes available?

We are one of the leading establishments in this area of research and if any new information relevant to this study becomes available the researchers will discuss this with you. You are free to withdraw from the study at any time. If you decide to withdraw your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

Also, on receiving new information the researchers might consider it to be in your best interests to withdraw you from the study. They will explain the reasons and arrange for your care to continue. At the end of the study the results will be presented to the scientific community.

Will my taking part in the study be kept confidential?

Once you have agreed to take part in this study, you will be allocated a study number which will be used at all times during your subsequent visits. This means that all information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will be anonymised and have your personal details removed so that you cannot be recognised from it.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions.

Please contact:

Dr Rebecca Moazzez

Rebecca.v.moazzez@kcl.ac.uk

0207 188 1856

If you have a complaint, you should talk to your research doctor who will do their best to answer your questions. If you remain unhappy, you may be able to make a formal complaint through the NHS complaints procedure. Details can be obtained through the Guy's and St Thomas' Patient Advisory Liaison Service (PALS) on 0207 1887188, address: PALS, KIC, Ground floor, north wing, St Thomas' Hospital, Westminster Bridge Road, London, SE1 7EH .

This trial is co-sponsored by King's College London and Guy's and St Thomas' NHS Foundation Trust. The sponsors will at all times maintain adequate insurance in relation to the study independently. Kings College London, through its own professional indemnity (Clinical Trials) and no fault compensation and the Trust having a duty of care to patients via NHS indemnity cover, in respect of any claims arising as a result of clinical negligence by its employees, brought by or on behalf of a study patient.

What will happen to the results of the research study?

The results of the study will be published in medical journals. Participants will not be identified in any report or publication.

Who has reviewed the study?

This study has been reviewed by an internal reviewer at King's College London and was given a favourable ethical opinion by Northampton REC, 14/EM/0183.

Contact for Further Information

Dr Rebecca Moazzez, Room 365, Floor 25, Tower Wing, Guy's Hospital, London Bridge.

0207 188 1856, rebecca.v.moazzez@kcl.ac.uk

Thank you for considering taking part and for taking time to read this sheet – please ask any questions if you need to.

You will be given a copy of the information sheet and a signed consent form to keep.

7.4 ICF for saliva collection

Informed Consent Form

Study title: Role of Saliva/pellicle in dental erosion and dental caries

Principal Investigator: Dr Rebecca Moazzez

	Please Initial box
I confirm that I have read and understood the information sheet (dated 27/07/2014, Version no.4) for the above study. I have had an opportunity to consider the information, ask questions and have these answered satisfactorily.	
I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
I understand that data collected during the study, may be looked at by responsible individuals from King's College clinical staff, regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give my permission for these individual to have access to my records.	
I understand that if the study is published, none of my personal details will be identifiable.	
I agree to take part in this study.	

_____ _____

Participant's Legal Name Date Signature

_____ _____

Name of person taking consent Date Signature

(if different from researcher)

_____ _____

Researcher Date Signature

7.5 in-vitro raw data

Non-contact surface profilometry results

Tukey's multiple comparisons test	Predicted (LS) mean diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
30					
Citric vs. Control	0.1044	-0.5156 to 0.7244	No	ns	0.9903
Citric with saliva vs. Control	0.01264	-0.6523 to 0.6776	No	ns	>0.9999
HCL vs. Control	0.4780	-0.1621 to 1.118	No	ns	0.2419
HCL with saliva vs. Control	1.817	1.152 to 2.482	Yes	****	<0.0001

Citric with saliva vs. Citric	-0.09175	-0.7718 to 0.5883	No	ns	0.9959
HCL vs. Citric	0.373<0.0001)	-0.2821 to 1.029	No	ns	0.5164
HCL with saliva vs. Citric without saliva	1.713	1.033 to 2.393	Yes	****	<0.0001
HCL vs. Citric with saliva	0.4654	-0.2330 to 1.164	No	ns	0.3543
HCL with saliva vs. Citric with saliva	1.805	1.083 to 2.526	Yes	****	<0.0001
HCL with saliva vs. HCL	1.339	0.6408 to 2.038	Yes	****	<0.0001
60					
Citric vs. Control	0.8796	0.2596 to 1.500	Yes	**	0.0013

Citric with saliva vs. Control	0.3388	-0.3262 to 1.004	No	ns	0.6238
HCL vs. Control	0.9242	0.2841 to 1.564	Yes	***	0.0010
HCL with saliva vs. Control	3.227	2.562 to 3.892	Yes	****	<0.0001
Citric with saliva vs. Citric	-0.5408	-1.221 to 0.1392	No	ns	0.1867
HCL vs. Citric	0.04453	-0.6112 to 0.7002	No	ns	0.9997
HCL with saliva vs. Citric	2.348	1.668 to 3.028	Yes	****	<0.0001
HCL vs. Citric with saliva	0.5853	-0.1131 to 1.284	No	ns	0.1460

HCL with saliva vs. Citric with saliva	2.889	2.167 to 3.610	Yes	****	<0.0001
HCL with saliva vs. HCL	2.303	1.605 to 3.002	Yes	****	<0.0001

Tukey's multiple comparisons test	mean diff.	95.00% CI of diff.	Significant?	Summary	Value
120					
CITRIC ACID vs. CONTROL	2.104	-1.408 to 5.615	No	ns	.548
CITRIC ACID+AEP vs. CONTROL	2.017	-1.750 to 5.783	No	ns	.674
HCL vs. CONTROL	1.816	-1.810 to 5.441	No	ns	.738
HCL+AEP vs. CONTROL	2.738	-1.028 to 6.504	No	ns	.310
GASTRIC JUICE vs. CONTROL	16.36	12.41 to 20.30	Yes	***	<.001
GASTRIC JUICE+AEP vs. CONTROL	20.31	16.36 to 24.25	Yes	***	<.001

CITRIC ACID+AEP vs. CITRIC ACID	-0.08698	-3.938 to 3.764	No	ns	>.999
HCL vs. CITRIC ACID	-0.2881	-4.002 to 3.425	No	ns	>.999
HCL+AEP vs. CITRIC ACID	0.6344	-3.217 to 4.486	No	ns	.999
GASTRIC JUICE vs. CITRIC ACID	14.25	10.22 to 18.28	Yes	***	<.001
GASTRIC JUICE+AEP vs. CITRIC ACID	18.20	14.18 to 22.23	Yes	***	<.001
HCL vs. CITRIC ACID+AEP	-0.2011	-4.156 to 3.754	No	ns	>.999
HCL+AEP vs. CITRIC ACID+AEP	0.7214	-3.364 to 4.806	No	ns	.998

GASTRIC JUICE vs. CITRIC ACID+AEP	14.34	10.09 to 18.59	Yes	***	< .001
GASTRIC JUICE+AEP vs. CITRIC ACID+AEP	18.29	14.04 to 22.54	Yes	***	< .001
HCL+AEP vs. HCL	0.9225	-3.033 to 4.878	No	ns	.992
GASTRIC JUICE vs. HCL	14.54	10.41 to 18.67	Yes	***	< .001
GASTRIC JUICE+AEP vs. HCL	18.49	14.37 to 22.62	Yes	***	< .001
GASTRIC JUICE vs. HCL+AEP	13.62	9.367 to 17.87	Yes	***	< .001
GASTRIC JUICE+AEP vs. HCL+AEP	17.57	13.32 to 21.82	Yes	***	< .001

GASTRIC JUICE+AEP vs. GASTRIC JUICE	3.952	-0.4606 to 8.364	No	ns	.110
300					
CITRIC ACID vs. CONTROL	2.305	-1.206 to 5.816	No	ns	.435
CITRIC ACID+AEP vs. CONTROL	1.395	-2.372 to 5.161	No	ns	.922
HCL vs. CONTROL	4.577	0.9522 to 8.202	Yes	**	.005
HCL+AEP vs. CONTROL	6.963	3.197 to 10.73	Yes	***	<.001
GASTRIC JUICE vs. CONTROL	27.11	23.17 to 31.06	Yes	***	<.001
GASTRIC JUICE+AEP vs. CONTROL	40.35	36.41 to 44.30	Yes	***	<.001

CITRIC ACID+AEP vs. CITRIC ACID	-0.9103	-4.762 to 2.941	No	ns	.992
HCL vs. CITRIC ACID	2.272	-1.441 to 5.986	No	ns	.522
HCL+AEP vs. CITRIC ACID	4.658	0.8069 to 8.510	Yes	**	.008
GASTRIC JUICE vs. CITRIC ACID	24.81	20.78 to 28.84	Yes	***	<.001
GASTRIC JUICE+AEP vs. CITRIC ACID	38.05	34.02 to 42.08	Yes	***	<.001
HCL vs. CITRIC ACID+AEP	3.183	-0.7726 to 7.138	No	ns	.200
HCL+AEP vs. CITRIC ACID+AEP	5.569	1.484 to 9.654	Yes	**	.002

GASTRIC JUICE vs. CITRIC ACID+AEP	25.72	21.47 to 29.97	Yes	***	< .001
GASTRIC JUICE+AEP vs. CITRIC ACID+AEP	38.96	34.71 to 43.21	Yes	***	< .001
HCL+AEP vs. HCL	2.386	-1.569 to 6.341	No	ns	. 539
GASTRIC JUICE vs. HCL	22.54	18.41 to 26.66	Yes	***	< .001
GASTRIC JUICE+AEP vs. HCL	35.78	31.65 to 39.90	Yes	***	< .001
GASTRIC JUICE vs. HCL+AEP	20.15	15.90 to 24.40	Yes	***	< .001
GASTRIC JUICE+AEP vs. HCL+AEP	33.39	29.14 to 37.64	Yes	***	< .001

GASTRIC JUICE+AEP vs. GASTRIC JUICE	13.24	8.829 to 17.65	Yes	***	< .001

Surface microhardness change results

Tukey's multiple comparisons test	Predicted (LS) mean diff.	95.00% CI of diff.	Significant?	Summary	Adjuste P Valu
30					
CA+AEP vs. DIW	50.72	46.40 to 55.04	Yes	****	<0.000
CA vs. DIW	42.80	38.36 to 47.24	Yes	****	<0.000
HCl vs. DIW	49.17	44.85 to 53.49	Yes	****	<0.000
HCl+AEP vs. DIW	62.63	58.31 to 66.95	Yes	****	<0.000
CA vs. CA+AEP	-7.921	-12.36 to -3.478	Yes	****	<0.000
HCl vs. CA+AEP	-1.550	-5.874 to 2.774	No	ns	0.855
HCl+AEP vs. CA+AEP	11.91	7.586 to 16.23	Yes	****	<0.000
HCl vs. CA	6.371	1.928 to 10.81	Yes	**	0.001
HCl+AEP vs. CA	19.83	15.39 to 24.27	Yes	****	<0.000
HCl+AEP vs. HCl	13.46	9.136 to 17.78	Yes	****	<0.000
60					

CA+AEP vs. DIW	69.50	65.18 to 73.82	Yes	****	<0.000
CA vs. DIW	50.80	46.36 to 55.25	Yes	****	<0.000
HCl vs. DIW	68.98	64.66 to 73.30	Yes	****	<0.000
HCl+AEP vs. DIW	83.80	79.48 to 88.12	Yes	****	<0.000
CA vs. CA+AEP	-18.70	-23.14 to -14.25	Yes	****	<0.000
HCl vs. CA+AEP	-0.5200	-4.844 to 3.804	No	ns	0.997
HCl+AEP vs. CA+AEP	14.30	9.976 to 18.62	Yes	****	<0.000
HCl vs. CA	18.18	13.73 to 22.62	Yes	****	<0.000
HCl+AEP vs. CA	33.00	28.55 to 37.44	Yes	****	<0.000
HCl+AEP vs. HCl	14.82	10.50 to 19.14	Yes	****	<0.000

Tukey's multiple comparisons test	Predicted (LS) mean diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value

120					
CA+AEP vs. DIW	86.03	77.06 to 95.00	Yes	***	<.001
CA vs. DIW	68.89	59.67 to 78.11	Yes	***	<.001
HCl vs. DIW	80.35	71.38 to 89.32	Yes	***	<.001
HCl+AEP vs. DIW	100.3	91.36 to 109.3	Yes	***	<.001
AGJ vs. DIW	11.90	2.010 to 21.78	Yes	**	.008
AGJ+AEP vs. DIW	15.75	5.861 to 25.63	Yes	***	<.001
CA vs. CA+AEP	-17.14	-26.36 to -7.924	Yes	***	<.001
HCl vs. CA+AEP	-5.680	-14.65 to 3.290	No	ns	.484
HCl+AEP vs. CA+AEP	14.30	5.330 to 23.27	Yes	***	<.001
AGJ vs. CA+AEP	-74.14	-84.02 to -64.25	Yes	***	<.001

AGJ+AEP vs. CA+AEP	-70.28	-80.17 to -60.40	Yes	***	<.001
HCl vs. CA	11.46	2.244 to 20.68	Yes	**	.005
HCl+AEP vs. CA	31.44	22.22 to 40.66	Yes	***	<.001
AGJ vs. CA	-57.00	-67.10 to -46.89	Yes	***	<.001
AGJ+AEP vs. CA	-53.14	-63.25 to -43.04	Yes	***	<.001
HCl+AEP vs. HCl	19.98	11.01 to 28.95	Yes	***	<.001
AGJ vs. HCl	-68.46	-78.34 to -58.57	Yes	***	<.001
AGJ+AEP vs. HCl	-64.60	-74.49 to -54.72	Yes	***	<.001
AGJ vs. HCl+AEP	-88.44	-98.32 to -78.55	Yes	***	<.001

AGJ+AEP vs. HCl+AEP	-84.58	-94.47 to -74.70	Yes	***	<.001
AGJ+AEP vs. AGJ	3.851	-6.871 to 14.57	No	ns	.933
300					
CA+AEP vs. DIW	113.9	105.0 to 122.9	Yes	***	<.001
CA vs. DIW	89.60	80.38 to 98.81	Yes	***	<.001
HCl vs. DIW	99.51	90.54 to 108.5	Yes	***	<.001
HCl+AEP vs. DIW	118.7	109.7 to 127.7	Yes	***	<.001
AGJ vs. DIW	28.49	18.60 to 38.37	Yes	***	<.001
AGJ+AEP vs. DIW	22.25	12.37 to 32.14	Yes	***	<.001
CA vs. CA+AEP	-24.34	-33.56 to -15.13	Yes	***	<.001
HCl vs. CA+AEP	-14.43	-23.40 to -5.460	Yes	***	<.001

HCl+AEP vs. CA+AEP	4.760	-4.210 to 13.73	No	ns	.687
AGJ vs. CA+AEP	-85.45	-95.34 to -75.57	Yes	***	<.001
AGJ+AEP vs. CA+AEP	-91.69	-101.6 to -81.80	Yes	***	<.001
HCl vs. CA	9.911	0.6952 to 19.13	Yes	*	.026
HCl+AEP vs. CA	29.10	19.89 to 38.32	Yes	***	<.001
AGJ vs. CA	-61.11	-71.22 to -51.00	Yes	***	<.001
AGJ+AEP vs. CA	-67.35	-77.46 to -57.24	Yes	***	<.001
HCl+AEP vs. HCl	19.19	10.22 to 28.16	Yes	***	<.001

AGJ vs. HCl	-71.02	-80.91 to -61.14	Yes	***	<.001
AGJ+AEP vs. HCl	-77.26	-87.14 to -67.37	Yes	***	<.001
AGJ vs. HCl+AEP	-90.21	-100.1 to -80.33	Yes	***	<.001
AGJ+AEP vs. HCl+AEP	-96.45	-106.3 to -86.56	Yes	***	<.001
AGJ+AEP vs. AGJ	-6.235	-16.96 to 4.487	No	ns	.587

7.6 Protocol for clinical study

IRAS ID: 235215

Protocol

PROTOCOL TITLE:
Erosive tooth wear related to Gastroesophageal Reflux Disease (GORD)
Questionnaire & clinical based study

Sponsor
Name: KCL Reza Rezavi
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Chief Investigator
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Name: Professor David Bartlett
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Name: Dr Rasha AlHarthi
Address: Floor 18, Tower Wing, Guy's Campus, SE19RT
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Title	The relationship between gastroesophygeal reflux disease to erosive tooth wear and the effect of saliva and acquired enamel pellicle in protection against the two conditions
Protocol Short Title/Acronym	GORD and dental erosion
Protocol Version number and Date	Version 1.0 26/01/2018
Study Phase if not mentioned in title	Questionnaire study
Study Duration	*2 years*
Methodology	*Questionnaire based study.*
Sponsor name	KCL
Chief Investigator	Dr Rebecca Moazzez
REC number	2325215
IRAS ID	
Medical condition or disease under investigation	Dental erosion
Purpose of clinical trial	*To determine the risk factors in GORD patients that causes the greatest wear*
Primary objective	To investigate the risk factors for erosive tooth wear in patients who suffer from Gastroesophageal Reflux Disease (GORD) symptoms
Secondary objective (s)	To investigate the protective role of saliva and acquired enamel pellicle (AEP) on the protection against erosive tooth wear in patients with gastro-oesophageal reflux disease (GORD)
Number of Subjects/Patients	*300*
Trial Design	Questionnaire on a convenient sample compared to matched control group
Endpoints	End of trial will be at the point where all the subjects have been recruited and all data and samples have been collected and analysed.
Main Inclusion Criteria	*GORD*
Statistical Methodology and Analysis	*Frequencies, Multivariate and univariate analysis*

Protocol for The relationship between gastroesophygeal reflux disease to erosive tooth wear and the effect of saliva and acquired enamel pellicle in protection against the two conditions

Background

For centuries tooth wear has affected mankind as a normal physiological ageing process. An annual tooth wear on the occlusal surface area of approximately 29µm for molars and about 15 µm for premolars is considered a normal physiological process due to age (Moslehifard et al., 2012). The aetiology of tooth wear is usually multifactorial and rarely is it due to a single pathological factor; hence dentists should appraise all related factors involved to establish the cause of the loss. The predominant aetiological factor controls the morphology of the tooth wear defect (Ganss, 2014).

ooth wear is becoming a common dental issue since patients are preserving their teeth longer and frequently consuming acidic foods and drink as part of their healthy diet (Al-Salehi, 2014). A study conducted by Bartlett to examine dental study models over 26 months stated a slow progression of tooth wear in that sample, informing that rate of progression of tooth wear is not predictable (Bartlett, D.W., 2013). Moreover, a systematic review on the prevalence of tooth wear indicated that adults with severe tooth wear shows an increase from 3% at age 20 years to 17% at age 70 years (Van't Spijker et al., 2009). In the United Kingdom, erosive tooth wear is acknowledged as the main aetiological factor in addition to recognizing attrition and abrasion as being important, and the common term used for such tooth surface loss is 'tooth wear' (Bartlett et al., 1999)

ental Erosion

Dental erosion is an irreversible lesion caused by a chemical process that does not include bacteria (1-2), it is caused by tooth tissue exposure to acid from either intrinsic or extrinsic origin, which can be determined by the location of the erosive lesion (Dukic et al., 2010). It is not the intention of this study to investigate dietary factors.

Endogenous acids:

There are number of causes for endogenous (gastric) acid reaching the oral cavity and these include gastroesophageal reflux disease (GORD), vomiting which might be either involuntary related to pregnancy sickness, migraines and stress or voluntary in the form of anorexia or bulimia nervosa (Milosevic and O'Sullivan, 2008). Another phenomenon called rumination can cause erosive tooth wear, where the oesophageal sphincter becomes relaxed and hence allows swallowed food to re-enter the oral cavity for re mastication and swallowing again (Bartlett, 2005). It commonly occurs during meal times in some cultures, although it is rare in Western society. It can be seen in individuals with learning disabilities, physiologically ill and depression related patients (Nolen-Hoeksema et al., 2008). Another factor, is that gastric juice with a PH of 1-3 is very acidic (Newton JL. et al, 2004) and the

component of hydrochloric acid within it is strong and highly erosive (Bartlett DW. et al, 2001)

Natural Saliva and severity of tooth wear:
Saliva and acquired enamel pellicle (AEP), both have a role in preventing dental erosion (2-6). Saliva is an important biological factor to consider in relation to tooth wear, the protective mechanism of which comes into play with an erosive challenge. It is responsible for clearance of the erosive agent from the mouth, buffering acids and decreases the rate of enamel dissolution by the calcium and phosphate content (Zero DT et al., 2000). The consistency and constituents of saliva can vary from patient to patient and as a reaction to taste stimulus; mucus saliva has a role in lubricating the food and serous saliva buffers acids and enhances food swallowing. If a medical condition, dehydrating lifestyle or xerostomic drugs are present, then it has the potential to affect the major salivary glands which controls the flow of serous saliva and hence its buffering capacity, which compromises the protection against endogenous and exogenous acids (Young and Khan, 2002). Minor glands on the other hand secrete the mucus saliva, which does not clear the food properly as it is viscous and does not contain bicarbonate buffer (Young, 2005).
Natural saliva has been used in in vitro studies to assess the process of demineralization and remineralization of dental erosion. Saliva contains almost 1,000 types of proteins and mineral contents such as calcium, phosphate and fluoride, which helps in the maintenance of the chemical and physical integrity of the tooth structure (8-9).

Acquired enamel pellicle
The acquired enamel pellicle (AEP) is a bacteria-free organic layer formed in vivo as a result of selective adsorption of salivary proteins on the surface of the enamel (Dawes, Jenkins, & Tongue, 1963), and forms within moments of brushing and reaches the equilibrium stage of saturation after 30 minutes and up to 2 hours (13-15). Due to its composition of minerals and proteins, the AEP forms a protective physical interface between the tooth surface and the oral cavity, reducing friction and abrasion, which modulates the mineralization/demineralization processes, modifying mineral precipitation and adherence of microorganisms to the dental surface (Buzalaf et al., 2012; Hannig & Joiner, 2006; Hara and Zero, 2010; Vukosavljevic et al., 2014), some studies correlated the barrier effect of the AEP to the mineral content (19-21), while others suggested that the protein contents are the reason behind the physical characteristics (22-24).The thickness of the acquired pellicle may control the distribution of the erosion pattern (Young and Khan, 2002), as sites with the thinnest pellicle in situ have proved to have the highest erosion in vitro (maxillary anterior palatal) after immersion in orange juice for 2 hours, while the sites with the thickest pellicle in situ showed the least erosion in vitro (mandibular lingual surface of both posterior and anterior teeth) (Lussi et al., 2004).

The saliva derived acquired pellicle may also have a function in enamel surface protection against erosive agents (Nekrashevych and Stosser, 2003).

Salivary factors that might influence the effect of gastric acid includes salivary glands hypofunction, there is no statistical significant difference in the stimulated salivary flow between a healthy group and the study group in a study done by Moazzez et al., but the study showed that gord patients with hoarsness has a decreased stimulated salivary flow. Another factor would be the buffering capacity, Gudmundson et al. suggested that when the buffering capacity is impaired, the acid exposure becomes more injurious to the oral tissues (Gudmundsson K et al., 1995)

objectives

1. To identify risk factors associated with presence of ETW in patients with GORD symptoms (n=300).
2. To compare presence of pepsin in saliva from patients with GORD and ETW and those with GORD but without ETW.
3. To compare the salivary flow rate and buffering capacity from patient with GORD and ETW and those with GORD but without ETW.
4. To compare the concentration of total protein in an in-vivo AEP from tooth surfaces with ETW and without ETW in the same patient suffering from GORD.
5. To compare the amount of mucin5b, albumin, CAVI and statherin in-vivo AEP from teeth with ETW and without ETW in the same patient suffering from GORD.

Method

The research will take place at the Oesophagyeal Laboratory & Breath Test Clinic at Guy's Hospital. Patients attending the Oesophageal Laboratory who are referred for investigation of GORD by manometry and 24-h oesophageal pH tests from a variety of medical sources will be asked to participate.
Participants will be given adequate time to consider the study and will have the opportunity to ask questions about the study at any time. Participants who change their mind will be able to do so without affecting their clinical care. If they are interested in taking part full informed written consent will be obtained.

Questionnaire

A Two validated questionnaire will be used. The first is a modified version used for patients suffering from dietary erosive wear. Participants will be asked to complete a medical history form, dental history form, diet history (To assess source of acids in the diet (To determine presence and source of any acid that could result in erosive tooth wear (wear of teeth by acids)). The second is RESQ-7 which is self-reported questionnaire used routinely for patients attending the Oesophageal Laboratory. It is used to assess frequency and intensity of gastroesophageal reflux disease symptoms. A 6-point Likert response format is used for both frequency and intensity. The questionnaire has been digitalized and programmed in a tablet format to assist with efficient completion and automatic analysis of the data obtained.

Erosive tooth wear assessment:
The severity of erosive tooth wear will be determined using the Basic Erosive Wear Examination (BEWE). This is a validated scoring system that has a 4-point scale (0-3), with 0 indicating no wear and 3 indicating severe wear affecting more than 50% of the tooth surface.

Table: Basic Erosive Wear Examination (BEWE)

Score	Criteria for wear classification
0	No loss of surface
1	Initial loss of surface texture Slight wear
2	Distinct defect, hard tissue loss <50% of the surface area Dentine is frequently involved (non carious dental lesion), representing moderate lesions
3	Hard tissue loss ≥50% of the surface area Dentine is frequently involved (non carious dental lesion), representing severe lesions

Saliva collection will be carried out by asking the patient to expectorate saliva in a pre-weighed universal tubes to asses the salivary flow and buffering capacity. AEP will be collected from tooth surfaces

Participants will be given a participant information sheet that clearly details their role in the study. They will be reassured that their decision to take part or not will not affect their clinical care in any way. Participants will be given adequate time to read through the Patient Information Sheet before deciding whether to take part and have the decision if they want to take the sheet home to consider participation in their following appointment. They will also have the opportunity to ask the research team any questions regarding the study they may have. If they decide to take part, written informed consent will be obtained. Ideally, the questionnaires and samples will be collected the same day as their medical appointment

All assessments will take place under ideal lighting, cleaning all tooth surfaces thoroughly using cotton wool buds and examining the cervical, buccal/labial, occlusal/ incisal and lingual/palatal surfaces of each tooth in the same manner with all participants.

Research flow chart:

	Screen Visit
Patient information and informed consent	X
Questionnaire	X
BEWE	X
Saliva collection	X
AEP collection	X
Adverse event monitoring	X

Inclusion criteria

1. Aged 18 to 75 years inclusive
2. Have a minimum of 20 natural uncrowned teeth (excluding 3rd molars) present
3. Give written informed consent
4. Be in good general health other than GORD symptoms
5. No diseases of the soft or hard tissues of the oral cavity
6. Diagnosed as a patient with Gastroesophygeal Reflux Disease (GORD)

Exclusion criteria

1. Pregnant or breast feeding
2. Medical history likely to impact on attendance or mobility
3. Presence of severe periodontal disease or active caries on more than one tooth.
4. Unable to speak or understand English
5. Salivary disease diagnoses (xerostomia)or medication which could affect salivary flow rate
6. Wearing an appliance
7. Restoration of the occlusal or incisal surfaces of upper anterior teeth and first molars.
8. no signs or symptoms of GORD

Clinical assessment

The clinical assessment of tooth wear is made through a visual grading on a 4 point scale ranging, from 0 to 3 and published by our team, and then statistical comparison made to the intake of acid(1). The assessment will occur under good lighting, dry teeth and all teeth scored on all surfaces. The scoring system has been utilized in a number of previously published studies.

Sample size

A total of 282 participants will be needed to identify a 10%-difference in the prevalence of any GORD parameter (i.e. symptom, etc.) between GORD patients with and without ETW, assuming a recruitment ratio of one-to-one (141 GORD patients with ETW and 141 without ETW), prevalence for the parameter of 15% and 5% in GORD patients with and without ETW respectively, 80% statistical power and 5% significance level. Sample size will be increased to 300 (150 in each group) to compensate for potential exclusions due of missing values (up to 6%). A sample of 300 participants will also give 82% statistical power to detect a standardised mean difference of 0.33 units in a continuous GORD parameter (i.e. duration, etc.) between GORD patients with and without ETW, assuming a recruitment ratio of one-to-one (150 GORD patients with ETW and 150 without ETW), a common standard deviation of 1 unit in both groups and 5% significance level.

Analysis

The data will be compared within and between groups, using non parametric analysis. In our experience with patient-based research these groups of analyses work well.

Outcome

This study will improve our understanding of risk factors, which will then be possible to inform dentists and the public about the relationship between erosive tooth wear and GORD.

The study will be conducted in the Oesophageal laboratory and respiratory clinic at Guy's Hospital.

Finance

No application for external funding has been made. The lead sponsor, King's College London, will take primary responsibility for ensuring that the design of the study meets appropriate standards and provides cover under its No Fault Compensation Insurance, which provides for payment of damages or compensation in respect of any claim made by a research subject for bodily injury arising out of participation in a clinical trial or healthy volunteer study (with certain restrictions).

Reference List

1. Bartlett DW, Coward PY. Comparison of the erosive potential of gastric juice and a carbonated drink in vitro. *Journal of Oral Rehabilitation* 2001;28(11):1045-47.
2. Moazzez R, Bartlett D. Intrinsic causes of erosion. *Monogr Oral Sci* 2014;25:180 96.
3. Nekrashevych Y, Hannig M, Stosser L. Assessment of enamel erosion and protective effect of salivary pellicle by surface roughness analysis and scanning electron microscopy. *Oral Health Prev Dent* 2004;2(1):5-11.

7.7 PIS for clinical study

Patient Information Sheet

Study Title:

Erosive tooth wear related to gastro-oesophageal reflux disease (GORD)

Investigator: Dr Rebecca Moazzez

You are being invited to take part in a research study. Before deciding whether take part it is important you understand why the research is being done and what it will involve.

Please take your time to read the following information carefully. Ask us if there is anything that is not clear. Talk to others about the research if you wish, or for independent advice the:

Guy's and St Thomas' Hospital NHS Foundation Trust Patient Advice and Liaison Service is available to you via phone: 020 7188 8801/ 020 7188 8803, or email: pals@gstt.nhs.uk.

It is up to you to decide whether to take part in this research or not. Choosing not to take part will not disadvantage you in any way. If you decide to take part you are free to withdraw from the study at any time and without giving a reason. You can withdraw

from the study at any point. If you do decide to take part you will be given this information sheet to keep and you will be asked to sign a consent form.

What is the purpose of the study?

The aim of this study is to better understand some of the risk factors associated with gastro-oesophageal reflux disease (GORD) symptoms, and its relation to the wear of the teeth known as (erosive tooth wear). Gastro-oesophageal reflux disease is known as (acid reflux), where the stomach contents come back up in the esophagus and can cause symptoms such taste of acid in the back of the mouth, heartburn, chest pain, husky voice and wear of teeth.

Erosive tooth wear is a condition where the teeth wear away faster than normal and is caused by acid erosion (from acidic foods/drinks or stomach acid). Erosive tooth wear is a relatively common condition that can affect anyone which appears to be becoming more widespread. Severe erosive tooth wear can cause teeth to become very sensitive, as well as causing cosmetic and chewing problems due to shortened teeth. In severe cases, Erosive tooth wear can cause tooth loss.

Some people with GORD suffer from tooth wear and others not. The scientific evidence for the risk factors for developing tooth wear in this group is lacking. This study will help us find out some of the risk factors in patients with GORD suffering from erosive tooth wear.

Do I have to take part?

No, You do not have to take part. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a

consent form. You are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive either now or in the future.

What will happen to me if I decide to take part?

At your first visit, you will be invited to join the study by a clinician and given this patient information sheet. You will be asked to sign a consent form if you decide to take part. You will be asked to answer a questionnaire. The questions will be about the type, intensity, frequency, duration and timing of any symptoms of GORD (reflux disease) you experience. You will then be asked to donate saliva (a 'spit' sample) into a bottle. You will have a dental examination to record any wear on the tooth surfaces. You may be asked to provide a sample of the surface coating on some of your teeth (acquired enamel pellicle). The clinician will inform you if this applies to you. The collected saliva and acquired enamel pellicle will be anonymised and transferred to the Biomaterials laboratory at the King's College London Dental Institute and will be analysed for this project. After the completion of the study the sample will be discarded.

What do I have to do?

There will be no changes to your clinical appointments. You just have to attend your set appointments as normal.

What are the side effects of any treatment received when taking part?

There are no side effects associated with this study.

What are the possible benefits of taking part?

This study will help in understanding the risk factors that causes erosive tooth wear in patients with GORD symptoms

. Although we do not anticipate immediate benefits, we are hoping that this research may improve treatment for patients in the future.

Will any genetic tests be carried out? No

What will happen if I don't want to carry on with the study?

If you would like to withdraw your participation in the research then you can do at any time. No further clinical or non-clinical interventions or procedures will be carried out on you under the study protocol. No new samples or personal data will be collected.

The tissue samples or data already collected from you may be retained and used for the purposes for which consent has already been given, provided they are effectively anonymised and securely stored on university computers which are encrypted and can only be accessed by the research investigators.

If you would prefer, you can request that all the data and samples collected in the study are disposed of and data deleted. You can make this request up until the study closure date of 01/02/2021.

What if relevant new information becomes available?

If any information relevant to this study becomes available, the researchers will discuss this with you. If you decide to continue in the study you will be asked to sign an updated consent form.

Also, on receiving new information the researchers might consider it to be in your best interests to withdraw you from the study. They will explain the reasons and arrange for your care to continue.

Will my taking part in the study be kept confidential?

Once you have agreed to take part in this study, you will be allocated a study number which will be used at all times during your subsequent visits. This means that all information which is collected about you during the research will be kept strictly confidential. Any information about you which leaves the hospital will be anonymised and have your personal details removed so that you cannot be recognised from it. Our procedures for handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998.

What will happen to the results of the research study?

The results of the study will be published in medical journals. Participants will not be identified in any report or publication.

When the study is completed, saliva will be discarded according to the protocol submitted to the Ethics Committee once all the studies are completed. The collection,

storage and disposal of saliva samples will be conducted in accordance with the Human Tissue Act (2004).

What if there is a problem? And contact details:

If you have any concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer their questions.

Dr Rebecca Moazzez

Rebecca.v.moazzez@kcl.ac.uk

0207 188 1856

If you remain unhappy and wish to complain formally, you can do this through the NHS complaints procedure. If taking part in this research project harms you there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay privately for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way that you have been approached or treated during the course of this study, the normal NHS complaints mechanisms should be available to you.

Details of how to complain can be obtained from the Volunteer Advice and Liaison Service (PALS)

Guy's and St Thomas' Hospital NHS Foundation Trust Patient Advice and Liaison Service

Telephone 020 7188 8801 or 020 7188 8803 email: pals@gstt.nhs.uk

Post: Patient information team, Knowledge and information centre, St Thomas' Hospital London, Westminster Bridge Road, SE1 7EH

Thank you for considering taking part and for taking time to read this information sheet – please ask any questions if you need to.

7.8 ICF for clinical study

Informed Consent Form

Study title:

Erosive tooth wear related to gastro-oesophageal reflux disease

Principal Investigator: Dr Rebecca Moazzez

	Please Initial box
I confirm that I have read and understood the information sheet (dated 26/01/2018, Version no 1.0) for the above study. I have had an opportunity to consider the information, ask questions and have these answered satisfactorily.	
I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
I understand that data collected during the study, may be looked at by responsible individuals from King's College clinical staff, regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give my permission for these individuals to have access to my medical notes.	

I understand that samples will be collected from me and used for research purposes.	
I understand that if the study is published, none of my personal details will be identifiable.	
I agree to take part in this study	

_____ _____

Participant's Legal Name Date Signature

_____ _____

Name of person taking consent Date Signature

(If different from researcher)

_____ _____

Researcher Date Signature

7.9 HRA approval

NHS
Health Research Authority

Dr Rebecca Moazzez

Reader/Hon Consultant in Restorative Dentistry Email: hra.approval@nhs.net

King's College London Dental Institute

King's College London Dental Institute

Room 365, floor 25, Tower Wing

Guy's Hospital, Great Maze Pond, London

SE1 9RT

20 March 2018

Dear Dr Moazzez

Letter of HRA Approval

Study title:	The relationship between Ga oesophygeal Reflux Disease (GORD) to erosive tooth and the effect of saliva and acq enamel pellicle (AEP) in protection ag the two conditions
IRAS project ID:	235215
REC reference:	18/NE/0099
Sponsor	King's College London

I am pleased to confirm that HRA Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting

documentation and any clarifications received. You should not expect to receive anything further from the HRA.

How should I continue to work with participating NHS organisations in England?

You should now provide a copy of this letter to all participating NHS organisations in England, as well as any documentation that has been updated as a result of the assessment.

This is a single site study where the site is the study co-sponsor. The R&D office will confirm to the CI when the study can start.

It is important that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details of the research management function for each organisation can be accessed here.

How should I work with participating NHS/HSC organisations in Northern Ireland, Scotland and Wales?

HRA Approval does not apply to NHS/HSC organisations within the devolved administrations of Northern Ireland, Scotland and Wales.

If you indicated in your IRAS form that you do have participating organisations in one or more devolved administration, the HRA has sent the final document set and the study wide governance report (including this letter) to the coordinating centre of each participating nation. You should work

with the relevant national coordinating functions to ensure any nation specific checks are complete, and with each site so that they are able to give management permission for the study to begin.

Please see IRAS Help for information on working with Northern Ireland, Scotland and Wales.

How should I work with participating non-NHS organisations?

HRA Approval does not apply to non-NHS organisations. You should work with your non-NHS organisations to obtain local agreement in accordance with their procedures.

What are my notification responsibilities during the study?

The document "*After Ethical Review – guidance for sponsors and investigators*", issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including: Registration of research

- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

I am a participating NHS organisation in England. What should I do once I receive this letter? You should work with the applicant and sponsor to complete any outstanding arrangements so you are able to confirm capacity and capability in line with the information provided in this letter.

The sponsor contact for this application is as follows:

Name: Professor Reza Razavi

Tel: 02078486960

Email: reza.razavi@kcl.ac.uk

Who should I contact for further information?

Please do not hesitate to contact me for assistance with this application. My contact details are below.

Your IRAS project ID is 235215. Please quote this on all correspondence.

Yours sincerely

Miss Lauren Allen
Assessor

Email: hra.approval@nhs.net

Copy to: Prof Reza Razavi
Mrs Jennifer Boston, Guy's & St.Thomas' Foundation NHS trust

List of Documents

The final document set assessed and approved by HRA Approval is listed below.

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only)		18 August 2017
IRAS Application Form [IRAS_Form_02032018]		02 March 2018
IRAS Application Form XML file [IRAS_Form_02032018]		02 March 2018

IRAS Application Form XML file [IRAS_Form_26022018]		26 February 2018
IRAS Checklist XML [Checklist_02032018]		02 March 2018
Letter from sponsor		10 November 2016
Non-validated questionnaire [Questionnaire Part A]	1.1	26 January 2018
Other [Prof Bartlett CV]		
Other [Dr Jafari CV]		
Other [PhD Student CV]		
Participant consent form	1.1	26 January 2018
Participant information sheet (PIS)	Version 1.3	14 March 2018
Research protocol or project proposal	1.2	06 March 2018
Summary CV for Chief Investigator (CI)		26 January 2018
Summary CV for student [Rasha AlHarthi CV]	1	23 February 2018
Validated questionnaire [RESQ-7]		

Summary of HRA assessment

The following information provides assurance to you, the sponsor and the NHS in England that the study, as assessed for HRA Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England to assist in assessing, arranging and confirming capacity and capability.

HRA assessment criteria

Section	HRA Assessment Criteria	Compliant with Standards?	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	No comments
3.1	Protocol assessment	Yes	A non-substantial amendment was made to the Protocol for assessment purposes only following REC favourable opinion to correct the funding arrangements.
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	An agreement will not be required as this is a single site study where the single site is also the study co-sponsor.

4.2	Insurance/indemnity arrangements assessed	Yes	The applicant has confirmed that the study will be covered by NHS insurance/indemnity, not the University's insurance as indicted in the IRAS form. Where applicable, independent contractors (e.g. General Practitioners) should ensure that the professional indemnity provided by their medical defence organisation covers the activities expected of them for this research study
4.3	Financial arrangements assessed	Yes	There is no external funding for the research.
Section	**HRA Assessment Criteria**	**Compliant with Standards?**	**Comments**
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	Arrangements for securely storing data and accessing medical records have been confirmed. Only the research team will have access to participants' medical records.
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Not Applicable	No comments

5.3	Compliance with any applicable laws or regulations	Yes	No comments
6.1	NHS Research Ethics Committee favourable opinion received for applicable studies	Yes	No comments
6.2	CTIMPS – Clinical Trials Authorisation (CTA) letter received	Not Applicable	No comments
6.3	Devices – MHRA notice of no objection received	Not Applicable	No comments
6.4	Other regulatory approvals and authorisations received	Not Applicable	No comments

Participating NHS Organisations in England

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.
This is a single site study where the single NHS site is also the study co-sponsor. If this study is subsequently extended to other NHS organisation(s) in England, an amendment should be submitted to the HRA, with a Statement of Activities and Schedule of Events for the newly participating NHS organisation(s) in England. The Chief Investigator or sponsor should share relevant study documents with participating NHS organisations in England in order to put arrangements in place to deliver the study. The documents should be sent to both the local study team, where applicable, and the office providing the research management function at the participating organisation. For NIHR CRN Portfolio studies, the Local LCRN contact should also be copied into this correspondence. For further guidance on working with participating NHS organisations please see the HRA website. If Chief Investigators, sponsors or Principal Investigators are asked to complete site level forms for participating NHS organisations in England which are not provided in IRAS or on the HRA website, the Chief Investigator, sponsor or Principal Investigator should notify the HRA immediately at hra.approval@nhs.net. The HRA will work with these organisations to achieve a consistent approach to information provision.

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and the minimum expectations for education, training and experience that PIs should meet (where applicable).
A Local Collaborator should be identified at the site to facilitate access arrangements for the external research team (where needed). GCP training is not a generic training expectation, in line with the HRA/MHRA statement on training expectations.

HR Good Practice Resource Pack Expectations

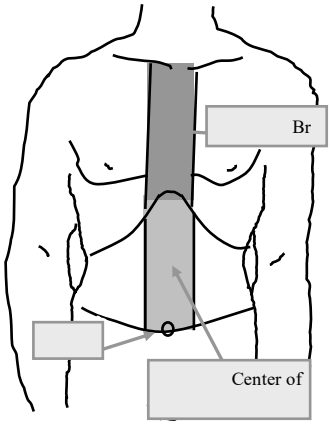
This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken
External staff (e.g. University) will be expected to obtain an Honorary Research Contract to conduct activity at the site. Where the activity is limited to administering questionnaires only then a Letter of Access will be appropriate. Disclosure and Barring Service and Occupational Health checks should be confirmed where an Honorary Research Contract or Letter of Access is expected.

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England to aid study set-up.
The applicant has indicated that they intend to apply for inclusion on the NIHR CRN Portfolio.

7.10 Questionnaire format

RESQ-7



Please answer the following questions to help us better understand the symptoms you have been experiencing over the past 7 days because of your reflux disease. For each question, please choose the answer that is most appropriate to you. Please answer each question by ticking <u>one</u> box per row.

1. **Thinking about your symptoms over the past 7 days, how often have you had the following?**

	Have not had	1 day	2 days	3-4 days	5-6 days	Daily
a. A burning feeling	☐	☐	☐	☐	☐	☐

behind your
breastbone

b. Pain behind your ☐ ☐ ☐ ☐ ☐ ☐
breastbone

c. A burning feeling ☐ ☐ ☐ ☐ ☐ ☐
in the centre of
the upper stomach

d. Pain in the centre ☐ ☐ ☐ ☐ ☐ ☐
of the upper stomach

e. An acid taste ☐ ☐ ☐ ☐ ☐ ☐
in your mouth

f. Unpleasant ☐ ☐ ☐ ☐ ☐ ☐
movement of
material upwards
from the stomach

g. Burping (gas coming ☐ ☐ ☐ ☐ ☐ ☐
from the stomach
through the mouth)

h. Hoarseness	☐	☐	☐	☐	☐	☐
i. Coughing	☐	☐	☐	☐	☐	☐
j. Difficulty swallowing	☐	☐	☐	☐	☐	☐
k. A bitter taste in your mouth	☐	☐	☐	☐	☐	☐
l. Stomach contents (liquid or food) moving upwards to your throat or mouth	☐	☐	☐	☐	☐	☐
m. Heartburn	☐	☐	☐	☐	☐	☐

2. Thinking about your symptoms over the past 7 days, how would you rate the intensity of the following?

	Did not have	Very mild	Mild	Moderate	Moderately severe	Severe
a. The burning feeling behind your breastbone	☐	☐	☐	☐	☐	☐
b. Pain behind your breastbone	☐	☐	☐	☐	☐	☐
c. The burning feeling in the centre of the upper stomach	☐	☐	☐	☐	☐	☐
d. Pain in the centre of the upper stomach	☐	☐	☐	☐	☐	☐
e. Acid taste in your mouth	☐	☐	☐	☐	☐	☐

f. Unpleasant movement of material upwards from the stomach ☐ ☐ ☐ ☐ ☐ ☐

g. Burping (gas coming from the stomach through the mouth) ☐ ☐ ☐ ☐ ☐ ☐

h. Hoarseness ☐ ☐ ☐ ☐ ☐ ☐

i. Coughing ☐ ☐ ☐ ☐ ☐ ☐

j. Swallowing difficulty ☐ ☐ ☐ ☐ ☐ ☐

k. The bitter taste in your mouth ☐ ☐ ☐ ☐ ☐ ☐

l. Stomach contents (liquid or food) moving upwards to your throat or mouth ☐ ☐ ☐ ☐ ☐ ☐

m. Heartburn	☐	☐	☐	☐	☐	☐

7.11 Questionnaire license

Licence Agreement for the Reflux symptom questionnaire, 7 day recall (RESQ-7)

This Licence Agreement (the "**Agreement**") is made effective as of the last date written below (the "**Effective Date**") by and between

(1) ASTRAZENECA AB, a company incorporated in Sweden under no. 556011-7482 with offices at SE-431 83 Mölndal, Sweden ("**AstraZeneca**"); and

(2) Dr. Rasha Said AlArabi AlHarthi of King's College London, a company incorporated in the United Kingdom, with registered offices at Guy's Hospital, Tower Wing London SE1 9RT ("**Licensee**").

Recitals

(A) WHEREAS, AstraZeneca owns and is in the possession of a certain questionnaire known as the Reflux symptom questionnaire, 7 day recall (the RESQ-7).

(B) WHEREAS, Licensee wishes to obtain, and AstraZeneca is willing to grant to Licensee, a right to use the RESQ-7 solely for the Study (as defined below) on the terms and conditions set forth below.

(C) WHEREAS, Licensee wishes to administer the RESQ-7 via PAPER

(D) NOW THEREFORE, the parties agree as follows.

Agreement

1 Licence

1.1 Upon payment of the Licence Fee and for the duration of the Term (as defined below), AstraZeneca grants to Licensee and its affiliates a non-exclusive, non-assignable and non-sublicenseable licence (the "**Licence**") to:

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4.1 This Agreement shall become effective on the Effective Date and shall continue in force until the completion of the Study (at which time this Agreement shall terminate automatically without

further notice), unless earlier terminated in accordance with this Section 4.1 (the "Term"). Either party may terminate this Agreement immediately by giving written notice to the other party if the other party should commit a material breach of any of its obligations under this Agreement and fail to rectify such breach within thirty (30) days after having been given a written request for such rectification.

4.2 Sections 1.2 and 3 and this Section 4.2 shall survive the termination of this Agreement.

5 Miscellaneous

5.1 The interpretation and construction of this Agreement shall be governed by the laws of Sweden excluding any conflicts or choice of law rule or principle that might otherwise refer construction or interpretation of this Agreement to the substantive law of another jurisdiction.

5.2 The parties hereby irrevocably and unconditionally consent to the exclusive jurisdiction of the Swedish courts for any action, suit or proceeding arising out of or relating to this Agreement, and agree not to commence any action, suit or proceeding related thereto except in such courts.

THIS AGREEMENT IS EXECUTED by authorised representatives of the parties.

Place: East Hartford, Connecticut, USA
Date: 21-Apr-2017
Name (Print): Amanda Rosett
CORPORATE TRANSLATIONS, INC. ON BEHALF OF:
ASTRAZENECA AB (PUBL)

Amanda Rosett
Signature

Place: King's College London, Guy's Hospital
Date: 21-04-17
Name (Print):
Dr. Rasha Said AlArabi AlHarthi

Signature

7.12 Questionnaire scoring instructions

Item re-coding

Frequency items 1a-1m should be re-coded as shown below before computing domain scores. Intensity items 2a-2m should not be re-coded.

Response option	Pre-coded item value		Final item value
Did not have	0	→	0
1 day	1	→	1
2 days	2	→	2
3-4 days	3	→	3.5
5-6 days	4	→	5.5
Daily	5	→	7

Computing domain scores

A mean value for the items in each domain should be calculated. For frequency (i.e. number of days) the min-max scores will be 0-7; for intensity the min-max scores will be 0-5.

Missing values

Sometimes respondents leave one or more of the items blank. We recommend that a domain score be calculated according to the 'half-scale' method, i.e. a domain score is computed if at least half if the items in a domain are answered. The missing items will then be imputed using the mean score of the non-missing item scores.

References:

Vakil N, Björck K, Denison H, Halling K, Karlsson M, Paty J, Silberg D, Rydén A. Validation of the Reflux Symptom Questionnaire Electronic Diary in partial responder to proton pump inhibitor. CTG 2012:3, e7;doi:10.1038/ctg.2012.1

Vakil N, Karlsson M, Denison H, Rydén A. A patient reported outcome instrument in partial responders to proton pump inhibitor therapy suggests new symptoms deserve consideration: Results from a validation study. Gut 2011;60 (Suppl 3), A266

Vakil N, Karlsson M, Denison H, Rydén A. The development of a patient reported outcome instrument in partial responders to proton pump inhibitor therapy suggests new symptoms deserve consideration: Results of a validation study. Gastroenterology 2011;140(5) (Suppl 1), P S-67

7.13 Patients data logger instruction sheet

pH-

Guy's and St Thomas' NHS
NHS Foundation Trust

INSTRUCTIONS FOR REFLUX MONITORING

Patient details:	Probe insertion Date: Time:
Investigator (initials):	Medication: ☐ On ☐ Off

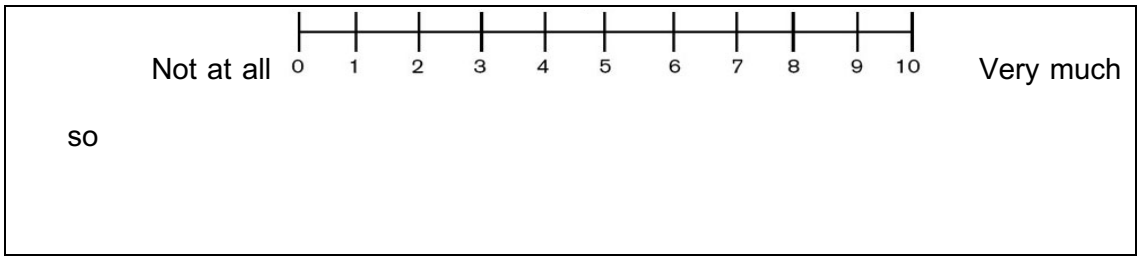
You need to return for probe removal on _____ at _____ AM/PM

At the end of this study please let us know:

1- Did you manage to have a **usual day** of your life? (Please circle)

Not at all 0 1 2 3 4 5 6 7 8 9 10 Very much so

2- Did you have your **typical symptoms**? (Please circle)



It is very important for the purpose of analysis to know when your symptoms happen, when you eat or drink and when you sleep and wake up. Therefore, please follow the instructions very carefully.

Pressing markers on the machine:

1. Please record your symptoms as you experience them by pressing the allocated button **only once.** (See image)
2. When there are symptoms not listed on the machine, use the diary sheet on last page to write down the time and type of the symptoms.
3. If a symptom is continuous, press the marker once and make a note on the diary in last page. Each time the symptom intensifies press the allocated button again.
4. Record the start and finishing times of your meal by pressing the allocated buttons.
5. Press the allocated buttons for sleeping and waking up. (see image)
6. Try not to sleep recline; using one pillow is fine. It does not matter if you sleep on your side or back.

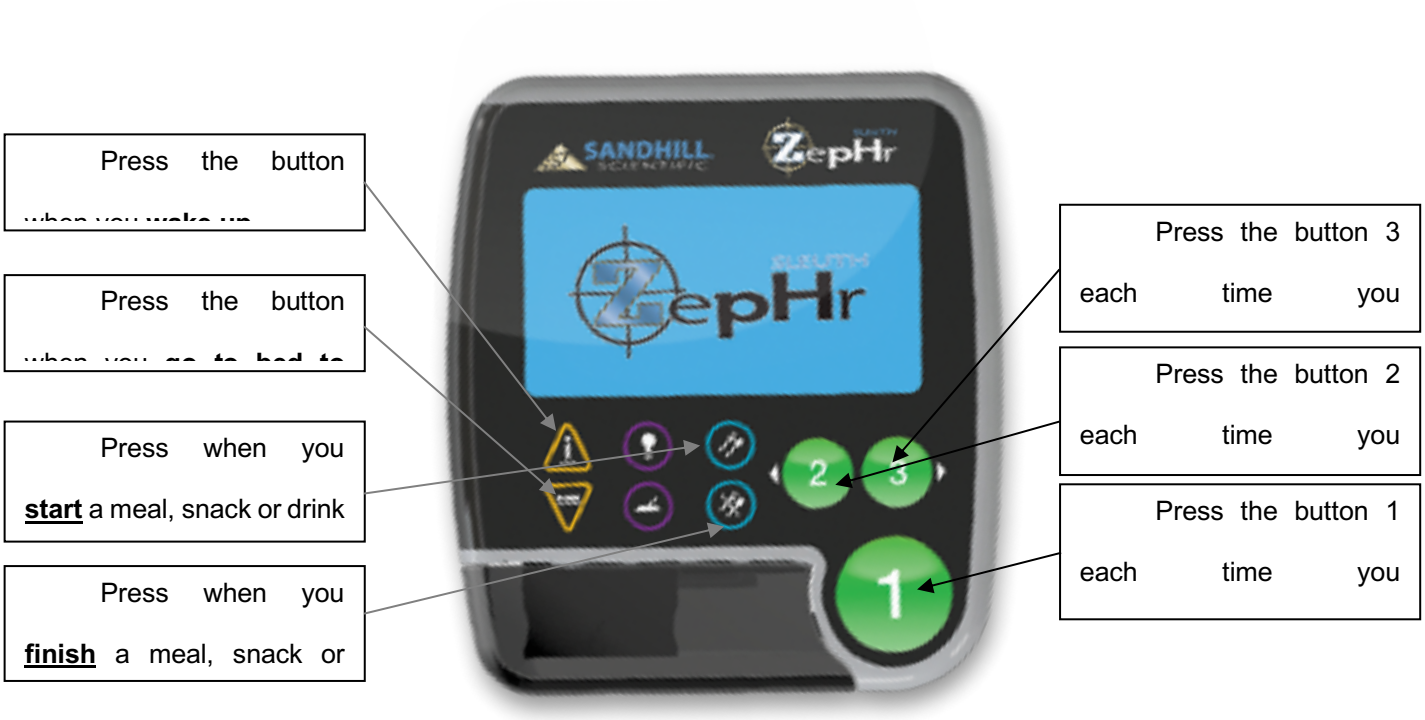
General recommendations:

1. We want to capture a usual day of your life. Be active and eat as normal as possible.
2. Keep the monitor on your body at all times. When going to bed, you can take it off your shoulder and put it under your pillow to avoid accidentally pulling the tube out. Never disconnect the tube from machine.
3. The recorder must not get wet. Do not take shower or a tub bath.
4. Try to have the meals you have normally.

5. Please report the start and finish time for use of alcoholic beverages. For other food and drinks, only pressing the meal buttons will be sufficient.
6. Even if you sip fizzy or flavored water, press meal buttons (still water is fine)
7. Do the activity or eat the food (in moderation) that will trigger your symptoms.
    - *The only restrictions are: No chewing gum, no hard candy*

Medications:

1. You may or may not be off your acid suppressant medications or antacids for this test. Do it as you are advised during your visit for the test.
2. If you are on other medications, take them as you normally would.



Date	Start time: hour/minute	End time: hour/minute	
`			

Diary: If you forget to press a button, please make a note here:

>> Use the time on the recorder.

Continue on a separate sheet if needed

References

1 The Concept of Prevention — An Introduction.

Ablal MA, Kaur JS, Cooper L, Jarad FD, Milosevic A, Higham SM, Preston AJ: The erosive potential of some alcopops using bovine enamel: an in vitro study. J Dent 2009;37:835-839.

Achem AC, Achem SR, Stark ME, DeVault KR: Failure of esophageal peristalsis in older patients: association with esophageal acid exposure. Am J Gastroenterol 2003;98:35-39.

Addy M, Hunter ML: Can tooth brushing damage your health? Effects on oral and dental tissues. Int Dent J 2003;53 Suppl 3:177-186.

Al-Hashimi I, Levine MJ: Characterization of in vivo salivary-derived enamel pellicle. Arch Oral Biol 1989;34:289-295.

Alaraudanjoki V, Laitala ML, Tjaderhane L, Pesonen P, Lussi A, Ronkainen J, Anttonen V: Influence of Intrinsic Factors on Erosive Tooth Wear in a Large-Scale Epidemiological Study. Caries Res 2016;50:508-516.

Alfaro EV, Aps JK, Martens LC: Oral implications in children with gastroesophageal reflux disease. Curr Opin Pediatr 2008;20:576-583.

Algarni AA, Mussi MC, Moffa EB, Lippert F, Zero DT, Siqueira WL, Hara AT: The impact of stannous, fluoride ions and its combination on enamel pellicle proteome and dental erosion prevention. PLoS One 2015;10:e0128196.

Alhabib KF, Vedal S, Champion P, Fitzgerald JM: The utility of ambulatory pH monitoring in patients presenting with chronic cough and asthma. Can J Gastroenterol 2007;21:159-163.

Alvarez Loureiro L, Fabruccini Fager A, Alves LS, Alvarez Vaz R, Maltz M: Erosive tooth wear among 12-year-old schoolchildren: a population-based cross-sectional study in Montevideo, Uruguay. Caries Res 2015;49:216-225.

Amaechi BT, Higham SM, Edgar WM, Milosevic A: Thickness of Acquired Salivary Pellicle as a Determinant of the Sites of Dental Erosion. Journal of Dental Research 1999a;78:1821-1828.

Amaechi BT, Higham SM, Edgar WM, Milosevic A: Thickness of acquired salivary pellicle as a determinant of the sites of dental erosion. J Dent Res 1999b;78:1821-1828.

Antunes LS, Veiga L, Nery VS, Nery CC, Antunes LA: Sports drink consumption and dental erosion among amateur runners. J Oral Sci 2017;59:639-643.

Ash A, Burnett GR, Parker R, Ridout MJ, Rigby NM, Wilde PJ: Structural characterisation of parotid and whole mouth salivary pellicles adsorbed onto DPI and QCMD hydroxyapatite sensors. Colloids Surf B Biointerfaces 2014;116:603-611.

Attin T: Methods for assessment of dental erosion. Monogr Oral Sci 2006;20:152-172.

Attin T, Becker K, Roos M, Attin R, Paque F: Impact of storage conditions on profilometry of eroded dental hard tissue. Clin Oral Invest 2009;13:473-478.

Attin T, Meyer K, Hellwig E, Buchalla W, Lennon AM: Effect of mineral supplements to citric acid on enamel erosion. Arch Oral Biol 2003;48:753-759.
Attin T, Wegehaupt FJ: Methods for assessment of dental erosion. Monogr Oral Sci 2014;25:123-142.
Attin T, Weiss K, Becker K, Buchalla W, Wiegand A: Impact of modified acidic soft drinks on enamel erosion. Oral Dis 2005;11:7-12.
Auad S, Moynihan P: Diet and dental erosion. Quintessence Int 2007;38:130-133.
Austin RS, Giusca CL, Macaulay G, Moazzez R, Bartlett DW: Confocal laser scanning microscopy and area-scale analysis used to quantify enamel surface textural changes from citric acid demineralization and salivary remineralization in vitro. Dent Mater 2016a;32:278-284.
Austin RS, Giusca CL, Macaulay G, Moazzez R, Bartlett DW: Confocal laser scanning microscopy and area-scale analysis used to quantify enamel surface textural changes from citric acid demineralization and salivary remineralization in vitro. Dent Mater 2016b;32:278-284.
Austin RS, Mullen F, Bartlett DW: Surface texture measurement for dental wear applications. Surface Topography: Metrology and Properties 2015;3:023002.
Austin RS, Stenhagen KS, Hove LH, Dunne S, Moazzez R, Bartlett DW, Tveit AB: A qualitative and quantitative investigation into the effect of fluoride formulations on enamel erosion and erosion-abrasion in vitro. Journal of Dentistry 2011;39:648-655.
Ayazi S, Lipham JC, Portale G, Peyre CG, Streets CG, Leers JM, Demeester SR, Banki F, Chan LS, Hagen JA, Demeester TR: Bravo catheter-free pH monitoring: normal values, concordance, optimal diagnostic thresholds, and accuracy. Clin Gastroenterol Hepatol 2009;7:60-67.
Bajaj D, Arola D: Role of prism decussation on fatigue crack growth and fracture of human enamel. Acta Biomater 2009;5:3045-3056.
Barbour ME, Lussi A, Shellis RP: Screening and prediction of erosive potential. Caries Res 2011;45 Suppl 1:24-32.
Barbour ME, Rees JS: The laboratory assessment of enamel erosion: a review. J Dent 2004;32:591-602.
Bardow A, Moe D, Nyvad B, Nauntofte B: The buffer capacity and buffer systems of human whole saliva measured without loss of CO2. Arch Oral Biol 2000;45:1-12.
Barham CP, Gotley DC, Miller R, Mills A, Alderson D: Pressure events surrounding oesophageal acid reflux episodes and acid clearance in ambulant healthy volunteers. Gut 1993;34:444-449.
Barriga-Rivera A, Elena M, Moya MJ, Lopez-Alonso M: The binomial symptom index: toward an optimal method for the evaluation of symptom association in gastroesophageal reflux. Neurogastroenterol Motil 2013;25:664-669.
Bartlett D, Dattani S, Mills I, Pitts N, Rattan R, Rochford D, Wilson NHF, Mehta S, O'Toole S: Monitoring erosive toothwear: BEWE, a simple tool to protect patients and the profession. Br Dent J 2019;226:930-932.
Bartlett D, Dugmore C: Pathological or physiological erosion--is there a relationship to age? Clin Oral Investig 2008;12 Suppl 1:S27-31.
Bartlett D, Ganss C, Lussi A: Basic Erosive Wear Examination (BEWE): a new scoring system for scientific and clinical needs. Clinical oral investigations 2008;12 Suppl 1:S65-68.
Bartlett D, O'Toole S: Tooth wear and aging. Aust Dent J 2019;64 Suppl 1:S59-S62.

Bartlett D, Phillips K, Smith B: A difference in perspective--the North American and European interpretations of tooth wear. Int J Prosthodont 1999;12:401-408.
Bartlett DW: Retrospective long term monitoring of tooth wear using study models. British Dental Journal 2003;194:211-213; discussion 204.
Bartlett DW, Coward PY: Comparison of the erosive potential of gastric juice and a carbonated drink in vitro. J Oral Rehabil 2001a;28:1045-1047.
Bartlett DW, Coward PY: Comparison of the erosive potential of gastric juice and a carbonated drink in vitro. Journal of Oral Rehabilitation 2001b;28:1045-1047.
Bartlett DW, Coward PY, Nikkah C, Wilson RF: The prevalence of tooth wear in a cluster sample of adolescent schoolchildren and its relationship with potential explanatory factors. Br Dent J 1998;184:125-129.
Bartlett DW, Fares J, Shirodaria S, Chiu K, Ahmad N, Sherriff M: The association of tooth wear, diet and dietary habits in adults aged 18-30 years old. J Dent 2011;39:811-816.
Bartlett DW, Lussi A, West NX, Bouchard P, Sanz M, Bourgeois D: Prevalence of tooth wear on buccal and lingual surfaces and possible risk factors in young European adults. Journal of Dentistry 2013;41:1007-1013.
Batista GR, Rocha Gomes Torres C, Sener B, Attin T, Wiegand A: Artificial Saliva Formulations versus Human Saliva Pretreatment in Dental Erosion Experiments. Caries Res 2016;50:78-86.
Baumann T, Kozik J, Lussi A, Carvalho TS: Erosion protection conferred by whole human saliva, dialysed saliva, and artificial saliva. Sci Rep 2016;6:34760.
Bennett JR: Heartburn and gastro-oesophageal reflux. Br J Clin Pract 1991;45:273-277.
Benz C, Weber J, Schlauch D, Riemann JF: Osophagusmotilitatsstorungen bei unklarem Thoraxschmerz. Med Klin;86:290-293.
Boeckxstaens G, El-Serag HB, Smout AJ, Kahrilas PJ: Symptomatic reflux disease: the present, the past and the future. Gut 2014;63:1185-1193.
Bolier EA, Kessing BF, Smout AJ, Bredenoord AJ: Systematic review: questionnaires for assessment of gastroesophageal reflux disease. Dis Esophagus 2015;28:105-120.
Braga SR, De Faria DL, De Oliveira E, Sobral MA: Morphological and mineral analysis of dental enamel after erosive challenge in gastric juice and orange juice. Microsc Res Tech 2011;74:1083-1087.
Bredenoord AJ: Mechanisms of reflux perception in gastroesophageal reflux disease: a review. American Journal of Gastroenterology 2012;107:8-15.
Bredenoord AJ, Fox M, Kahrilas PJ, Pandolfino JE, Schwizer W, Smout AJ, International High Resolution Manometry Working G: Chicago classification criteria of esophageal motility disorders defined in high resolution esophageal pressure topography. Neurogastroenterology & Motility 2012;24 Suppl 1:57-65.
Bredenoord AJ, Weusten BL, Curvers WL, Timmer R, Smout AJ: Determinants of perception of heartburn and regurgitation. Gut 2006;55:313-318.
Brevik SC, Lussi A, Rakhmatullina E: A new optical detection method to assess the erosion inhibition by in vitro salivary pellicle layer. J Dent 2013;41:428-435.
Broderick R, Fuchs KH, Breithaupt W, Varga G, Schulz T, Babic B, Lee A, Musial F, Horgan S: Clinical Presentation of Gastroesophageal Reflux Disease: A Prospective Study on Symptom Diversity and Modification of Questionnaire Application. Dig Dis 2019:1-8.
Buzalaf MA, Hannas AR, Kato MT: Saliva and dental erosion. J appl oral sci 2012a;20:493-502.

Buzalaf MA, Hannas AR, Kato MT: Saliva and dental erosion. J Appl Oral Sci 2012b;20:493-502.
Bytzer P, Jones R, Vakil N, Junghard O, Lind T, Wernersson B, Dent J: Limited ability of the proton-pump inhibitor test to identify patients with gastroesophageal reflux disease. Clin Gastroenterol Hepatol 2012;10:1360-1366.
Calabrese C, Marzano V, Urbani A, Lazzarini G, Valerii MC, Liguori G, Di Molfetta S, Rizzello F, Gionchetti P, Campieri M, Spisni E: Distinct proteomic profiles characterise non-erosive from erosive reflux disease. Aliment Pharmacol Ther 2011;34:982-993.
Calabrese C, Trere D, Liguori G, Gabusi V, Vici M, Cenacchi G, Derenzini M, Di Febo G: Esophageal cell proliferation in gastroesophageal reflux disease: clinical-morphological data before and after pantoprazole. World J Gastroenterol 2009;15:936-941.
Carlen A, Borjesson AC, Nikdel K, Olsson J: Composition of pellicles formed in vivo on tooth surfaces in different parts of the dentition, and in vitro on hydroxyapatite. Caries research 1998;32:447-455.
Carpenter G, Cotroneo E, Moazzez R, Rojas-Serrano M, Donaldson N, Austin R, Zaidel L, Bartlett D, Proctor G: Composition of enamel pellicle from dental erosion patients. Caries Res 2014;48:361-367.
Carpenter GH: The secretion, components, and properties of saliva. Annu Rev Food Sci Technol 2013;4:267-276.
Carvalho TS, Colon P, Ganss C, Huysmans MC, Lussi A, Schlueter N, Schmalz G, Shellis RP, Tveit AB, Wiegand A: Consensus report of the European Federation of Conservative Dentistry: erosive tooth wear-diagnosis and management. Clin Oral Invest 2015;19:1557-1561.
Cheaib Z, Lussi A: Impact of acquired enamel pellicle modification on initial dental erosion. Caries Res 2011;45:107-112.
Cheaib Z, Lussi A: Role of amylase, mucin, IgA and albumin on salivary protein buffering capacity: a pilot study. J Biosci 2013;38:259-265.
Cheng R, Yang H, Shao MY, Hu T, Zhou XD: Dental erosion and severe tooth decay related to soft drinks: a case report and literature review. J Zhejiang Univ Sci B 2009a;10:395-399.
Cheng ZJ, Wang XM, Cui FZ, Ge J, Yan JX: The enamel softening and loss during early erosion studied by AFM, SEM and nanoindentation. Biomed Mater 2009b;4.
Cheng ZJ, Wang XM, Cui FZ, Ge J, Yan JX: The enamel softening and loss during early erosion studied by AFM, SEM and nanoindentation. Biomed Mater 2009c;4:015020.
Chiocca JC, Olmos JA, Salis GB, Soifer LO, Higa R, Marcolongo M, Argentinean Gastro-Oesophageal Reflux Study G: Prevalence, clinical spectrum and atypical symptoms of gastro-oesophageal reflux in Argentina: a nationwide population-based study. Aliment Pharmacol Ther 2005;22:331-342.
Cho YS, Choi MG, Jeong JJ, Chung WC, Lee IS, Kim SW, Han SW, Choi KY, Chung IS: Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Asan-si, Korea. Am J Gastroenterol 2005;100:747-753.
Clouse RE, Prakash C, Haroian LR: Symptom association tests are improved by the extended ambulatory pH recording time with the bravo capsule. Gastroenterology 2003;124:A537.
Colas-Atger E, Bonaz B, Papillon E, Gueddah N, Rolachon A, Bost R, Fournet J: Relationship between acid reflux episodes and gastroesophageal reflux symptoms is very inconstant. Dig Dis Sci 2002;47:645-651.

Correa MC, Lerco MM, Cunha Mde L, Henry MA: Salivary parameters and teeth erosions in patients with gastroesophageal reflux disease. Arq Gastroenterol 2012;49:214-218.
Costantini M, Crookes PF, Bremner RM, Hoeft SF, Ehsan A, Peters JH, Bremner CG, DeMeester TR: Value of physiologic assessment of foregut symptoms in a surgical practice. Surgery 1993;114:780-786; discussion 786-787.
Cuy JL, Mann AB, Livi KJ, Teaford MF, Weihs TP: Nanoindentation mapping of the mechanical properties of human molar tooth enamel. Arch Oral Biol 2002;47:281-291.
Davies SJ, Gray RJM, Qualtrough AJE: Management of tooth surface loss. British dental journal 2002;192:11.
Dawes C: Physiological factors affecting salivary flow rate, oral sugar clearance, and the sensation of dry mouth in man. J Dent Res 1987;66 Spec No:648-653.
Dawes C, Pedersen AML, Villa A, Ekström J, Proctor GB, Vissink A, Aframian D, McGowan R, Aliko A, Narayana N, Sia YW, Joshi RK, Jensen SB, Kerr AR, Wolff A: The functions of human saliva: A review sponsored by the World Workshop on Oral Medicine VI. Archives of Oral Biology 2015;60:863-874.
Dent J, El-Serag HB, Wallander MA, Johansson S: Epidemiology of gastro-oesophageal reflux disease: a systematic review. Gut 2005;54:710-717.
Dent J, Vakil N, Jones R, Bytzer P, Schoning U, Halling K, Junghard O, Lind T: Accuracy of the diagnosis of GORD by questionnaire, physicians and a trial of proton pump inhibitor treatment: the Diamond Study. Gut 2010;59:714-721.
Derceli Jdos R, Faraoni JJ, Pereira-da-Silva MA, Palma-Dibb RG: Analysis of the Early Stages and Evolution of Dental Enamel Erosion. Braz Dent J 2016;27:313-317.
Desjardin M, Luc G, Collet D, Zerbib F: 24-hour pH-impedance monitoring on therapy to select patients with refractory reflux symptoms for antireflux surgery. A single center retrospective study. Neurogastroenterol Motil 2016;28:146-152.
Di Fede O, Di Liberto C, Occhipinti G, Vigneri S, Lo Russo L, Fedele S, Lo Muzio L, Campisi G: Oral manifestations in patients with gastro-oesophageal reflux disease: a single-center case-control study. J Oral Pathol Med 2008;37:336-340.
Diamant NE: Pathophysiology of gastroesophageal reflux disease. GI Motility online 2006.
Dixon B, Sharif MO, Ahmed F, Smith AB, Seymour D, Brunton PA: Evaluation of the basic erosive wear examination (BEWE) for use in general dental practice. Br Dent J 2012;213:E4.
Dodds WJ, Dent J, Hogan WJ, Helm JF, Hauser R, Patel GK, Egide MS: Mechanisms of gastroesophageal reflux in patients with reflux esophagitis. N Engl J Med 1982;307:1547-1552.
Douglas WH, Reeh ES, Ramasubbu N, Raj PA, Bhandary KK, Levine MJ: Statherin: a major boundary lubricant of human saliva. Biochem Biophys Res Commun 1991;180:91-97.
Eisenburger M, Addy M, Hughes JA, Shellis RP: Effect of time on the remineralisation of enamel by synthetic saliva after citric acid erosion. Caries Res 2001;35:211-215.
Eusebi LH, Ratnakumaran R, Yuan Y, Solaymani-Dodaran M, Bazzoli F, Ford AC: Global prevalence of, and risk factors for, gastro-oesophageal reflux symptoms: a meta-analysis. Gut 2018;67:430-440.
Everett CF, Morice AH: Clinical history in gastroesophageal cough. Respir Med 2007;101:345-348.

Faller RV, Eversole SL, Tzeghai GE: Enamel protection: a comparison of marketed dentifrice performance against dental erosion. Am J Dent 2011;24:205-210.
Fass R, Naliboff B, Higa L, Johnson C, Kodner A, Munakata J, Ngo J, Mayer EA: Differential effect of long-term esophageal acid exposure on mechanosensitivity and chemosensitivity in humans. Gastroenterology 1998;115:1363-1373.
Fass R, Navarro-Rodriguez T: Noncardiac chest pain. J Clin Gastroenterol 2008;42:636-646.
Featherstone JD, Behrman JM, Bell JE: Effect of whole saliva components on enamel demineralization in vitro. Crit Rev Oral Biol Med 1993;4:357-362.
Field JC, German MJ, Waterhouse PJ: Qualifying the lapped enamel surface: a profilometric, electron microscopic and microhardness study using human, bovine and ovine enamel. Arch Oral Biol 2014;59:455-460.
Field JC, Waterhouse PJ, German MJ: The Early Erosive and Abrasive Challenge: A Profilometric, Electron Microscopic and Microhardness Study Using Human, Bovine and Ovine Enamel. Eur J Prosthodont Restor Dent 2017;25:93-100.
Filipi K, Halackova Z, Filipi V: Oral health status, salivary factors and microbial analysis in patients with active gastro-oesophageal reflux disease. Int Dent J 2011;61:231-237.
Finke M, Jandt KD, Parker DM: The Early Stages of Native Enamel Dissolution Studied with Atomic Force Microscopy. J Colloid Interface Sci 2000;232:156-164.
Finke M, Parker DM, Jandt KD: Influence of soft drinks on the thickness and morphology of in situ acquired pellicle layer on enamel. J Colloid Interface Sci 2002;251:263-270.
Francis CA, Hector MP, Proctor GB: Precipitation of specific proteins by freeze-thawing of human saliva. Arch Oral Biol 2000;45:601-606.
Friesen LR, Bohaty B, Onikul R, Walker MP, Abraham C, Williams KB, Cocjin JT, Cocjin EL, Friesen CA: Is histologic esophagitis associated with dental erosion: a cross-sectional observational study? BMC Oral Health 2017;17:116.
Fujiwara Y, Higuchi K, Watanabe Y, Shiba M, Watanabe T, Tominaga K, Oshitani N, Matsumoto T, Nishikawa H, Arakawa T: Prevalence of gastroesophageal reflux disease and gastroesophageal reflux disease symptoms in Japan. J Gastroenterol Hepatol 2005;20:26-29.
Ganss C, Klimek J, Schwarz N: A comparative profilometric in vitro study of the susceptibility of polished and natural human enamel and dentine surfaces to erosive demineralization. Arch Oral Biol 2000;45:897-902.
Ganss C, Lussi A: Current erosion indices--flawed or valid? Clin Oral Investig 2008;12 Suppl 1:S1-3.
Ganss C, Lussi A: Diagnosis of erosive tooth wear. Monogr Oral Sci 2014;25:22-31.
Ganss C, Lussi A, Klimek J: Comparison of Calcium/Phosphorus Analysis, Longitudinal Microradiography and Profilometry for the Quantitative Assessment of Erosive Demineralisation. Caries Research 2005;39:178-184.
Ganss C, Lussi A, Scharmann I, Weigelt T, Hardt M, Klimek J, Schlueter N: Comparison of calcium analysis, longitudinal microradiography and profilometry for the quantitative assessment of erosion in dentine. Caries Res 2009;43:422-429.
Ganss C, Lussi A, Schlueter N: Dental erosion as oral disease. Insights in etiological factors and pathomechanisms, and current strategies for prevention and therapy. Am J Dent 2012;25:351-364.
Greabu M, Battino M, Mohora M, Totan A, Didilescu A, Spinu T, Totan C, Miricescu D, Radulescu R: Saliva--a diagnostic window to the body, both in health and in disease. J Med Life 2009;2:124-132.

Gregory-Head BL, Curtis DA, Kim L, Cello J: Evaluation of dental erosion in patients with gastroesophageal reflux disease. J Prosthet Dent 2000;83:675-680.
Grenby TH: Lessening dental erosive potential by product modification. European Journal of Oral Sciences 1996;104:221-228.
Grobler SR, Senekal PJ, Laubscher JA: In vitro demineralization of enamel by orange juice, apple juice, Pepsi Cola and Diet Pepsi Cola. Clin Prev Dent 1990;12:5-9.
Gudmundsson K, Kristleifsson G, Theodors A, Holbrook WP: Tooth erosion, gastroesophageal reflux, and salivary buffer capacity. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1995;79:185-189.
Gutschow CA, Leers JM, Schroder W, Prenzel KL, Fuchs H, Bollschweiler E, Bludau M, Holscher AH: Effect of aging on esophageal motility in patients with and without GERD. Ger Med Sci 2011;9:Doc22.
Gyawali CP, Fass R: Management of Gastroesophageal Reflux Disease. Gastroenterology 2018;154:302-318.
Gyawali CP, Kahrilas PJ, Savarino E, Zerbib F, Mion F, Smout A, Vaezi M, Sifrim D, Fox MR, Vela MF, Tutuian R, Tack J, Bredenoord AJ, Pandolfino J, Roman S: Modern diagnosis of GERD: the Lyon Consensus. Gut 2018;67:1351-1362.
Hahn Berg IC, Lindh L, Arnebrant T: Intraoral lubrication of PRP-1, statherin and mucin as studied by AFM. Biofouling 2004;20:65-70.
Hall AF, Sadler JP, Strang R, de Josselin de Jong E, Foye RH, Creanor SL: Application of transverse microradiography for measurement of mineral loss by acid erosion. Advances in Dental Research 1997;11:420-425.
Hannig C, Hannig M: The oral cavity--a key system to understand substratum-dependent bioadhesion on solid surfaces in man. Clin Oral Investig 2009;13:123-139.
Hannig M: Ultrastructural investigation of pellicle morphogenesis at two different intraoral sites during a 24-h period. Clin Oral Investig 1999a;3:88-95.
Hannig M: Ultrastructural investigation of pellicle morphogenesis at two different intraoral sites during a 24-h period. Clin Oral Invest 1999b;3:88-95.
Hannig M: The protective nature of the salivary pellicle. Int Dent J 2002;52:417-423.
Hannig M, Balz M: Influence of in vivo formed salivary pellicle on enamel erosion. Caries Res 1999;33:372-379.
Hannig M, Balz M: Protective properties of salivary pellicles from two different intraoral sites on enamel erosion. Caries Res 2001;35:142-148.
Hannig M, Fiebiger M, Guntzer M, Dobert A, Zimehl R, Nekrashevych Y: Protective effect of the in situ formed short-term salivary pellicle. Archives of Oral Biology 2004a;49:903-910.
Hannig M, Fiebiger M, Guntzer M, Dobert A, Zimehl R, Nekrashevych Y: Protective effect of the in situ formed short-term salivary pellicle. Archives of Oral Biology 2004b;49:903-910.
Hannig M, Fiebiger M, Güntzer M, Döbert A, Zimehl R, Nekrashevych Y: Protective effect of the in situ formed short-term salivary pellicle. Archives of Oral Biology 2004c;49:903-910.
Hannig M, Hannig C: The pellicle and erosion. Monographs in Oral Science 2014;25:206-214.
Hannig M, Hess NJ, Hoth-Hannig W, De Vrese M: Influence of salivary pellicle formation time on enamel demineralization--an in situ pilot study. Clin Oral Invest 2003a;7:158-161.
Hannig M, Hess NJ, Hoth-Hannig W, De Vrese M: Influence of salivary pellicle formation time on enamel demineralization--an in situ pilot study. Clin Oral Investig 2003b;7:158-161.

Hannig M, Joiner A: The structure, function and properties of the acquired pellicle. Monogr Oral Sci 2006a;19:29-64.
Hannig M, Joiner A: The structure, function and properties of the acquired pellicle. Monographs in Oral Science 2006b;19:29-64.
Hannig M, Khanafer AK, Hoth-Hannig W, Al-Marrawi F, Acil Y: Transmission electron microscopy comparison of methods for collecting in situ formed enamel pellicle. Clin Oral Investig 2005a;9:30-37.
Hannig M, Khanafer AK, Hoth-Hannig W, Al-Marrawi F, Acil Y: Transmission electron microscopy comparison of methods for collecting in situ formed enamel pellicle. Clin Oral Invest 2005b;9:30-37.
Hara AT, Ando M, Gonzalez-Cabezas C, Cury JA, Serra MC, Zero DT: Protective effect of the dental pellicle against erosive challenges in situ. J Dent Res 2006a;85:612-616.
Hara AT, González-Cabezas C, Creeth J, Zero DT: The effect of human saliva substitutes in an erosion-abrasion cycling model. Eur J Oral Sci 2008;116:552-556.
Hara AT, Lussi A, Zero DT: Biological factors. Monographs in Oral Science 2006b;20:88-99.
Hara AT, Zero DT: Analysis of the erosive potential of calcium-containing acidic beverages. Eur J Oral Sci 2008;116:60-65.
Hara AT, Zero DT: The potential of saliva in protecting against dental erosion. Monogr Oral Sci 2014;25:197-205.
Harvey NM, Carpenter GH, Proctor GB, Klein J: Normal and frictional interactions of purified human statherin adsorbed on molecularly-smooth solid substrata. Biofouling 2011;27:823-835.
Hellwig E, Lussi A, Goetz F: Influence of human saliva on the development of artificial erosions. Caries Res 2013;47:553-558.
Helm JF, Dodds WJ, Pelc LR, Palmer DW, Hogan WJ, Teeter BC: Effect of esophageal emptying and saliva on clearance of acid from the esophagus. N Engl J Med 1984;310:284-288.
Hemingway CA, Parker DM, Addy M, Barbour ME: Erosion of enamel by non-carbonated soft drinks with and without toothbrushing abrasion. Br Dent J 2006;201:447-450; discussion 439; quiz 466.
Hemingway CA, Shellis RP, Parker DM, Addy M, Barbour ME: Inhibition of hydroxyapatite dissolution by ovalbumin as a function of pH, calcium concentration, protein concentration and acid type. Caries Research 2008;42:348-353.
Hemingway CA, White AJ, Shellis RP, Addy M, Parker DM, Barbour ME: Enamel erosion in dietary acids: inhibition by food proteins in vitro. Caries Res 2010;44:525-530.
Hemmink GJ, Bredenoord AJ, Weusten BL, Monkelbaan JF, Timmer R, Smout AJ: Esophageal pH-impedance monitoring in patients with therapy-resistant reflux symptoms: 'on' or 'off' proton pump inhibitor? Am J Gastroenterol 2008;103:2446-2453.
Hershcovici T, Fass R: Pharmacological management of GERD: where does it stand now? Trends in Pharmacological Sciences 2011;32:258-264.
Heurich E, Beyer M, Jandt KD, Reichert J, Herold V, Schnabelrauch M, Sigusch BW: Quantification of dental erosion--a comparison of stylus profilometry and confocal laser scanning microscopy (CLSM). Dent Mater 2010;26:326-336.

Hjortsjo C, Jonski G, Young A, Saxegaard E: Effect of acidic fluoride treatments on early enamel erosion lesions-A comparison of calcium and profilometric analyses. Archives of Oral Biology 2010;55:229-234.
Holbrook WP, Arnadottir IB, Hloethversson SO, Arnarsdottir E, Jonsson SH, Saemundsson SR: The Basic Erosive Wear Examination (BEWE) applied retrospectively to two studies. Clin Oral Investig 2014;18:1625-1629.
Holbrook WP, Furuholm J, Gudmundsson K, Theodórs A, Meurman JH: Gastric reflux is a significant causative factor of tooth erosion. J Dent Res 2009;88:422-426.
Holloway RH: Questionnaires in gastroesophageal reflux disease: What do the answers mean? Journal of Gastroenterology and Hepatology 2009;24:1311-1312.
Holme B, Hove LH, Tveit AB: Using white light interferometry to measure etching of dental enamel. Measurement 2005;38:137-147.
Hove L, Holme B, Ogaard B, Willumsen T, Tveit AB: The protective effect of TiF4, SnF2 and NaF on erosion of enamel by hydrochloric acid in vitro measured by white light interferometry. Caries Res 2006;40:440-443.
Hove LH, Holme B, Young A, Tveit AB: The protective effect of TiF4, SnF2 and NaF against erosion-like lesions in situ. Caries Res 2008;42:68-72.
Hove LH, Young A, Tveit AB: An in vitro study on the effect of TiF(4) treatment against erosion by hydrochloric acid on pellicle-covered enamel. Caries Res 2007;41:80-84.
Howden GF: Erosion as the presenting symptom in hiatus hernia. A case report. British Dental Journal 1971;131:455-456.
Huew R, Waterhouse P, Moynihan P, Kometa S, Maguire A: Dental caries and its association with diet and dental erosion in Libyan schoolchildren. Int J Paediatr Dent 2012;22:68-76.
Hughes JA, West NX, Parker DM, van den Braak MH, Addy M: Effects of pH and concentration of citric, malic and lactic acids on enamel, in vitro. J Dent 2000;28:147-152.
Humphrey SP, Williamson RT: A review of saliva: normal composition, flow, and function. The Journal of prosthetic dentistry 2001;85:162-169.
Imfeld T: Dental erosion. Definition, classification and links. Eur J Oral Sci 1996;104:151-155.
Ing AJ, Ngu MC, Breslin AB: Pathogenesis of chronic persistent cough associated with gastroesophageal reflux. Am J Respir Crit Care Med 1994;149:160-167.
Ireland AJ, McGuinness N, Sherriff M: An investigation into the ability of soft drinks to adhere to enamel. Caries Research 1995;29:470-476.
Irwin RS: Chronic cough due to gastroesophageal reflux disease: ACCP evidence-based clinical practice guidelines. Chest 2006;129:80S-94S.
Irwin RS, Corrao WM, Pratter MR: Chronic persistent cough in the adult: the spectrum and frequency of causes and successful outcome of specific therapy. Am Rev Respir Dis 1981;123:413-417.
Iso M: Setsuwa to ongaku denshō, ed Shohan. Ōsaka-shi, Izumi Shoin, 2000.
Isolauri J, Laippala P: Prevalence of symptoms suggestive of gastro-oesophageal reflux disease in an adult population. Ann Med 1995;27:67-70.
Jaeggi T, Lussi A: Toothbrush abrasion of erosively altered enamel after intraoral exposure to saliva: an in situ study. Caries Res 1999;33:455-461.
Jaeggi T, Lussi A: Prevalence, incidence and distribution of erosion. Monogr Oral Sci 2014;25:55-73.

Jarvinen V, Meurman JH, Hyvarinen H, Rytomaa I, Murtomaa H: Dental erosion and upper gastrointestinal disorders. Oral Surg Oral Med Oral Pathol 1988;65:298-303.
Jarvinen VK, Rytomaa, II, Heinonen OP: Risk factors in dental erosion. J Dent Res 1991;70:942-947.
Javorkova N, Varechova S, Pecova R, Tatar M, Balaz D, Demeter M, Hyrdel R, Kollarik M: Acidification of the oesophagus acutely increases the cough sensitivity in patients with gastro-oesophageal reflux and chronic cough. Neurogastroenterol Motil 2008;20:119-124.
Jensdottir T, Arnadottir IB, Thorsdottir I, Bardow A, Gudmundsson K, Theodors A, Holbrook WP: Relationship between dental erosion, soft drink consumption, and gastroesophageal reflux among Icelanders. Clin Oral Invest 2004;8:91-96.
Jensdottir T, Bardow A, Holbrook P: Properties and modification of soft drinks in relation to their erosive potential in vitro. Journal of Dentistry 2005a;33:569-575.
Jensdottir T, Bardow A, Holbrook P: Properties and modification of soft drinks in relation to their erosive potential in vitro. J Dent 2005b;33:569-575.
Jensen JL, Lamkin MS, Oppenheim FG: Adsorption of human salivary proteins to hydroxyapatite: a comparison between whole saliva and glandular salivary secretions. J Dent Res 1992;71:1569-1576.
Johansson AK: On dental erosion and associated factors. Swed Dent J Suppl 2002:1-77.
Johnson LF, Demeester TR: Twenty-four-hour pH monitoring of the distal esophagus. A quantitative measure of gastroesophageal reflux. The American journal of gastroenterology 1974;62:325-332.
Johnson LF, DeMeester TR: Development of the 24-hour intraesophageal pH monitoring composite scoring system. J Clin Gastroenterol 1986;8 Suppl 1:52-58.
Jones MP, Sloan SS, Rabine JC, Ebert CC, Huang CF, Kahrilas PJ: Hiatal hernia size is the dominant determinant of esophagitis presence and severity in gastroesophageal reflux disease. American Journal of Gastroenterology 2001;96:1711-1717.
Jones R, Coyne K, Wiklund I: The gastro-oesophageal reflux disease impact scale: a patient management tool for primary care. Aliment Pharmacol Ther 2007;25:1451-1459.
Jones R, Junghard O, Dent J, Vakil N, Halling K, Wernersson B, Lind T: Development of the GerdQ, a tool for the diagnosis and management of gastro-oesophageal reflux disease in primary care. Aliment Pharmacol Ther 2009;30:1030-1038.
Jordao HWT, Coleman HG, Kunzmann AT, McKenna G: The association between erosive toothwear and gastro-oesophageal reflux-related symptoms and disease: A systematic review and meta-analysis. J Dent 2020:103284.
Joshi M, Joshi N, Kathariya R, Angadi P, Raikar S: Techniques to Evaluate Dental Erosion: A Systematic Review of Literature. J Clin Diagn Res 2016;10:ZE01-ZE07.
Kahrilas PJ, Bredenoord AJ, Fox M, Gyawali CP, Roman S, Smout AJ, Pandolfino JE: The Chicago Classification of esophageal motility disorders, v3.0. Neurogastroenterol Motil 2015a;27:160-174.
Kahrilas PJ, Bredenoord AJ, Fox M, Gyawali CP, Roman S, Smout AJPM, Pandolfino JE: The Chicago Classification of esophageal motility disorders, v3.0. Neurogastroenterology & Motility 2015b;27:160-174.
Kahrilas PJ, Quigley EM: Clinical esophageal pH recording: a technical review for practice guideline development. Gastroenterology 1996;110:1982-1996.

Kahrilas PJ, Sifrim D: High-resolution manometry and impedance-pH/manometry: valuable tools in clinical and investigational esophagology. Gastroenterology 2008;135:756-769.
Karlaftis A, Karamanolis G, Triantafyllou K, Polymeros D, Gaglia A, Triantafyllou M, Papanikolaou IS, Ladas SD: Clinical characteristics in patients with non-cardiac chest pain could favor gastroesophageal reflux disease diagnosis. Ann Gastroenterol 2013;26:314-318.
Kaufman E, Lamster IB: The diagnostic applications of saliva--a review. Crit Rev Oral Biol Med 2002;13:197-212.
Klimek J, Hellwig E, Ahrens G: Fluoride taken up by plaque, by the underlying enamel and by clean enamel from three fluoride compounds in vitro. Caries Res 1982;16:156-161.
Kosoric J, Williams R, Hector M, Anderson P: A Synthetic Peptide Based on a Natural Salivary Protein Reduces Demineralisation in Model Systems for Dental Caries and Erosion. International Journal of Peptide Research and Therapeutics 2007;13:497-503.
Kumar S, Keeling A, Osnes C, Bartlett D, O'Toole S: The sensitivity of digital intraoral scanners at measuring early erosive wear. Journal of Dentistry 2019;81:39-42.
Lamkin MS, Oppenheim FG: Structural features of salivary function. Critical Reviews in Oral Biology & Medicine 1993;4:251-259.
Leach RK: Fundamental principles of engineering nanometrology, ed Second edition. Amsterdam, Elsevier, William Andrew, 2014.
Lee YH, Zimmerman JN, Custodio W, Xiao Y, Basiri T, Hatibovic-Kofman S, Siqueira WL: Proteomic evaluation of acquired enamel pellicle during in vivo formation. PLoS ONE [Electronic Resource] 2013;8:e67919.
Leinonen J, Kivela J, Parkkila S, Parkkila AK, Rajaniemi H: Salivary carbonic anhydrase isoenzyme VI is located in the human enamel pellicle. Caries Res 1999a;33:185-190.
Leinonen J, Kivelä J, Parkkila S, Parkkila AK, Rajaniemi H: Salivary carbonic anhydrase isoenzyme VI is located in the human enamel pellicle. Caries Res 1999b;33:185-190.
Levy FM, Magalhaes AC, Gomes MF, Comar LP, Rios D, Buzalaf MA: The erosion and abrasion-inhibiting effect of TiF(4) and NaF varnishes and solutions on enamel in vitro. Int J Paediatr Dent 2012;22:11-16.
Li J, Helmerhorst EJ, Yao Y, Nunn ME, Troxler RF, Oppenheim FG: Statherin is an in vivo pellicle constituent: identification and immuno-quantification. Archives of oral biology 2004;49:379-385.
Li W, Liu J, Chen S, Wang Y, Zhang Z: Prevalence of dental erosion among people with gastroesophageal reflux disease in China. J Prosthet Dent 2017;117:48-54.
Li Y, Yu F, Niu L, Hu W, Long Y, Tay FR, Chen J: Associations among Bruxism, Gastroesophageal Reflux Disease, and Tooth Wear. J Clin Med 2018;7.
Lin M, Triadafilopoulos G: Belching: dyspepsia or gastroesophageal reflux disease? The American Journal of Gastroenterology 2003;98:2139-2145.
Lin WT, Kitasako Y, Nakashima S, Tagami J: A comparative study of the susceptibility of cut and uncut enamel to erosive demineralization. Dent Mater J 2017;36:48-53.
Lippert F, Parker DM, Jandt KD: In situ remineralisation of surface softened human enamel studied with AFM nanoindentation. Surface Science 2004a;553:105-114.

Lippert F, Parker DM, Jandt KD: In vitro demineralization/remineralization cycles at human tooth enamel surfaces investigated by AFM and nanoindentation. J Colloid Interface Sci 2004b;280:442-448.
Loffeld RJ: Incisor teeth status in patients with reflux oesophagitis. Digestion 1996;57:388-390.
Ludviksdottir D, Bjornsson E, Janson C, Boman G: Habitual coughing and its associations with asthma, anxiety, and gastroesophageal reflux. Chest 1996;109:1262-1268.
Lussi A: Dental erosion clinical diagnosis and case history taking. Eur J Oral Sci 1996;104:191-198.
Lussi A, Carvalho TS: Erosive tooth wear: a multifactorial condition of growing concern and increasing knowledge. Monogr Oral Sci 2014;25:1-15.
Lussi A, Jaeggi T: Chemical factors. Monogr Oral Sci 2006;20:77-87.
Lussi A, Jaeggi T, Zero D: The role of diet in the aetiology of dental erosion. Caries Research 2004;38 Suppl 1:34-44.
Lussi A, Kohler N, Zero D, Schaffner M, Megert B: A comparison of the erosive potential of different beverages in primary and permanent teeth using an in vitro model. Eur J Oral Sci 2000;108:110-114.
Lussi A, Megert B, Shellis RP, Wang X: Analysis of the erosive effect of different dietary substances and medications. Br J Nutr 2012a;107:252-262.
Lussi A, Megert B, Shellis RP, Wang XJ: Analysis of the erosive effect of different dietary substances and medications. Brit J Nutr 2012b;107:252-262.
Lussi A, Schaffner M: Progression of and risk factors for dental erosion and wedge-shaped defects over a 6-year period. Caries Res 2000;34:182-187.
Lussi A, Schlueter N, Rakhmatullina E, Ganss C: Dental erosion--an overview with emphasis on chemical and histopathological aspects. Caries Research 2011;45 Suppl 1:2-12.
Mann C, Ranjitkar S, Lekkas D, Hall C, Kaidonis JA, Townsend GC, Brook AH: Three-dimensional profilometric assessment of early enamel erosion simulating gastric regurgitation. Journal of Dentistry 2014;42:1411-1421.
Margaritis V, Mamai-Homata E, Koletsi-Kounari H, Polychronopoulou A: Evaluation of three different scoring systems for dental erosion: a comparative study in adolescents. J Dent 2011;39:88-93.
Marsh PD, Do T, Beighton D, Devine DA: Influence of saliva on the oral microbiota. Periodontology 2000 2016;70:80-92.
Martinez SD, Malagon IB, Garewal HS, Cui H, Fass R: Non-erosive reflux disease (NERD)--acid reflux and symptom patterns. Alimentary Pharmacology & Therapeutics 2003;17:537-545.
Martini T, Rios D, Cassiano LPS, Silva CMS, Taira EA, Ventura TMS, Pereira H, Magalhaes AC, Carvalho TS, Baumann T, Lussi A, Oliveira RB, Palma-Dibb RG, Buzalaf MAR: Proteomics of acquired pellicle in gastroesophageal reflux disease patients with or without erosive tooth wear. J Dent 2019;81:64-69.
Masson LF, McNeill G, Tomany JO, Simpson JA, Peace HS, Wei L, Grubb DA, Bolton-Smith C: Statistical approaches for assessing the relative validity of a food-frequency questionnaire: use of correlation coefficients and the kappa statistic. Public Health Nutr 2003;6:313-321.
Mayhall CW: Concerning the composition and source of the acquired enamel pellicle of human teeth. Arch Oral Biol 1970;15:1327-1341.

McColl E, Junghard O, Wiklund I, Revicki D: Assessing Symptoms in Gastroesophageal Reflux Disease: How Well Do Clinicians' Assessments Agree with Those of Their Patients? The American journal of gastroenterology 2005;100:11-18.
McNally PR, Maydonovitch CL, Prosek RA, Collette RP, Wong RK: Evaluation of gastroesophageal reflux as a cause of idiopathic hoarseness. Dig Dis Sci 1989;34:1900-1904.
Menezes MA, Herbella FAM: Pathophysiology of Gastroesophageal Reflux Disease. World J Surg 2017;41:1666-1671.
Meurman JH, Frank RM: Scanning electron microscopic study of the effect of salivary pellicle on enamel erosion. Caries Res 1991;25:1-6.
Meurman JH, ten Cate JM: Pathogenesis and modifying factors of dental erosion. European Journal of Oral Sciences 1996;104:199-206.
Michishige F, Kanno K, Yoshinaga S, Hinode D, Takehisa Y, Yasuoka S: Effect of saliva collection method on the concentration of protein components in saliva. J Med Invest 2006;53:140-146.
Milani DC, Venturini AP, Callegari-Jacques SM, Fornari F: Gastro-oesophageal reflux disease and dental erosions in adults: influence of acidified food intake and impact on quality of life. Eur J Gastroenterol Hepatol 2016;28:797-801.
Milosevic A: Sports drinks hazard to teeth. Br J Sports Med 1997;31:28-30.
Milosevic A, Brodie DA, Slade PD: Dental erosion, oral hygiene, and nutrition in eating disorders. Int J Eat Disord 1997;21:195-199.
Milosevic A, O'Sullivan E, Royal College of Surgeons of E: Diagnosis, prevention and management of dental erosion: summary of an updated national guideline. Prim Dent care 2008;15:11-12.
Mistry M, Zhu S, Moazzez R, Donaldson N, Bartlett DW: Effect of Model Variables on in vitro Erosion. Caries Res 2015a;49:508-514.
Mistry M, Zhu SS, Moazzez R, Donaldson N, Bartlett DW: Effect of Model Variables on in vitro Erosion. Caries Research 2015b;49:508-514.
Mita H, Kitasako Y, Takagaki T, Sadr A, Tagami J: Development and evaluation of a low-erosive apple juice drink with Phosphoryl-Oligosaccharides of Calcium. Dent Mater J 2013;32:212-218.
Moazzez R, Anggiansah A, Botha AJ, Bartlett D: Association of achalasia and dental erosion. Gut 2005;54:1665-1666.
Moazzez R, Bartlett D: Intrinsic causes of erosion. Monographs in Oral Science 2014;25:180-196.
Moazzez R, Bartlett D, Anggiansah A: Dental erosion, gastro-oesophageal reflux disease and saliva: how are they related? J Dent 2004a;32:489-494.
Moazzez R, Bartlett D, Anggiansah A: Dental erosion, gastro-oesophageal reflux disease and saliva: how are they related? Journal of Dentistry 2004b;32:489-494.
Moazzez RV, Austin RS, Rojas-Serrano M, Carpenter G, Cotroneo E, Proctor G, Zaidel L, Bartlett DW: Comparison of the Possible Protective Effect of the Salivary Pellicle of Individuals with and without Erosion. Caries Research 2014;48:57-62.
Mouli VP, Ahuja V: Questionnaire based gastroesophageal reflux disease (GERD) assessment scales. Indian J Gastroenterol 2011;30:108-117.
Mulic A, Tveit AB, Wang NJ, Hove LH, Espelid I, Skaare AB: Reliability of two clinical scoring systems for dental erosive wear. Caries Res 2010;44:294-299.
Mullan F, Austin RS, Parkinson CR, Bartlett DW: An in-situ pilot study to investigate the native clinical resistance of enamel to erosion. J Dent 2018a;70:124-128.

Mullan F, Bartlett D, Austin RS: Measurement uncertainty associated with chromatic confocal profilometry for 3D surface texture characterization of natural human enamel. Dent Mater 2017a;33:e273-e281.
Mullan F, Bartlett D, Austin RS: Measurement uncertainty associated with chromatic confocal profilometry for 3D surface texture characterization of natural human enamel. Dent Mater 2017b;33:e273-e281.
Mullan F, Mylonas P, Parkinson C, Bartlett D, Austin RS: Precision of 655nm Confocal Laser Profilometry for 3D surface texture characterisation of natural human enamel undergoing dietary acid mediated erosive wear. Dent Mater 2018b;34:531-537.
Munoz JV, Herreros B, Sanchiz V, Amoros C, Hernandez V, Pascual I, Mora F, Minguez M, Bagan JV, Benages A: Dental and periodontal lesions in patients with gastro-oesophageal reflux disease. Dig Liver Dis 2003;35:461-467.
Mutahar M, Carpenter G, Bartlett D, German M, Moazzez R: The presence of acquired enamel pellicle changes acid-induced erosion from dissolution to a softening process. Sci Rep 2017a;7:10920.
Mutahar M, Carpenter G, Bartlett D, German M, Moazzez R: The presence of acquired enamel pellicle changes acid-induced erosion from dissolution to a softening process. Sci Rep-Uk 2017b;7.
Mutahar M, O'Toole S, Carpenter G, Bartlett D, Andiappan M, Moazzez R: Reduced statherin in acquired enamel pellicle on eroded teeth compared to healthy teeth in the same subjects: An in-vivo study. PLoS One 2017c;12:e0183660.
Mylonas P, Austin RS, Moazzez R, Joiner A, Bartlett DW: In vitro evaluation of the early erosive lesion in polished and natural human enamel. Dental Materials 2018;34:1391-1400.
Nakata K, Nikaido T, Ikeda M, Foxton RM, Tagami J: Relationship between fluorescence loss of QLF and depth of demineralization in an enamel erosion model. Dent Mater J 2009;28:523-529.
Nasi A, Queiroz NSF, Michelsohn NH: Prolonged Gastroesophageal Reflux Monitoring by Impedance-Phmetry: A Review of the Subject Pondered with Our Experience with 1,200 Cases. Arq Gastroenterol 2018;55Suppl 1:76-84.
Navazesh M: Methods for collecting saliva. Ann N Y Acad Sci 1993;694:72-77.
Navazesh M, Christensen CM: A comparison of whole mouth resting and stimulated salivary measurement procedures. J Dent Res 1982;61:1158-1162.
Nekrashevych Y, Hannig M, Stosser L: Assessment of enamel erosion and protective effect of salivary pellicle by surface roughness analysis and scanning electron microscopy. Oral Health Prev Dent 2004;2:5-11.
Nekrashevych Y, Stosser L: Protective influence of experimentally formed salivary pellicle on enamel erosion. An in vitro study. Caries Research 2003a;37:225-231.
Nekrashevych Y, Stosser L: Protective influence of experimentally formed salivary pellicle on enamel erosion. An in vitro study. Caries Res 2003b;37:225-231.
Neto RML, Herbella FAM, Schlottmann F, Patti MG: Does DeMeester score still define GERD? Dis Esophagus 2019;32.
Newton JL, James OF, Williams GV, Allen A: The diurnal profile of gastric pepsin activity is reduced with Helicobacter pylori infection. Dig Dis Sci 2004;49:1103-1108.
Nieuw Amerongen AV, Oderkerk CH, Driessen AA: Role of mucins from human whole saliva in the protection of tooth enamel against demineralization in vitro. Caries Res 1987;21:297-309.

Nilsson M, Johnsen R, Ye W, Hveem K, Lagergren J: Prevalence of gastro-oesophageal reflux symptoms and the influence of age and sex. Scand J Gastroenterol 2004;39:1040-1045.
Nirwan JS, Hasan SS, Babar Z-U-D, Conway BR, Ghori MU: Global Prevalence and Risk Factors of Gastro-oesophageal Reflux Disease (GORD): Systematic Review with Meta-analysis. Sci Rep-Uk 2020;10:5814.
O'Toole S, Bartlett DW, Moazzez R: Efficacy of sodium and stannous fluoride mouthrinses when used before single and multiple erosive challenges. Aust Dent J 2016;61:497-501.
O'Toole S, Bernabe E, Moazzez R, Bartlett D: Timing of dietary acid intake and erosive tooth wear: A case-control study. J Dent 2017;56:99-104.
O'Toole S, Mistry M, Mutahar M, Moazzez R, Bartlett D: Sequence of stannous and sodium fluoride solutions to prevent enamel erosion. J Dent 2015a;43:1498-1503.
O'Toole S, Mistry M, Mutahar M, Moazzez R, Bartlett D: Sequence of stannous and sodium fluoride solutions to prevent enamel erosion. Journal of Dentistry 2015b;43:1498-1503.
Oginni A, Agbakwuru A, Ndububa D: The prevalence of dental erosion in Nigerian patients with gastro-oesophageal reflux disease. BMC oral health 2005a;5:1.
Oginni AO, Agbakwuru EA, Ndububa DA: The prevalence of dental erosion in Nigerian patients with gastro-oesophageal reflux disease. BMC Oral Health 2005b;5:1.
Okunseri C, Wong MC, Yau DT, McGrath C, Szabo A: The relationship between consumption of beverages and tooth wear among adults in the United States. J Public Health Dent 2015;75:274-281.
Olley RC, Wilson R, Bartlett D, Moazzez R: Validation of the Basic Erosive Wear Examination. Caries Res 2014;48:51-56.
Orr WC: Sleep issues in gastroesophageal reflux disease: beyond simple heartburn control. Rev Gastroenterol Disord 2003;3 Suppl 4:S22-29.
Orr WC: Therapeutic options in the treatment of nighttime gastroesophageal reflux. Digestion 2005;72:229-238.
Orr WC: Review article: sleep-related gastro-oesophageal reflux as a distinct clinical entity. Alimentary pharmacology & therapeutics 2010;31:47-56.
Orr WC, Johnson LF, Robinson MG: Effect of sleep on swallowing, esophageal peristalsis, and acid clearance. Gastroenterology 1984;86:814-819.
Osailan S, Pramanik R, Shirodaria S, Challacombe SJ, Proctor GB: Investigating the relationship between hyposalivation and mucosal wetness. Oral Dis 2011;17:109-114.
Ott DJ, McManus CM, Ledbetter MS, Chen MY, Gelfand DW: Heartburn correlated to 24-hour pH monitoring and radiographic examination of the esophagus. Am J Gastroenterol 1997;92:1827-1830.
Pace F, Pallotta S, Tonini M, Vakil N, Bianchi Porro G: Systematic review: gastro-oesophageal reflux disease and dental lesions. Aliment Pharmacol Ther 2008;27:1179-1186.
Paepegaey AM, Barker ML, Bartlett DW, Mistry M, West NX, Hellin N, Brown LJ, Bellamy PG: Measuring enamel erosion: A comparative study of contact profilometry, non-contact profilometry and confocal laser scanning microscopy. Dent Mater 2013;29:1265-1272.
Park S, Quinn JB, Romberg E, Arola D: On the brittleness of enamel and selected dental materials. Dent Mater 2008;24:1477-1485.

Passos VF, Melo MA, Vasconcellos AA, Rodrigues LK, Santiago SL: Comparison of methods for quantifying dental wear caused by erosion and abrasion. Microsc Res Tech 2013;76:178-183.
Patel A, Sayuk GS, Gyawali CP: Parameters on Esophageal pH-Impedance Monitoring That Predict Outcomes of Patients With Gastroesophageal Reflux Disease. Clinical Gastroenterology and Hepatology 2015;13:884-891.
Paterson W, Goyal RK, Habib FI: Esophageal motility disorders. GI Motility online 2006.
Pauwels A: Dental erosions and other extra-oesophageal symptoms of gastro-oesophageal reflux disease: Evidence, treatment response and areas of uncertainty. United European Gastroenterol J 2015;3:166-170.
Peres KG, Armenio MF, Peres MA, Traebert J, De Lacerda JT: Dental erosion in 12-year-old schoolchildren: a cross-sectional study in Southern Brazil. Int J Paediatr Dent 2005;15:249-255.
Peters Y, Al-Kaabi A, Shaheen NJ, Chak A, Blum A, Souza RF, Di Pietro M, Iyer PG, Pech O, Fitzgerald RC, Siersema PD: Barrett oesophagus. Nat Rev Dis Primers 2019;5:35.
Piangprach T, Hengtrakool C, Kukiattrakoon B, Kedjarune-Leggat U: The effect of salivary factors on dental erosion in various age groups and tooth surfaces. J Am Dent Assoc 2009;140:1137-1143.
Picos A, Lasserre JF, Chisnoiu AM, Berar AM, d'Incau E, Picos AM, Chira A, des Varannes SB, Dumitrascu DL: Factors associated with dental erosions in gastroesophageal reflux disease: a cross-sectional study in patients with heartburn. Med Pharm Rep 2020;93:23-29.
Pretty IA, Edgar WM, Higham SM: The erosive potential of commercially available mouthrinses on enamel as measured by Quantitative Light-induced Fluorescence (QLF). J Dent 2003;31:313-319.
Proctor GB: The physiology of salivary secretion. Periodontol 2000 2016;70:11-25.
Rakhmatullina E, Beyeler B, Lussi A: Inhibition of enamel erosion by stannous fluoride containing rinsing solutions. Schweiz Monatsschr Zahnmed 2013;123:296-302.
Ramachandran A, Raja Khan SI, Vaitheeswaran N: Incidence and Pattern of Dental Erosion in Gastroesophageal Reflux Disease Patients. J Pharm Bioallied Sci 2017;9:S138-S141.
Rantonen PJ, Meurman JH: Correlations between total protein, lysozyme, immunoglobulins, amylase, and albumin in stimulated whole saliva during daytime. Acta Odontologica Scandinavica 2000;58:160-165.
Rauber BF, Milani DC, Callegari-Jacques SM, Fornari L, Bonadeo NM, Fornari F: Predictors of dental erosions in patients evaluated with upper digestive endoscopy: a cross-sectional study. Odontology 2020.
Rayment SA, Liu B, Soares RV, Offner GD, Oppenheim FG, Troxler RF: The effects of duration and intensity of stimulation on total protein and mucin concentrations in resting and stimulated whole saliva. J Dent Res 2001;80:1584-1587.
Record Owner NLM: Excess gastroesophageal reflux in patients with hiatus hernia is caused by mechanisms other than transient LES relaxations.
Richter JE: Chest pain and gastroesophageal reflux disease. J Clin Gastroenterol 2000;30:S39-41.
Richter JE, Castell DO: The Esophagus, ed 5th. Chicester, John Wiley & Sons, 2011.
Rios D, Honorio HM, Magalhaes AC, Silva SM, Delbem AC, Machado MA, Buzalaf MA: Scanning electron microscopic study of the in situ effect of salivary stimulation on erosion and abrasion in human and bovine enamel. Braz Oral Res 2008;22:132-138.

Ritchie J, Lewis J: Qualitative research practice : a guide for social science students and researchers. London, SAGE, 2003.
Robinson C, Kirkham J, Brookes SJ, Shore RC: The role of albumin in developing rodent dental enamel: a possible explanation for white spot hypoplasia. J Dent Res 1992;71:1270-1274.
Rodriguez JM, Austin RS, Bartlett DW: In vivo measurements of tooth wear over 12 months. Caries Res 2012;46:9-15.
Rodriguez JM, Bartlett DW: A comparison of two-dimensional and three-dimensional measurements of wear in a laboratory investigation. Dent Mater 2010;26:e221-225.
Roesch-Ramos L, Roesch-Dietlen F, Remes-Troche JM, Romero-Sierra G, Mata-Tovar Cde J, Azamar-Jacome AA, Barranca-Enriquez A: Dental erosion, an extraesophageal manifestation of gastroesophageal reflux disease. The experience of a center for digestive physiology in Southeastern Mexico. Rev Esp Enferm Dig 2014;106:92-97.
Roman S, Gyawali CP, Savarino E, Yadlapati R, Zerbib F, Wu J, Vela M, Tutuian R, Tatum R, Sifrim D, Keller J, Fox M, Pandolfino JE, Bredenoord AJ: Ambulatory reflux monitoring for diagnosis of gastro-esophageal reflux disease: Update of the Porto consensus and recommendations from an international consensus group. Neurogastroenterol Motil 2017a;29:1-15.
Roman S, Gyawali CP, Savarino E, Yadlapati R, Zerbib F, Wu J, Vela M, Tutuian R, Tatum R, Sifrim D, Keller J, Fox M, Pandolfino JE, Bredenoord AJ, group Gc: Ambulatory reflux monitoring for diagnosis of gastro-esophageal reflux disease: Update of the Porto consensus and recommendations from an international consensus group. Neurogastroenterol Motil 2017b;29:1-15.
Ronkainen J, Aro P, Storskrubb T, Johansson SE, Lind T, Bolling-Sternevald E, Vieth M, Stolte M, Talley NJ, Agreus L: Prevalence of Barrett's esophagus in the general population: an endoscopic study. Gastroenterology 2005;129:1825-1831.
Ruff JC, Koch MO, Perkins S: Bulimia: dentomedical complications. Gen Dent 1992;40:22-25.
Rydén A, Denison H, Karlsson M, Vakil N: Development and validation of a patient-reported outcome instrument in partial responders to proton pump inhibitors. Scand J Gastroenterol 2013;48:1018-1026.
Saritas Yuksel E, Vaezi MF: Extraesophageal manifestations of gastroesophageal reflux disease: cough, asthma, laryngitis, chest pain. Swiss Med Wkly 2012;142:w13544.
Savarino E, Bazzica M, Zentilin P, Pohl D, Parodi A, Cittadini G, Negrini S, Indiveri F, Tutuian R, Savarino V, Ghio M: Gastroesophageal reflux and pulmonary fibrosis in scleroderma: a study using pH-impedance monitoring. Am J Respir Crit Care Med 2009;179:408-413.
Schipper RG, Silletti E, Vingerhoeds MH: Saliva as research material: Biochemical, physicochemical and practical aspects. Archives of Oral Biology 2007a;52:1114-1135.
Schipper RG, Silletti E, Vingerhoeds MH: Saliva as research material: biochemical, physicochemical and practical aspects. Archives of oral biology 2007b;52:1114-1135.
Schlueter N, Amaechi BT, Bartlett D, Buzalaf MAR, Carvalho TS, Ganss C, Hara AT, Huysmans M, Lussi A, Moazzez R, Vieira AR, West NX, Wiegand A, Young A, Lippert F: Terminology of Erosive Tooth Wear: Consensus Report of a Workshop

Organized by the ORCA and the Cariology Research Group of the IADR. Caries Res 2020;54:2-6.
Schlueter N, Ganss C, Hardt M, Schegietz D, Klimek J: Effect of pepsin on erosive tissue loss and the efficacy of fluoridation measures in dentine in vitro. Acta Odontol Scand 2007;65:298-305.
Schlueter N, Glatzki J, Klimek J, Ganss C: Erosive-abrasive tissue loss in dentine under simulated bulimic conditions. Arch Oral Biol 2012;57:1176-1182.
Schlueter N, Hara A, Shellis RP, Ganss C: Methods for the measurement and characterization of erosion in enamel and dentine. Caries Res 2011a;45 Suppl 1:13-23.
Schlueter N, Hara A, Shellis RP, Ganss C: Methods for the measurement and characterization of erosion in enamel and dentine. Caries Research 2011b;45 Suppl 1:13-23.
Schlueter N, Hardt M, Klimek J, Ganss C: Influence of the digestive enzymes trypsin and pepsin in vitro on the progression of erosion in dentine. Archives of Oral Biology 2010;55:294-299.
Schlueter N, Klimek J, Ganss C: Effect of a chitosan additive to a Sn2+-containing toothpaste on its anti-erosive/anti-abrasive efficacy--a controlled randomised in situ trial. Clin Oral Investig 2014;18:107-115.
Schlueter N, Luka B: Erosive tooth wear - a review on global prevalence and on its prevalence in risk groups. Br Dent J 2018;224:364-370.
Schroeder PL, Filler SJ, Ramirez B, Lazarchik DA, Vaezi MF, Richter JE: Dental erosion and acid reflux disease. Ann Intern Med 1995;122:809-815.
Schweigel H, Wicht M, Schwendicke F: Salivary and pellicle proteome: A datamining analysis. Sci Rep 2016;6:38882.
Shaker R: Protective mechanisms against supraesophageal GERD. J Clin Gastroenterol 2000;30:S3-8.
Shaw MJ, Talley NJ, Beebe TJ, Rockwood T, Carlsson R, Adlis S, Fendrick AM, Jones R, Dent J, Bytzer P: Initial validation of a diagnostic questionnaire for gastroesophageal reflux disease. Am J Gastroenterol 2001;96:52-57.
Shelat D: Book review: Understanding Dental Caries: From Pathogenesis to Prevention and Therapy. British Dental Journal 2017;222:912-912.
Shellis RP, Barbour ME, Jesani A, Lussi A: Effects of buffering properties and undissociated acid concentration on dissolution of dental enamel in relation to pH and acid type. Caries Res 2013;47:601-611.
Shellis RP, Featherstone JD, Lussi A: Understanding the chemistry of dental erosion. Monogr Oral Sci 2014;25:163-179.
Shellis RP, Finke M, Eisenburger M, Parker DM, Addy M: Relationship between enamel erosion and liquid flow rate. Eur J Oral Sci 2005;113:232-238.
Shellis RP, Ganss C, Ren Y, Zero DT, Lussi A: Methodology and models in erosion research: discussion and conclusions. Caries Res 2011;45 Suppl 1:69-77.
Sieber KR, Schmidt C, Baumann T, Lussi A, Carvalho TS: Acquired Enamel Pellicle Modification with Casein and Mucin in Different Concentrations and its Impact on Initial Dental Erosion. Caries Res 2019;53:457-466.
Sifrim D: Esophageal impedance monitoring and pathophysiology of gastroesophageal reflux disease. Verh K Acad Geneeskd Belg 2006;68:151-178.
Sifrim D, Dupont L, Blondeau K, Zhang X, Tack J, Janssens J: Weakly acidic reflux in patients with chronic unexplained cough during 24 hour pressure, pH, and impedance monitoring. Gut 2005;54:449-454.

Sifrim D, Holloway R, Silny J, Xin Z, Tack J, Lerut A, Janssens J: Acid, nonacid, and gas reflux in patients with gastroesophageal reflux disease during ambulatory 24-hour pH-impedance recordings. Gastroenterology 2001;120:1588-1598.
Sifrim D, Zerbib F: Diagnosis and management of patients with reflux symptoms refractory to proton pump inhibitors. Gut 2012;61:1340-1354.
Silman AJ, Macfarlane GJ: Epidemiological studies : a practical guide, ed 2nd ed. Cambridge, Cambridge University Press, 2002.
Silva MA, Damante JH, Stipp AC, Tolentino MM, Carlotto PR, Fleury RN: Gastroesophageal reflux disease: New oral findings. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001;91:301-310.
Singh S, Richter JE, Bradley LA, Haile JM: The symptom index. Differential usefulness in suspected acid-related complaints of heartburn and chest pain. Dig Dis Sci 1993;38:1402-1408.
Siqueira WL, Custodio W, McDonald EE: New Insights into the Composition and Functions of the Acquired Enamel Pellicle. Journal of Dental Research 2012;91:1110-1118.
Siqueira WL, Helmerhorst EJ, Zhang W, Salih E, Oppenheim FG: Acquired enamel pellicle and its potential role in oral diagnostics. Ann N Y Acad Sci 2007a;1098:504-509.
Siqueira WL, Helmerhorst EJ, Zhang W, Salih E, Oppenheim FG: Acquired enamel pellicle and its potential role in oral diagnostics. Ann N Y Acad Sci 2007b;1098:504-509.
Siqueira WL, Margolis HC, Helmerhorst EJ, Mendes FM, Oppenheim FG: Evidence of Intact Histatins in the in vivo Acquired Enamel Pellicle. Journal of Dental Research 2010;89:626-630.
Siqueira WL, Zhang W, Helmerhorst EJ, Gygi SP, Oppenheim FG: Identification of Protein Components in in vivo Human Acquired Enamel Pellicle Using LC−ESI−MS/MS. J Proteome Res 2007c;6:2152-2160.
Siqueira WL, Zhang W, Helmerhorst EJ, Gygi SP, Oppenheim FG: Identification of protein components in in vivo human acquired enamel pellicle using LC-ESI-MS/MS. J Proteome Res 2007d;6:2152-2160.
Skoczylas T, Yandrapu H, Poplawski C, Asadi M, Wallner G, Sarosiek J: Salivary bicarbonate as a major factor in the prevention of upper esophageal mucosal injury in gastroesophageal reflux disease. Dig Dis Sci 2014;59:2411-2416.
Smith BG, Knight JK: An index for measuring the wear of teeth. Br Dent J 1984;156:435-438.
Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC: Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150:76-85.
Sonju Clasen AB, Hannig M, Skjorland K, Sonju T: Analytical and ultrastructural studies of pellicle on primary teeth. Acta Odontol Scand 1997;55:339-343.
Stenhagen KR, Hove LH, Holme B, Taxt-Lamolle S, Tveit AB: Comparing different methods to assess erosive lesion depths and progression in vitro. Caries Res 2010;44:555-561.
Stenhagen KR, Hove LH, Holme B, Tveit AB: Enamel erosion depths measured on impressions by a white light interferometer. Acta Odontol Scand 2013;71:398-403.
Stoltey J, Reeba H, Ullah N, Sabhaie P, Gerson L: Does Barrett's oesophagus develop over time in patients with chronic gastro-oesophageal reflux disease? Alimentary Pharmacology & Therapeutics 2007;25:83-91.

Svendsen IE, Arnebrant T, Lindh L: Validation of mechanically-assisted sodium dodecyl-sulphate elution as a technique to remove pellicle protein components from human enamel. Biofouling 2008;24:227-233.
Tabak LA: Structure and function of human salivary mucins. Critical Reviews in Oral Biology & Medicine 1990;1:229-234.
Taira EA, Ventura TMS, Cassiano LPS, Silva CMS, Martini T, Leite AL, Rios D, Magalhaes AC, Buzalaf MAR: Changes in the Proteomic Profile of Acquired Enamel Pellicles as a Function of Their Time of Formation and Hydrochloric Acid Exposure. Caries Res 2018;52:367-377.
Tefera L, Fein M, Ritter MP, Bremner CG, Crookes PF, Peters JH, Hagen JA, DeMeester TR: Can the combination of symptoms and endoscopy confirm the presence of gastroesophageal reflux disease? Am Surg 1997;63:933-936.
Teixeira L, Manso MC, Manarte-Monteiro P: Erosive tooth wear status of institutionalized alcoholic patients under rehabilitation therapy in the north of Portugal. Clin Oral Investig 2017;21:809-819.
ten Bruggen Cate HJ: Dental erosion in industry. Br J Ind Med 1968;25:249-266.
Tokayer AZ: Gastroesophageal reflux disease and chronic cough. Lung 2008;186 Suppl 1:S29-34.
Tonami K, Ericson D: Protein profile of pepsin-digested carious and sound human dentine. Acta Odontol Scand 2005;63:17-20.
Triadafilopoulos G, Tandon A, Shetler KP, Clarke J: Clinical and pH study characteristics in reflux patients with and without ineffective oesophageal motility (IEM). BMJ Open Gastroenterol 2016;3:e000126.
Tschoppe P, Neumann K, Mueller J, Kielbassa AM: Effect of fluoridated bleaching gels on the remineralization of predemineralized bovine enamel in vitro. J Dent 2009;37:156-162.
Tucker K, Adams M, Shaw L, Smith AJ: Human enamel as a substrate for in vitro acid dissolution studies: influence of tooth surface and morphology. Caries Res 1998;32:135-140.
Turner RJ, Sugiya H: Understanding salivary fluid and protein secretion. Oral Dis 2002;8:3-11.
Tuttle SG, Rufin F, Bettarello A: The physiology of heartburn. Ann Intern Med 1961;55:292-300.
Vaezi MF: Use of symptom indices in the management of GERD. Gastroenterol Hepatol (N Y) 2012;8:185-187.
Vaezi MF, Hicks DM, Abelson TI, Richter JE: Laryngeal signs and symptoms and gastroesophageal reflux disease (GERD): a critical assessment of cause and effect association. Clin Gastroenterol Hepatol 2003;1:333-344.
Vakil N: Epigastric pain in dyspepsia and reflux disease. Rev Gastroenterol Disord 2003;3 Suppl 4:S16-21.
Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R: The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. Am J Gastroenterol 2006a;101:1900-1920; quiz 1943.
Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R, Global Consensus G: The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. Am J Gastroenterol 2006b;101:1900-1920; quiz 1943.
Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R, Globale K: [The Montreal definition and classification of gastroesophageal reflux disease: a global, evidence-based consensus paper]. Z Gastroenterol 2007a;45:1125-1140.

Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R, Konsensusgruppe DG: The Montreal definition and classification of gastroesophageal reflux disease: A global, evidence-based consensus paper. Z Gastroenterol 2007b;45:1125-1140.
van 't Spijker A, Kreulen CM, Creugers NH: Attrition, occlusion, (dys)function, and intervention: a systematic review. Clin Oral Implants Res 2007;18 Suppl 3:117-126.
van ‘t Hof W, Veerman ECI, Nieuw Amerongen AV, Ligtenberg AJM: Antimicrobial Defense Systems in Saliva.
Van't Spijker A, Rodriguez JM, Kreulen CM, Bronkhorst EM, Bartlett DW, Creugers NH: Prevalence of tooth wear in adults. International Journal of Prosthodontics 2009;22:35-42.
Vela MF, Camacho-Lobato L, Srinivasan R, Tutuian R, Katz PO, Castell DO: Simultaneous intraesophageal impedance and pH measurement of acid and nonacid gastroesophageal reflux: effect of omeprazole. Gastroenterology 2001;120:1599-1606.
Ventura T, Cassiano LPS, Souza ESCM, Taira EA, Leite AL, Rios D, Buzalaf MAR: The proteomic profile of the acquired enamel pellicle according to its location in the dental arches. Arch Oral Biol 2017;79:20-29.
Vukosavljevic D, Custodio W, Buzalaf MA, Hara AT, Siqueira WL: Acquired pellicle as a modulator for dental erosion. Archives of Oral Biology 2014;59:631-638.
Walsh NP, Montague JC, Callow N, Rowlands AV: Saliva flow rate, total protein concentration and osmolality as potential markers of whole body hydration status during progressive acute dehydration in humans. Arch Oral Biol 2004;49:149-154.
Wan Nik WN, Banerjee A, Moazzez R: Gastro-oesophageal reflux disease symptoms and tooth wear in patients with Sjogren's syndrome. Caries Res 2011;45:323-326.
Wang JH, Luo JY, Dong L, Gong J, Zuo AL: Composite score of reflux symptoms in diagnosis of gastroesophageal reflux disease. World J Gastroenterol 2004;10:3332-3335.
Wang X, Lussi A: Functional foods/ingredients on dental erosion. Eur J Nutr 2012;51 Suppl 2:S39-48.
Ward BW, Wu WC, Richter JE, Lui KW, Castell DO: Ambulatory 24-hour esophageal pH monitoring. Technology searching for a clinical application. J Clin Gastroenterol 1986;8 Suppl 1:59-67.
Watanabe S, Dawes C: Salivary flow rates and salivary film thickness in five-year-old children. J Dent Res 1990;69:1150-1153.
Wei Z, Du Y, Zhang J, Tai B, Du M, Jiang H: Prevalence and Indicators of Tooth Wear among Chinese Adults. PLoS One 2016;11:e0162181.
West NX, Hughes JA, Addy M: Erosion of dentine and enamel in vitro by dietary acids: the effect of temperature, acid character, concentration and exposure time. J Oral Rehabil 2000;27:875-880.
West NX, Hughes JA, Addy M: The effect of pH on the erosion of dentine and enamel by dietary acids in vitro. Journal of oral rehabilitation 2001;28:860-864.
West NX, Lussi A, Seong J, Hellwig E: Dentin hypersensitivity: pain mechanisms and aetiology of exposed cervical dentin. Clin Oral Invest 2013a;17 Suppl 1:S9-19.
West NX, Sanz M, Lussi A, Bartlett D, Bouchard P, Bourgeois D: Prevalence of dentine hypersensitivity and study of associated factors: a European population-based cross-sectional study. J Dent 2013b;41:841-851.
Wetselaar P, Faris A, Lobbezoo F: A plea for the development of an universally accepted modular tooth wear evaluation system. BMC Oral Health 2016;16:115.

Wetselaar P, Lobbezoo F: The tooth wear evaluation system: a modular clinical guideline for the diagnosis and management planning of worn dentitions. Journal of Oral Rehabilitation 2016;43:69-80.
Wetton S, Hughes J, Newcombe RG, Addy M: The effect of saliva derived from different individuals on the erosion of enamel and dentine. A study in vitro. Caries Res 2007;41:423-426.
Wetton S, Hughes J, West N, Addy M: Exposure time of enamel and dentine to saliva for protection against erosion: a study in vitro. Caries research 2006;40:213-217.
White AJ, Yorath C, ten Hengel V, Leary SD, Huysmans MC, Barbour ME: Human and bovine enamel erosion under 'single-drink' conditions. Eur J Oral Sci 2010;118:604-609.
White DA, Tsakos G, Pitts NB, Fuller E, Douglas GV, Murray JJ, Steele JG: Adult Dental Health Survey 2009: common oral health conditions and their impact on the population. Br Dent J 2012;213:567-572.
White SN, Luo W, Paine ML, Fong H, Sarikaya M, Snead ML: Biological organization of hydroxyapatite crystallites into a fibrous continuum toughens and controls anisotropy in human enamel. J Dent Res 2001;80:321-326.
Wiener GJ, Koufman JA, Wu WC, Cooper JB, Richter JE, Castell DO: Chronic hoarseness secondary to gastroesophageal reflux disease: documentation with 24-h ambulatory pH monitoring. Am J Gastroenterol 1989;84:1503-1508.
Wiener GJ, Morgan TM, Copper JB, Wu WC, Castell DO, Sinclair JW, Richter JE: Ambulatory 24-hour esophageal pH monitoring. Reproducibility and variability of pH parameters. Dig Dis Sci 1988;33:1127-1133.
Wiklund I, Carlsson J, Vakil N: Gastroesophageal reflux symptoms and well-being in a random sample of the general population of a Swedish community. Am J Gastroenterol 2006;101:18-28.
Wilder-Smith CH, Wilder-Smith P, Kawakami-Wong H, Voronets J, Osann K, Lussi A: Quantification of dental erosions in patients with GERD using optical coherence tomography before and after double-blind, randomized treatment with esomeprazole or placebo. Am J Gastroenterol 2009;104:2788-2795.
Wolcott RB, Yager J, Gordon G: Dental sequelae to the binge-purge syndrome (bulimia): report of cases. J Am Dent Assoc 1984;109:723-725.
Woltgens JH, Vingerling P, de Blieck-Hogervorst JM, Bervoets DJ: Enamel erosion and saliva. Clin Prev Dent 1985;7:8-10.
Yacavone RF, Locke GR, 3rd, Provenzale DT, Eisen GM: Quality of life measurement in gastroenterology: what is available? Am J Gastroenterol 2001;96:285-297.
Yao Y, Berg EA, Costello CE, Troxler RF, Oppenheim FG: Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. J Biol Chem 2003;278:5300-5308.
Yao Y, Grogan J, Zehnder M, Lendenmann U, Nam B, Wu Z, Costello CE, Oppenheim FG: Compositional analysis of human acquired enamel pellicle by mass spectrometry. Archives of oral biology 2001;46:293-303.
Yoshikawa H, Furuta K, Ueno M, Egawa M, Yoshino A, Kondo S, Nariai Y, Ishibashi H, Kinoshita Y, Sekine J: Oral symptoms including dental erosion in gastroesophageal reflux disease are associated with decreased salivary flow volume and swallowing function. J Gastroenterol 2012;47:412-420.
Young A, Tenuta LM: Initial erosion models. Caries Res 2011;45 Suppl 1:33-42.
Young WG, Khan F: Sites of dental erosion are saliva-dependent. Journal of Oral Rehabilitation 2002;29:35-43.

Zheng J, Xiao F, Zheng L, Qian LM, Zhou ZR: Erosion behaviors of human tooth enamel at different depth. Tribology International 2010;43:1262-1267.