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1 Introduction

Erosive tooth wear is a common oral condition which if not treated can compromise the longevity of teeth and a person's quality of life [1]. Understanding what happens in the early stages of this condition, particularly to the integrity of the enamel surface, should enable a better understanding of prevention [2]. The time acidic foods or drinks are exposed in the mouth during eating and or drinking is not precisely known, due to individual variation, but it's likely to be only for a few seconds. But how long it takes for acidic challenge to change the enamel surface is unknown. It is apparent that frequency of consumption of acidic foods and drinks creates a greater risk of developing erosion but they are cleared quickly from the mouth [3]. Current understanding of erosive tooth wear suggests that following a short duration (less than 5 minutes) acid exposure the enamel surface may soften to a depth of between 0.2 μm and 5 μm [4,5]. This softening is believed to reflect partial loss of surface minerals leading to increasing surface roughness and decreased surface microhardness [5–8]. But these times are derived from laboratory investigations which are partly influenced by the model, presence of artificial or natural saliva, the biofilm, but also by the sensitivity of the measuring technique. Clinically, we appreciate that irreversible loss of tooth structure occurs if acid exposure is prolonged, and increases if further attritive or abrasive factors are introduced [4,9–12]. The definition of what is 'short duration' acid-erosion, in the literature, has not been specified to one particular time period or to the number of acid erosion cycles used, but rather by the duration of acid exposure utilised; typically lasting for several seconds or minutes, and which do not exceed 5 minutes duration [4,11,13–15].

The physical properties of the early erosive lesion are ill-defined due to the difficulty in detecting quantifiable changes to the enamel structure within short acid erosion time periods [16]. This is primarily a consequence of the sensitivity of most methods to measure any change. Studies have evaluated the formation of early erosive lesions with a range of acid immersion periods and/or number of immersion cycles and varies from 5 seconds [17] up to 2 hours [7] for 1 acid cycle

[6,7,11,18–24] to greater than 5 cycles of acid erosion with varying immersion times [17,23,25–27]. Most early enamel erosion studies have been conducted on polished enamel surfaces to ensure consistency of sample preparation, and allow reproducible and accurate measurement of effects occurring from acid exposure [16,18,28,29]. Laboratory simulation of the early erosive lesion on natural, unpolished enamel has been challenging due to its topography and morphology [30].

Previous erosion studies have utilised surface profilometry, to measure step height loss [17,22,27,31] and surface microhardness to determine surface and sub surface softening [6,18,27,29]. Moreover, the equipment and acquisition parameters utilised, varied between studies, and thus it is difficult to generalise the overall detection capabilities of specific measurement techniques. However, these techniques have produced varying degrees of success when detecting the earliest changes following acid erosion. To complicate matters further different measuring systems produce different data, although it appears that the comparative changes are consistent between them [31,32]. A recent study concluded that chromatic non-contacting optical profilometry had measurement uncertainty of 0.49 μm [33] suggesting enamel loss from early enamel erosion may not be reliably detected below 0.4 microns. Whilst another study reported areal textural changes after 30 seconds exposure to citric acid, utilising non-contacting optical microscopy [27]. Surface microhardness has been shown to be sensitive in detecting early changes due to acid erosion after 30 seconds exposure to citric acid [27], and this technique may be useful for times less than this on polished or natural enamel surfaces. Scanning electron microscopy (SEM), has been used to evaluate morphological changes following exposure to acid, however this technique alters the sample surface to allow imaging[21,24]. Tandem scanning microscopy (TSM), involves no sample preparation and high image acquisition, has previously been evaluated for dentine occlusion studies[34,35] but its use in evaluating enamel erosion has yet to be considered. Optical coherence tomography (OCT) has been used to determine surface [16,36] and subsurface [16,37,38] changes that occur to enamel after erosion but to different degrees of success. A recent study determined that alterations in enamel surface reflectivity could be utilised to study the early erosive lesion with

change detectable after 1 minute acid exposure in polished bovine enamel surfaces[36]. However, longer erosive time periods of 60 minutes[38] up to 6 hours[37] were required in order for subsurface changes in natural enamel to be detectable.

The aim of the study was to determine the minimum time that acid exposure causes change on the enamel surface can be measured by profilometry, surface roughness, surface microhardness, OCT, and TSM. Our null hypothesis was that the formation of an early erosive lesion is independent of time

2 Methods

Enamel slabs (n=120) were sectioned from the mid-buccal surfaces of previously extracted caries-free human molar teeth (REC ref 12/LO/1836), using a water-cooled 300 µm diamond wafering blade (XL 12205, Benetec Ltd., London, UK). Each slab was mounted, unpolished enamel surface down, in self-cured bisacryl material (Protemp™4, 3M ESPE, Seefeld, Germany) using a custom-made silicone mould. Sixty slabs were polished using successively finer silicon-carbide discs (Versocit, Struers A/S, Copenhagen, Denmark) of grit 500, 1200, 2000, and 4000 for 25s, 30s, and 60s respectively, using a water-cooled rotating polishing machine at 150 RPM and 10N constant pressure (LaboPol-30, Struers ApS, Ballerup, Denmark) removing approximately 300 µm of enamel and achieving flatness tolerance $\pm 0.2\mu\text{m}$. The newly polished surfaces were then ultrasonicated (GP-70, Nusonics, Lakewood, USA) in 100ml deionised water (pH 5.8) for 15 minutes to remove smear layer and air-dried for 24 hours at room temperature. PVC adhesive tape was placed on the polished enamel slab/bis-acryl embedding material surface such that each the polished enamel surface had a 1mm x 3mm window of exposed enamel, protected by two zones of reference tape either side; allowing for comparison of eroded and protected enamel regions after erosion and after tape removal[27]

Natural surfaces were not cleaned using the same method as for the polished surfaces because this did not result in a sufficiently cleaned enamel surface upon which to conduct the acid erosion

challenge. As a result, to ensure they were free from surface debris and organic contamination, all natural enamel surfaces (n=60) underwent a standardised cleaning regime consisting of: a 10-minute immersion in 4.7% sodium hypochlorite solution (Coventry Chemicals Ltd, Coventry, UK), followed by 30-minute ultrasonic cleaning in deionised water and air dried for 1 hour followed by a 2-minute clean with ethanol and cotton wipes, and final air dry for 1 hour. They were examined under TSM before and after cleaning to ensure surface cleanliness which was determined as the visible lack of surface material after cleaning and clear visualisation of enamel surface topography.

All samples were then randomly assigned to one of six treatment groups according to period of acid immersion: 0, 10, 30, 60, 120, or 300 seconds; 10 natural and 10 polished surfaces per group. Citric acid (0.3%w/w) was prepared, using previously published protocols, by adding anhydrous citric acid powder (Sigma Aldrich, Poole, Dorset UK) to deionised water, and the pH adjusted to 3.2 using 0.1 M sodium hydroxide (Sigma Aldrich, Poole, Dorset UK)[27]. The titratable acidity was calculated according to the volume of sodium hydroxide required to increase the solution pH to 7, which was 18.0 ml[23,27]. Each group of teeth were immersed in 100ml 0.3 % citric acid solution (10ml per sample) and agitated (62.5 rpm) using an orbital shaker (Stuart mini-Orbital Shaker SSM1, Bibby Scientific, England), at differing times for only 1 erosion cycle. All surfaces were washed with deionised water (pH 5.8) and left to dry overnight before data collection to ensure consistent profilometry scanning of all surfaces under the same scanning conditions [27,33].

Baseline surface microhardness was tested for all polished surfaces utilising a Knoop microhardness tester (Duramin-5, Struers Ltd, Rotherham, UK) and those surfaces outside the range 270 – 400 KHN were rejected [22]. After acid immersion and drying, adhesive tape for polished enamel surfaces was carefully removed and surfaces assessed. Post erosion microhardness was conducted from 6 indentations produced 100 μ m apart, selected conveniently in the eroded and uneroded enamel regions, at 981.2 mN load and 10 s press time and the mean change calculated from the difference between the them according to previously published protocols and ASTM E384 17 [22,27,39].

Profilometric measurement was conducted using a 2 μm laser spot sized red light confocal scanning profilometer (Taicaan, XYRIS 2000, UK) with 0.01 μm z height resolution and (x,y) scanning interval of 10 μm , using previously published protocols [23,32]. Two reference marks made using indelible pen on the bis-acryl material of each sample was used to allow scan co-localisation. Analysis for mean 3D step height was conducted using surface metrology software (MountainsMap®, Digital surf, France) using the ISO 5436-1 standard [40]. Surface roughness was measured according to previously published protocol [33] using five representative areas, each 0.04 mm^2 , over the sample were imaged, scanning occurred in a raster pattern, at scanning interval of 4 μm . A 25 μm Gaussian filter was applied to each scan in order to determine 3D roughness (S_a) data for each sample, according to previously published protocols and ISO 25178-2 [27,33].

OCT was conducted by a swept-source multi-beam clinical OCT machine (VivoSight™ Michelson Diagnostics, Kent, UK) utilising a near infra-red laser (1305 nm), with <7.5 μm optical resolution [38], on representative samples of polished enamel surfaces for each erosion group. The refractive index of sound enamel has been previously approximated as 1.65 [36], the resolution of this system for measuring sound enamel is 4.06 μm (x direction) and 3.32 μm (z direction) in air respectively. Each sample was scanned in a raster pattern to produce B-stack volumes consisting of 500 B-stacks of x,z dimension (6 x 1 mm) with y-dimension of 4 μm between each scan, . OCT data were semi-quantitatively analysed by using a stack analysis algorithm that was custom designed for this study to extract single full profile peak intensity average images from each B-stack volume. These images were semi-quantitatively analysed using image processing software (ImageJ, Abramoff et al [41]) by assessing the peak intensity of the eroded enamel compared with non-eroded reference enamel in polished enamel surfaces. This method could not be used for the natural surfaces and thus they were only evaluated qualitatively with TSM.

TSM (Noran Instruments; Middleton, WI, USA) used a x20 objective lens (x20/0.35 NA objective) filtered light projection (green, 550 nm), to acquire representative 2D images of surfaces before and

after acid erosion. A camera (iXon 885 EM-CCD Andor Technology; Northern Ireland, UK) and image acquisition software Micromanager v1.4.22 (Open Imaging; Inc. San Francisco, CA, USA), was used together with image processing software (ImageJ, Abramoff et al [41]) to qualitatively analyse the 2D images [42]. The images produced using TSM were evaluated qualitatively to determine any visual changes between eroded and uneroded/protected enamel in polished surfaces, and between co-localised images of before/after eroded natural enamel surfaces. Co-localisation was conducted by means of utilising distinct surface features on each natural sample as fiducial markers for comparison of each sample against itself before/after erosion; in accordance with previous protocol [42].

2.1 Statistical analysis

Statistical analysis was conducted using OriginPro 8.5 Statistical Software (OriginPro version 8.5, OriginLab Corp, MA, United States). Kolmogorov-Smirnov test confirmed data conformed to normal distribution, therefore means and standard deviations of each acid immersion group were calculated. Intra-group analysis compared with baseline values for uneroded enamel utilising dependent T-tests, whilst inter-group analysis was conducted with one-way ANOVA; $p < 0.05$ was considered statistically significant. Non-linear regression analysis and Spearman rank correlation coefficient comparing 3D step height and surface microhardness (mean KHN) was conducted, whilst linear regression analysis and Pearson correlation coefficient was conducted to compare surface roughness (mean SA) with both 3D step height and surface microhardness; $p < 0.05$ was considered statistically significant and R^2 values expressed for all correlation measures and r values for the association between the variables.

3 Results

Surface microhardness results for the polished surfaces are shown in Table 1. Surface microhardness changes after 10 s, 30 s, 60 s, 120 s, and 300 s citric acid immersion were 40.9 (2.03) KHN, 60.7 (1.79) KHN, 91.7 (3.08) KHN, 100.1 (2.07) KHN, and 119.9 (4.34) KHN and were statistically significant for all groups compared to baseline ($p < 0.01$). Polished enamel surfaces became statistically softer with increasing citric acid immersion ($p < 0.01$). Microhardness data could not be derived from natural surfaces. The mean (SD) step height change for 10, 30, 60, 120, and 300 s acid immersion groups for the polished surfaces were, 0.16(0.04) μm , 0.20(0.1) μm , 0.24(0.1) μm , 1.16(0.71) μm , and 2.01(0.47) μm respectively and these were statistically different compared to baseline (Table 1). Mean (SD) step height change was detected at all times but the number of surfaces that could be analysed was not consistent, the number of analysable surfaces for 10, 30, 60, 120, and 300 s were 5, 3, 7, 8, and 9 respectively. Results below 60 s were considered unreliable and discounted. Step height data was not obtainable from the natural surfaces.

The mean surface roughness for polished enamel for 10, 30, 60, 120, and 300 s acid immersion groups the mean (SD) surface roughness were, 0.27(0.024) μm , 0.30(0.028) μm , 0.51(0.068) μm , 0.95(0.201) μm , and 1.28(0.146) μm respectively and were statistically significant at all citric acid immersion time points compared to before erosion ($p < 0.05$). The mean (SD) surface roughness for the natural enamel decreased for all citric acid immersion time points compared to baseline (Table 1), but were only statistically significant at 120 s and 300 s; 0.83(0.125) μm and 0.80(0.140) μm respectively ($p < 0.01$). Intergroup analysis revealed no statistically significant differences in mean surface roughness between immersion groups ($p > 0.05$).

Correlation analysis between surface microhardness, surface profilometry, and surface roughness can be seen in Figure 1. There was a negative curvilinear relationship ($r = -0.7676$) and positive correlation ($R^2 = 0.6593$) between surface microhardness mean KHN and 3D step height (Figure 1A), positive linear relationship ($r = 0.854$) and positive correlation ($R^2 = 0.7293$) between surface roughness and 3D step height (Figure 1B), and negative linear relationship ($r = -0.8811$) and positive

correlation (0.7764) between surface roughness and surface microhardness (Figure 1C); the correlation between each measurement output/method was highly significant ($p < 0.0001$).

Percentage peak intensity analysis of representative polished enamel surfaces using OCT revealed significant differences in surface reflectivity between eroded and non-eroded regions for different citric acid immersion groups (see Figure 2). Percentage peak intensity change after 10s, 30s, 60s, 120s, and 300s were 88.8%, 86.8%, 77.9%, 69.2%, and 49.1% respectively and were statistically significant for all groups compared with baseline ($p < 0.01$). Surface reflectivity analysis could not be determined for the natural surfaces.

Micrographic analyses of polished and natural enamel surfaces revealed differences between eroded and non-eroded surfaces for each acid erosion group (Figures 3 and 4). The honeycomb structure of polished enamel could be seen after 10s erosion (Figure 3 B) and progressed with increased erosion immersion time. The eroded regions became subsequently darker, and scratch marks present after the polishing process progressively were lost. This indicated that the erosive process resulted in further loss of enamel with increasing erosion time. Changes to the surface of natural enamel surfaces were more subtle, with changes appearing to occur in the prism-inter prism interface after 10 s (Figure 4) and progressed to further generalised destruction of enamel prism structure and surface topography after 300s (Figure 4).

4 Discussion

The surface microhardness results indicate surface softening of polished enamel surfaces occurred after 10 seconds, which is much earlier than previously reported in the literature [27]. This suggests that the effects of citric acid on polished enamel, involving the processes of phosphate leaching and calcium chelation from the enamel surface, occur rapidly during acid exposure resulting in surface softening [16]. It was not possible to obtain any accurate, reproducible, or measurable Knoop indentations on natural enamel surfaces to ascertain the minimum time that changes in surface

microhardness occurred. This is partly due to the curvature of the enamel surface but also the variations in surface topography and profile that exist in all natural enamel surfaces, making the measurement of a standardised Knoop diamond indent impossible to conduct; which is why this technique is used only on polished uniformly flat enamel surfaces [16,43].

The findings from this study demonstrated that the current NCLP scanning settings and method was sufficient to measure the formation of the early erosive lesion in polished enamel (after 10s), and in natural enamel (after 120s) but only using surface roughness parameters . The surface roughness (Sa) data indicated that in polished enamel statistically significant ($p < 0.05$) changes in Sa from baseline could be detected after just 10s citric acid erosion; whilst these changes could only be detected after 120 s in natural enamel (Table 1). The disparity in in the erosive characteristics between polished and natural enamel is likely due to the presence of the aprismatic enamel surface layer present in the natural enamel surfaces which is removed and thus missing in all the polished surfaces. The surface layer contains a higher concentration of fluoride and phosphate, and fewer impurities such as magnesium and carbonate, and has been previously shown to be more highly resistant to acid dissolution [18,29]. In a study by Zheng et al [18] the mechanical properties of enamel differed according to its distance from the dentino-enamel junction (DEJ); erosion resistance decreased as the DEJ was reached and subsequent wear loss of eroded enamel was significantly lower for surface layer enamel versus enamel close to the DEJ. Further study of the natural enamel surface and the implications of changes in surface roughness following acid attack are required.

Within the scanning protocol and specifications of the non-contacting optical profilometer used in this study, we were able to detect significant changes in surface roughness after 10 s of acid exposure, which is much earlier when compared with studies utilising longer erosion time periods [13,22,24,27,29]. Current findings are consistent with a previous report which demonstrated that changes in surface roughness, as a measurement output, could be detected after 10 s [21], however this was conducted on polished bovine enamel using pH 2 hydrochloric acid and atomic force

microscopy as the measurement technique and Ra as the amplitude parameter for surface roughness. The earliest reported change in surface roughness for polished human enamel occurred after 30 s citric acid exposure [27]. Enamel surfaces in the current study were polished to higher flatness tolerance of $\pm 0.2 \mu\text{m}$ compared with some previous studies which utilised $0.4 \mu\text{m}$ [27] and $0.25 \mu\text{m}$ [24]. This may have helped in the detection of the earliest deviations in the polished enamel surface due to acid erosion in the current study. This is consistent with suggestions from previous reports indicating the importance of sample preparation and its potential influence on surface texture feature detection [27]. The detection with non-contacting profilometry of changes in surface roughness after 10 s citric acid exposure suggest that the physiological processes occurring during early erosive attack, such as calcium and phosphate release from the enamel surface, occur relatively quickly [17,24,27].

The surface roughness of natural enamel surfaces decreased as acid immersion time increased, indicating smoothening of the aprismatic enamel surface, and contrasts to the results from polished surfaces which became rougher with increasing erosion. This difference in the wear behaviour and surface characteristics between polished and natural human enamel after erosion was also observed in a study by Mullan et al [44] who found the median (IQR) surface roughness (Sa) of natural enamel reduced significantly ($p < 0.0001$) (baseline Sa of $1.45(2.58) \mu\text{m}$ to $0.38(0.35) \mu\text{m}$) after three 15-minute cycles of orange juice mediated erosion, whilst the median (IQR) surface roughness (Sa) for polished enamel increased (baseline $0.04(0.17) \mu\text{m}$ to $0.27(0.08) \mu\text{m}$) after the same erosion period [44]. This supports what is observed clinically in patients who suffer from erosive tooth wear, where natural enamel surfaces become progressively smoother and shinier due to loss of surface structure and texture [1].

In the polished enamel group, there was a lack of consistent outputs for 3D step height formation in surfaces with citric acid exposure below 60 s. This could indicate that below this time the integrity of the enamel remains unchanged or was not detected using our non-contacting optical profilometry

methods. The barrier method of choice, PVC taping in 1:3 ratio to leave an exposed region of enamel, has been widely published previously has been shown to not influence the effect of acid-mediated erosion on enamel [22,23,27,42,44] . Additionally the calculation of 3D step height using ISO 5435-1 utilises three relatively flat regions, two in the reference regions and one in the central portion of the eroded region; and thus any influence from the use of taping to protect the reference regions such as left over adhesive at the eroded-uneroded region or slight diffusion of acid under the tape is unlikely to affect 3D step height calculation [32,40]. The progression from early erosive lesion to erosive tooth wear with measurable loss of enamel would appear to occur in the presence of prolonged citric acid attack greater than 60 s duration. In natural enamel however, 3D step height change was undetectable. This was due to a number of technical challenges faced in trying to measure the natural enamel surface: the PVC tape barriers would not adhere to the natural enamel, which meant a referenced region of exposed enamel could not be produced. In addition, utilising a non-contacting optical profilometers to measure 3D step height in natural enamel is very difficult due to the curvature and non-uniform surface topography of the natural enamel surface [33]. These comparisons were conducted without either natural or artificial saliva and their impact is unknown. This study's aim was to assess the sensitivity of the measuring systems, the next will be to determine the influence of saliva.

To the authors knowledge this is the first study to correlate the use of changes in surface profilometry (3D step height), surface roughness (Sa) and surface microhardness (KHN) when measuring the same co-localised eroded regions to characterise the early erosive lesion within an in-vitro early-erosion model. Results indicated there was a strong positive correlation between all three measurement methods, whilst the association between each variable differed. The negative curvilinear relationship between surface microhardness and 3D step height indicates as bulk loss of enamel occurs, the microhardness of the underlying enamel reduces. Additionally, there was a positive linear relationship between surface roughness – of the enamel surface at the base of the erosion trough – and 3D step height formation. Both these findings may be explained by the fact

that surface microhardness and surface roughness was conducted on the acid- softened enamel left behind after bulk enamel loss occurred. This acid-softened enamel is formed as a result of the softening process that occurs during citric-acid mediated dental erosion where penetration of the acid into the enamel subsurface occurs before bulk superficial enamel is lost due to prolonged acidic attack [45,46]. The negative linear relationship between surface roughness and surface microhardness indicates that in polished enamel surfaces, surface roughness may be a surrogate marker for the enamel softening that occurs following citric acid erosion. Future studies will need to be conducted to correlate surface roughness (Sa) changes with associated calcium release analysis to determine whether surface roughness can be used as a surrogate measurement for the chemical changes occurring on the eroded enamel surface.

OCT did not produce quantifiable data for enamel subsurface/depth changes for any of the acid exposure times. However, OCT allowed surface intensity changes of the profile of eroded surfaces to be quantified and analysed. Differences in surface reflectivity between the eroded and non-eroded (reference) region of each sample, denoted by gradual decrease in percentage peak intensity, indicated that early acid erosion did result in changes in surface optical properties of enamel. This is likely due to the loss of calcium and phosphate from the enamel surface resulting in an increase in surface roughness and hence change in the optical properties of enamel producing a less reflective and more optically diffuse surface [38]. Aden et al [36] used OCT to conduct quantitative analysis of mean local pixel intensities on polished bovine enamel specimens in-vitro with increasing acid exposure time. Their results indicated pixel intensity decreased with increased acid erosion, suggesting that surface change due to acid exposure could be detected after 1, 2, and 5 minutes [36]. However, longer acid exposure times were required to measure sub-surface changes using OCT in natural enamel in-vivo, for example, Austin et al [38] demonstrated sub-surface changes in the superficial 33 μm of enamel after 60 minutes rinsing with orange juice *in-vivo*. Although saliva and salivary pellicle may affect the erosive process in-vivo [38], this suggests that short acid immersion periods produce alterations in the enamel surface characteristics with minimal subsurface changes

whilst more extended acid exposure times are required before subsurface changes in enamel occur. Significant additional changes to subsurface enamel may also be required before quantitative changes in 3D step height can be detected with the OCT as supported by the work of Chan et al [37] who reported that polarisation-sensitive OCT detected subsurface enamel changes after 6 hours immersion in a pH 4.5 demineralisation solution cycled over 2 days [37].

TSM images allowed qualitative assessment of the eroded zone for each erosion period, and as time increased the presence of the honeycomb-like structure of eroded enamel became more prominent and was best visualised after 300 s of citric acid exposure. This supports the work by Zheng et al [29] who reported that after 5 minutes acid erosion, the formation of a honeycomb-like structure could be distinguished, which was due to the preferential dissolution of the prism inter-prism interface [29]. SEM could have also been used in our study to further corroborate surface structural changes; however, TSM allows for sample scanning without further surface modification.

The *in vitro* methods used in the current study demonstrate the formation of erosive lesions in polished enamel. Using polished enamel specimens, it was possible to detect relatively small changes in enamel surface characteristics following short acid exposures and sensitive enough to discriminate between different acid exposure times. Thus, polished enamel specimens are appropriate for studies that aim to investigate the formation of erosive lesions and studies that aim to investigate the prevention or repair of erosive lesions. Indeed, such specimens are commonly used in the evaluation of enamel remineralisation agents and formulations [16,22,23,26]. This study did not consider the impact of the acquired salivary pellicle on the formation of the early erosive lesion, as it was necessary to overcome the significant challenges in developing a working dry-field model first before additional influencing factors were introduced. The presence of saliva and/or the use of artificial saliva has been previously shown to influence the erosive patterns observed following acid mediated erosion of enamel for more extensive erosion periods, greater than 5 and

10 minutes, however there is a paucity of literature on the effect of the AEP on the formation and progression of the early erosive lesion; this will be the focus of future work in this area [27,48–50].

One potential limitation of this study is that the resolution of the optical profilometer used to determine 3D step height change may not have been sufficient enough to detect changes consistently/reliably below 60 seconds in polished enamel surfaces. Any enamel loss which may be occurring during the initial 60 s citric acid exposure may not have been detected by the current non-contacting optical profilometer, and therefore whether or not enamel bulk material loss is occurring at such early erosion times may not be entirely excluded without further analysis, such as with atomic force microscopy. Additionally, this study sought to determine the level of dental erosion which could be detected using current and previously utilised scanning parameters/techniques and did not consider how different data acquisition variables may affect the NCLP detection performance. Fleming et al [51] explored the minimum data acquisition variables for contact profilometry on the measurement of artificial wear scars created on resin-based composite samples, and determined the minimum x- and y- axis spacing required to ensure accurate quantification of mean total volumetric wear. Whilst their results may be extrapolated to non-contacting white profilometry, it is unknown whether this would be case for the NCLP used in this study which used a red-laser mono-chromatic based displacement sensor. Future study is therefore required to consider the effect of altering the scanning parameters on the accuracy of the measurements obtained using NCLP's that utilise this type of displacement sensor.

Using the current methods utilised, neither 3D step height nor surface microhardness data could be obtained in natural enamel surfaces. Future studies may consider using different measurement techniques to obtain quantifiable data for determining changes that occur in natural enamel surfaces after citric acid demineralisation.

5 Conclusion

Changes in surface roughness, surface microhardness and qualitative image analysis were evident for polished enamel surfaces and demonstrated that 0.3% citric acid (pH 3.2) alters the surface after only 10 s of citric acid exposure. Changes in surface roughness (Sa) and surface microhardness (KHN) were sensitive enough to allow the determination of the early erosive lesion; and their use in early enamel erosion studies is recommended. Natural enamel surfaces, however, required much longer erosion periods before any measurable change could be quantified; neither profilometric changes nor microhardness measurements were possible using the specific measurement equipment and data acquisition methods selected for this study

362 **6 Conflict of interest statement**

363 Andrew Joiner is employed by Unilever Plc. There are no other conflicts of interest.

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Table 1 – Descriptive statistics for 3D step height change (μm), mean(SD) surface roughness (μm), and the number of analysable samples for each characterisation method for both polished and natural enamel groups. Data is represented as mean(SD) after citric acid erosion. Statistical significance is compared to baseline according to asterisk assignment * $p < 0.05$, ** $p < 0.01$

	Polished Enamel Group						Natural Enamel Group					
Immersion Period (s)	3D Step Height change (μm)	Analysable Samples	Mean (SD) Surface Roughness (μm)	Analysable Samples	Mean (SD) Microhardness Change (KHN)	Analysable Samples	3D Step Height (μm)	Analysable Samples	Mean (SD) Surface Roughness (μm)	Analysable Samples	Mean (SD) Microhardness Change (KHN)	Analysable Samples
0	0	0	0.18 (0.001)	10	4.4 (1.44)	10	-	0	0.99 (0.265)	10	-	0
10	0.16 (0.04)	5	0.27 (0.002) *	10	40.9 (2.03) *	10	-	0	0.95 (0.098)	10	-	0
30	0.20 (0.10)	3	0.30 (0.028) *	10	60.7 (1.79) *	10	-	0	0.88 (0.241)	10	-	0
60	0.24 (0.10) **	7	0.51 (0.068) *	10	91.7 (3.08) *	10	-	0	0.86 (0.481)	10	-	0
120	1.16 (0.71) **	8	0.95 (0.201) *	10	100.1 (2.07) *	10	-	0	0.83 (0.125) **	10	-	0
300	2.01 (0.47) **	9	1.28 (0.146) *	10	119.9 (4.34) *	10	-	0	0.80 (0.140) **	10	-	0

Figure 1 - [A] Negative curvilinear relationship and large correlation is demonstrated between surface microhardness (mean KHN) and 3D step height (μm) for all samples evaluated. [B] Positive linear relationship and large correlation is demonstrated between surface roughness (mean Sa, μm) and 3D step height (μm) for all samples evaluated. [C] Negative linear relationship and large correlation is demonstrated between surface roughness (mean Sa, μm) and surface microhardness (mean KHN) for all samples evaluated.

Revised version

Figure 2 – Optical Coherence Tomography, surface reflectivity percentage peak intensity change for representative samples from each acid erosion group compared with baseline/before acid erosion. A decrease in enamel surface reflectivity is evident after acid erosion and continues to decrease with increasing acid erosion.

Figure 3– Tandem scanning confocal microscopic assessment of changes in appearance of the enamel surface of polished enamel samples: 0s (A), 10s (B), 30s (C), 60s (D), 120s (E), 300s (F). Early erosive changes include appearance of honeycomb structure, progressive darkening of eroded region, and progressive loss of post-polishing scratch marks.

Figure 4 – Tandem scanning confocal microscopic assessment of changes in appearance of the enamel surface of natural enamel samples: before citric acid immersion (top left and bottom left); after citric acid immersion 10 s (top right) and 300 s (bottom right). Early changes include initial breakdown of prism-interprism interfaces(indicated with red arrows), further increasing size of the prisms relative to their 'Before' acid erosion image, loss of superficial and deeper topographical features.