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IN VITRO ENAMEL THICKNESS MEASUREMENTS WITH ULTRASOUND

Dr Khalid Hussain Sindi¹, Dr Nigel Lawrence Bubb¹, Miss Diana Lynn Gutteridge¹, Dr Joseph Anthony Evans²

¹ School of Dentistry, Faculty of Medicine and Health, University of Leeds, LS2 9LU, UK

² School of Medicine, Faculty of Medicine and Health, University of Leeds, LS2 9LU, UK

Corresponding author:

Dr Khalid Hussain Sindi

School of Dentistry

Faculty of Medicine and Health,

University of Leeds

LS2 9LU

Mobile: +4479369494877

Fax: +441133436548

drsindi@gmail.com

Abstract

This work investigates the agreement between ultrasound and histological measurements of enamel thickness in vitro. Fifteen extracted human premolars were sectioned coronally to produce 30 sections. The enamel thickness of each specimen was measured with a 15 MHz hand-held ultrasound probe and verified with histology. The speed of sound (SOS) in enamel was established. Bland-Altman analysis, intra-class correlation (ICC) coefficient and Wilcoxon sign rank test was used to assess agreement. The mean SOS in enamel was $6191 \pm 199 \text{ ms}^{-1}$. Bland-Altman limits of agreement were -0.16 to 0.18 mm when the SOS for each specimen was used, and -0.17 to 0.21 mm when the mean SOS was used. ICC agreement was 0.97 and the Wilcoxon sign rank test gave a p-value of 0.55. Using the SOS of each specimen results in more accurate enamel thickness measurement. Ultrasound measurements showed good agreement with histology which highlights its potential for monitoring the progressive loss of enamel thickness in erosive tooth surface loss (TSL).

Keywords: ultrasound; erosion; tooth wear; tooth surface loss; non-destructive; enamel thickness measurement

Introduction

Ultrasound is a non-invasive, non-destructive imaging tool that has been used in medicine since the 1940s. Its first use as an imaging tool in dentistry was by Baum and co-workers (1963) who used an ultrasound device, originally designed for ophthalmology, to scan teeth in B-mode. However, the images produced were not of sufficient clarity to render the ultrasound device usable in the dental surgery. Later, research by Lees and Barber (1968) attempted to use ultrasound for examining teeth, with more encouraging results. Recently, Huysmans and Thijssen (2000) demonstrated that ultrasound can be used to measure enamel thickness in a sample of 9 extracted human incisors. Tagtekin et al. (2005) investigated ultrasound for monitoring occlusal enamel on worn molars in vitro and concluded that ultrasound was a promising tool for that task. Indeed, several studies have compared ultrasound measurements with histology, the gold standard in the field, but with mixed results (Harput et al. 2011; Louwerse et al. 2004; Slak et al. 2011; Tagtekin et al. 2005). One factor that may explain the variation between these studies is the assumed SOS in enamel. This value is used to derive the enamel thickness. The variation in the SOS within the enamel tissue of teeth is well established with a range between 4500 ms^{-1} and 6500 ms^{-1} . Table 1 summarises the various reports. The variations in SOS occur both within single teeth and between different subjects and it is likely that much of the variation is due to the orientation of the enamel rods with respect to the incident ultrasound beam (John 2005). Sound travels faster in enamel rods that are parallel to the ultrasound beam and the opposite holds true.

However, the reliability of the measurement itself is also influenced by the orientation of the ultrasound transducer with respect to the enamel surface. Ideally the measurements would be carried out at normal incidence. Dwyer-Joyce and co-workers (2010) investigated the incidence angle after which no echo was seen from the ADJ and found that in human molar

teeth, this angle was 10°. In a preliminary study investigating the echo amplitude from the external surface of synthetic incisors, we found that 50% of the echo amplitude plummeted when the incidence angle was $\geq 25^\circ$ (Sindi 2013). This angle discrepancy between the two studies might be due to the non-planar nature of molar teeth compared to incisors. Of course, if the transducer is normal to the enamel surface when taking a measurement this will also ensure a consistent orientation of the beam relative to the enamel rods.

For absolute measurement of enamel thickness, knowledge of the SOS is essential and hence it might be assumed that the SOS uncertainties preclude the use of ultrasound in routine clinical applications of enamel thickness assessment. On the other hand, if the enamel SOS does not change in a particular tooth over the time, then changes in enamel thickness can be monitored without knowledge of the SOS. This is the case in erosive tooth surface loss (TSL), a multifactorial disease that has increasing prevalence (Lussi and Jaeggi 2008). It is defined as the loss of hard dental tissues due to acids of non-bacterial origins (intrinsic, extrinsic or both) which causes enamel demineralisation. Erosive TSL causes poor aesthetics, deterioration of dental function, hypersensitivity and diagnosis is made by obtaining medical and dental history with thorough investigation of dietary intake. Early detection and monitoring of erosion is crucial to prevent its progression and avoid the aforementioned complications.

To date, there is no in vivo dental tool available that can aid dentists in diagnosing and monitoring the progression (or stabilisation) of the erosive process reproducibly and quantitatively (Amaechi and Higham 2005). The currently used methods for monitoring erosive TSL are sequential study casts (Wickens 1999), silicone putty index (Shaw and Smith 1999), photographs and erosive TSL indices (Bartlett et al. 2008; Eccles 1979; Linkosalo and Markkanen 1985; Larsen et al. 2000; O'Brien 1994; O'Sullivan and Curzon 2000), which are

subjective, not reproducible and do not measure enamel in a sub-millimetre level. Laboratory-based methods, such as profilometry (Bartlett 2003), are costly and cannot be used in the dental surgery. Profilometry also requires an impression of the teeth from which replicas are made but it has been shown that impressions can lead to inaccurate measurements (Rodriguez and Bartlett 2011).

One important question which arises is the extent to which it is possible to take a single assumed value of the SOS and use it to get a useful measure of enamel thickness. Hence the aim of this work was to assess the agreement between enamel thickness measurements by ultrasound and histology using the same SOS value for each tooth (selected as being the mean of our sample) and compare it with the agreement obtained when using individualised SOS values. This is important clinically since it would open the possibility of a routine clinical tool using a standard value.

Materials and Methods

Tooth Selection and Storage

Fifteen extracted human premolar teeth were randomly chosen from the Skeletal Tissue Bank, University of Leeds, after obtaining ethical approval (130109/DS/19) from the Dental Research and Ethics Committee, University of Leeds, according to the Human Tissues Act 2004 (UK). The teeth were kept hydrated in 0.1% thymol (Sigma Aldrich, MO, USA) solution and stored in the laboratory refrigerator at 5 °C.

Sectioning of the Premolar Teeth and Storage Media

The crowns of all premolars were inspected for near planar areas (buccally, palatally, mesially and distally) so that the cut sections could include these acoustically preferential regions. All 15 premolars were sectioned coronally using a cutting machine employing a

250 μm water cooled diamond cut-off wheel (Accutom, Struers, Denmark). Two disc shaped specimens with a thickness of 2.50 ± 0.02 mm were obtained from each premolar's crown (an 'occlusal' and 'cervical' specimen) (Fig. 1A), which resulted in a total of 30 specimens. The specimen thickness was determined with a digital micrometer (293-766-30, Mitutoyo, Japan). The specimens were stored in labelled vials filled with Hanks Balanced Salt Solution (HBSS) (Thermoscientific, Hyclone Laboratories Inc., USA) in a refrigerator at 5 °C for subsequent ultrasound measurements.

Marking Specimens

Each specimen was marked with a permanent marker (Twin tip, Sharpie™, Newell Rubbermaid, Inc., USA) at two locations on the enamel surface (V and T in Fig. 1B). For each specimen, the V marked area was used to determine the SOS in that specimen. The T marked area was used to measure enamel thickness with ultrasound, which was then validated with histological measurements. Marks were made on the most planar areas of the specimens. The specimens were kept hydrated in HBSS at all times, except when measurements were performed.

Ultrasound Setup

Dental boxing wax (00609, Kerr, CA, USA) was used to secure the specimens on a microscopic glass slide in order to prevent moving or rocking while ultrasound readings (in the z plane in Figure 1B) were made from the pre-marked areas. A direct contact pulse-echo technique using a 15 MHz focussed transducer (VR-260, Olympus® Inc., Waltham, USA) with a replaceable Perspex delay line that had a 2 mm tip was coupled to the enamel surface with a water drop (Huysmans and Thijssen 2000). The transducer was excited with a pulser-receiver unit (PR-5742, Olympus®, MA, USA), and the waveforms were displayed on a

digital oscilloscope (LT-342, Lecroy[®], USA). When recognisable enamel layer echoes were displayed on the oscilloscope, 1000 sample traces were averaged and saved in ASCII format on a computer coupled to the oscilloscope. The calibration of the ultrasonic setup was checked using a Perspex block (assumed SOS 2700 ms⁻¹) prior to tooth measurements.

SOS Measurements in Enamel at V Marked areas

The SOS was calculated using the range equation (Slak et al. 2011). In order to satisfy this equation and derive the SOS (v), the TOF (t) and the enamel thickness (d) at the V marked area in Figure 1B should be known.

$$v = \frac{2d}{t}$$

Equation 1

where

d: enamel thickness

v: SOS in enamel

t: TOF

Time of Flight Calculation at V Marked Areas

The TOF measurements were made while the specimen was held with one hand and the transducer in the other (Tagtekin et al. 2005). The transducer tip was placed perpendicular to the V marked area on the enamel surface, while ensuring intimate contact between the tip and the surface (Fig. 2).

Three repeat measurements were obtained from each V marked area of each section. In the repeat measurements, the transducer was removed from the enamel surface and reapplied. The signal from each repeat measurement was averaged 1000 times by the oscilloscope before saving (Fig. 3). Figure 3 depicts a representative waveform with the first echo from the enamel surface and the second echo from the ADJ. Generally the ADJ echo was a smaller version of the enamel surface echo but there is clearly some uncertainty in the choice of the reference points in the waveform. A total of 90 TOF measurements were obtained from all sections. The TOF was calculated from the first peak corresponding to the Perspex-enamel interface and the second peak representing the amelo-dentinal junction (ADJ). When there were multiple consecutive peaks, the first peak was chosen. Once all TOF's were carried out for the 30 sections, thickness measurements of the V marked site was completed as discussed in the following section.

Thickness Measurement at V and T Marked Areas

Each tooth section was placed under a stereo microscope (Nippon, Kogako, Tokyo), equipped with a 20 W fibre-optic light source (Leica L2, Leica Microsystems GmbH, Wetzlar, Germany) and viewed at 20x magnification. A computer controlled digital microscope camera (Moticam 2300, Motic[®], Inc. Ltd., China) with a resolution of 3 Megapixels was mounted on one of the ocular eye pieces via an eye piece adapter, so that images could be taken without moving the setup. Before images were captured, a calibration slide provided by the manufacturer was used in order to calibrate the camera's software (Motek Images Plus, version 2.0 ML).

Once the V marked area of the specimen was in the field of view and the enamel layer was in sharp focus, a digital image was taken and saved. The software that captured the images had a built-in line-measurement tool which was used to measure enamel thickness (d).

Three radial measurements were made at the V marked area and the mean was taken. The line measurement tool cursor was placed on the external enamel surface and was extended to the ADJ. The ADJ was sometimes ambiguous and therefore the contrast was adjusted until the boundary became clearer for a measurement to be taken. Care was exercised not to spend unnecessary 'dry time' for the specimen.

SOS in Enamel at V Marked Areas

The SOS in enamel at the V marked area on all 30 specimens was obtained by incorporating the TOF and thickness values from this area in Equation 1.

Enamel Thickness Measurements with Ultrasound at T Marked Areas

To avoid bias in enamel thickness measurements, a different area (T in Fig. 1B) was chosen within the same section to measure enamel thickness, but this was not in the V marked area from which SOS measurements were made (because thickness was already known in that area). The enamel thickness at the T marked area in Figure 1B was calculated from the measured time of flight using the range formula. The enamel thickness at the T marked area was first measured using the mean SOS for all specimens and then using the SOS for each specimen, to know if there was any difference between the two SOSs when performing thickness measurements with ultrasound.

The ultrasonic enamel thickness values at the T marked areas were verified by measuring enamel thickness at these areas using a stereo microscope following the method described previously.

Statistical Methods

The aim of the analysis was to examine the agreement in enamel thickness between histology and ultrasound, and also to examine SOS in enamel. This was done using three

different statistical approaches. The first method examined agreement using the Bland-Altman limits of agreement method (Bland and Altman 1986). In order to achieve an overall evaluation of the agreement, the intra-class correlation coefficient (ICC) test was used as a second method (Gilligan et al. 2011). This method involves dividing the total variability in enamel thicknesses into two components, the variation between different teeth, and the variation within measurements of the same teeth (i.e. measurements of the same teeth by different methods). The ICC is the proportion of the total variability between teeth. If the method is reproducible, then the majority of variation should be between teeth, with little variation between repeat measurements of the same teeth (within teeth). This would give an ICC value close to 1.

The difference in SOS values between tooth sections was also examined. As the specimens came in pairs from the same teeth, the paired t-test was used for analysis.

The third statistical method used was a hypothesis test.

- The null hypothesis was: there is no difference between measurements made with ultrasound and histology.
- The alternative hypothesis was: there is a difference between measurements made with ultrasound and histology.

Prior to hypothesis testing, the Shapiro-Wilk test was used to check if the data was normally distributed. The significance level for the normality test was set at $\alpha = 0.05$. If the p-value for the test was < 0.05 then the data were not normally distributed and a non-parametric test, such as the Wilcoxon sign rank test was used instead of the paired t-test. However, if the p-value was > 0.05 then the data were deemed normally distributed and the paired t-test was used. The significance level for the hypothesis test was set at $\alpha = 0.05$.

Results

Premolar teeth were sectioned successfully yielding 30 sections and stored in HBSS. Permanent marks (at V and T areas) were successfully placed in each of the 30 sections (Fig. 4). A total of 180 ASCII data files were successfully obtained from the ultrasound measurements. Validation of the ultrasound measurements was checked by comparing the measurements with the true enamel thickness obtained from histology.

SOS Measurement in Enamel at V Marked Areas

The SOS values obtained from the 30 specimens have SOS values ranging from 5187 to 7222 ms^{-1} ($\text{SD} \pm 199 \text{ ms}^{-1}$)

The mean SOS in enamel was determined in this work at $6191 \pm 199 \text{ ms}^{-1}$. The mean values obtained for the occlusal and cervical sections were 6267 ms^{-1} and 6131 ms^{-1} respectively ($\text{SD} \pm 210, 188 \text{ ms}^{-1}$). The paired t-test was used to compare the differences in SOS between sections. These showed no significant difference using a paired t-test ($p = 0.12$)

Verifying Ultrasonic Enamel Thickness Measurements

The mean ultrasonic enamel thickness for 30 specimens (at T marked areas) using SOS of each specimen was $1.05 \pm 0.03 \text{ mm}$ (range 0.60-1.96 mm) and the mean ultrasonic thickness using mean SOS of all specimens was $1.05 \pm 0.04 \text{ mm}$ (range 0.59-1.73 mm). The mean histologic enamel thickness was $1.04 \pm 0.03 \text{ mm}$ (range 0.60-1.70 mm).

Agreement between Ultrasound and Histology

Analyses were performed to examine the agreement in enamel thickness between ultrasound and histology. The initial analysis considered the agreement in terms of the actual difference (in mm). The Bland-Altman method was used for the analysis, and the results

showed that the mean difference between ultrasound and histology (using SOS of each specimen) was 0.01 ± 0.09 mm with 95% Bland-Altman Limits of Agreement from -0.16 to 0.18 mm (Fig. 5) The mean difference between ultrasound and histology (using SOS of all specimens) was 0.02 ± 0.10 mm with 95% Bland-Altman Limits of Agreement from -0.17 to 0.21 mm. The ICC agreement analysis was 0.97 which means there is almost excellent agreement between the methods. The Shapiro-Wilk test result was < 0.05 (p-value = 0.00) which indicated that the data was not normally distributed. The Wilcoxon sign rank test of ultrasound and histology gave a p-value of 0.55, which indicated that there were no statistically significant difference between ultrasound and histology.

Discussion

Ultrasound has been proposed as a potential tool for direct, non-destructive enamel thickness measurements. The majority of ultrasonic enamel thickness measurements reported in the literature assumed a constant SOS within the tooth and across other teeth. This attracts an element of uncertainty in the enamel thickness measurements because the SOS varies within and across teeth. A solution for this problem is to use information in the relative TOFs in the serial measurements. When the ultrasound transducer is placed on the enamel surface to take a measurement, what is being measured is the TOF, not the thickness (which requires an SOS to be calculated using the range equation). The TOF will be the point of interest here, because if it decreases, it means that some enamel has been lost, provided the transducer is perpendicular to the enamel surface.

SOS Measurement in Enamel at V Marked Areas

In this work, the mean SOS value for the 30 sections, 6191 ($SD \pm 199 \text{ ms}^{-1}$), was in very good agreement with the reported literature values (see Fig. 6). There is a wide range in

SOS across the specimens ($5187\text{-}7222\text{ ms}^{-1}$), which was not surprising as the SOS has been reported to be different across different teeth and within the same tooth. John (2005) has demonstrated that SOS in a tooth can vary depending on how parallel the ultrasound wave is to the enamel rods. Enamel rods vary in orientation along the enamel layer where some rods lie perpendicular to the ADJ others lie parallel to it (Lees and Rollins 1972) which explains the higher SOS values shown in Figure 6. This also means that V marked areas might not necessarily have the same SOS present in T marked areas. Furthermore, anisotropy in teeth has been reported by several studies as a primary obstacle for the utilisation of diagnostic ultrasound in dentistry (Harput et al. 2011; Huysmans and Thijssen 2000; Louwse et al. 2004; Slak et al. 2011; Tagtekin et al. 2005).

However our results showed no statistically significant difference ($p\text{-value} = 0.12$) in SOS values between the two sections (occlusal and cervical), which suggests that the site of measurement on the individual tooth is not critical although this might be due to the relatively close proximity of both sections.

Enamel Thickness Measurements with Ultrasound at T Marked Areas

The results from this work showed very good agreement between ultrasound and histology in measuring enamel thickness in premolars, which means that the ultrasonic system was accurate and effective in measuring enamel thickness. The results indicated that in all analyses there was a relatively small mean difference between the two methods. This means there is no consistent trend of ultrasound over- or under-predicting enamel thickness. The mean percentage difference between ultrasound and histology was $1.05 \pm 6.34\%$. This is better than the *in vitro* findings of Slak and co-workers (2011) which reported a $\sim 12.00\%$ difference in enamel thickness between ultrasound and histology from a sample of 4 human central incisors.

It is well established that the accuracy of ultrasound in measuring the true enamel thickness depends on the SOS in enamel, which varies across different individuals and different teeth. Since Slak and co-workers (2011) have determined the SOS in enamel in one of the teeth and used its mean SOS value in subsequent measurements for other teeth, it is not surprising to see a difference between the measured value and the true value. This explains the higher accuracy achieved in this work because the SOS was determined for each specimen and a mean SOS value was not used for all specimens. Also, the paired t-test results demonstrated that there was no statistically significant difference between ultrasound and histology (p-value = 0.46). It also confirms that the second ultrasound echo actually arises from the same location as the histological ADJ.

Assuming an SOS of 6191m s^{-1} the wavelength at 15MHz is 0.41mm. The enamel thicknesses measured ranged from 0.50 to 1.50 mm. Hence it is at least feasible in principle that the two reflecting surfaces can be separately distinguished. Some authors (Longo et al. 2010) have outlined methods by which the SOS can be determined simultaneously and this may be developed further in the future. Using the mean difference as a measure of accuracy between two methods is acceptable for assessing the 'overall' accuracy of the measurements which shows how ultrasound under/overestimates the histology measurements. However, if the aim was to assess for each tooth how ultrasound agrees with the histology measurements, then it is best to calculate the 95% limits of agreement (Bland and Altman 1986) and report this value as the accuracy of the ultrasonic technique. Using this approach, the results showed that the majority of the ultrasound measurements are within approximately 10% of the histology measurements. The 10% difference in the measurements may have arisen due to the non-planar nature of premolars which means that the transducer tip was not perpendicular to the enamel surface while taking measurement. This results in smaller echoes that are difficult to recognise in the waveform (Slak et al. 2011). It seems clear that the enamel thickness

cannot be measured everywhere (i.e. non-planar areas), but on the other hand, erosive tooth surface loss is known to affect the buccal surface of anterior teeth (incisors) more readily because of the direct contact with the acidic foodstuff and drinks one consumes. A study by Sindi and co-workers (2014) showed that central incisors generally are amenable to monitoring enamel thickness clinically with a reproducibility of better than 0.10 mm. It is likely that measurements on molars and premolars will be poorer and may not become usable clinically because of their curvaceous nature.

The ICC obtained in this work (0.96) means that the majority of the differences were between teeth and not within measurements of the same teeth (measurements of the same teeth by ultrasound and histology). Therefore there is a high level of agreement between ultrasound and histology measurements.

An *in vitro* study has measured enamel thickness on worn cusps of molar teeth with ultrasound before and after abrading the cusps with abrasive paper (Tagtekin et al. 2005) They verified the results with histological sections and found a moderate correlation between both methods (ultrasound and histology) but not perfect agreement. This could be due to the use of one SOS value ($6132 \pm 2.5 \text{ ms}^{-1}$) for all teeth which may have caused inaccuracies in their results.

To investigate this further, the mean SOS for all the specimens in this work was used to calculate the enamel thickness using the same TOF data used earlier. The 95% limits of agreement increased to -0.17 to 0.21 mm compared to -0.16 to 0.18 mm. This demonstrates that using the specific SOS rather than mean values results in a more accurate measurement. This is an important distinction between previous work by other researchers and our work, where the enamel thickness obtained for each section was based on its own SOS measurement.

For the purpose of measuring progressive loss of enamel thickness the important thing is the ability to reproducibly measure the **change** in thickness from baseline rather than the **remaining** enamel thickness value. If the current system was to be used on maxillary central incisors it would be expected to produce more accurate results, because planar and larger central incisors would reflect stronger echoes that are easier to identify.

It is important to note that there is scope for improving the agreement between ultrasound and histological measurements. This could be achieved by signal processing, to increase the signal-to-noise ratio which renders reflected echoes easier to locate.

Harput and co-workers (2011) used the fractional Fourier transform (FrFt) to improve the definition of the enamel surface and ADJ echoes. They investigated signal loss in human teeth and used a custom-made wave excitation technique known as linear frequency modulated (LFM) chirp excitation that is tailored for individual teeth. This allows most of the ultrasound wave to be targeted into the tooth and separated from overlapping echoes which makes their detection easier. However, implementing this technique requires a solid background in signal analysis and programming, which is beyond the scope of this article. Nevertheless, it would be an interesting method to learn and adopt in future experiments involving ultrasound and dental applications.

Conclusions

Ultrasound shows promise as a non-destructive technique for measuring enamel and monitoring erosive TSL. The ultrasound technique used here was accurate and within 10% of histological measurements, which renders the technique promising for serial monitoring of enamel thickness.

Tables

Table 1. Speed of sound (SOS) in enamel as reported in several published studies (ms^{-1}).

Study	SOS (ms^{-1})	Tooth type
Huysmans and Thijssen (2000)	6500	Human incisors
Ng <i>et al.</i> (1989)	6450	Human incisors and molars
Barber (1969), Blodgett (2002)	6250	Human incisors
Hamano <i>et al.</i> (2003)	6244	Human molars
Ghorayeb and Valle (2002)	6200	Human molars
Bozkurt <i>et al.</i> (2005)	6132	Human premolars
Slak <i>et al.</i> (2011)	6100	Human incisors
Lees and Barber (1971)	6000	Human molars
Maev <i>et al.</i> (2002)	5900	Human molars
Hedrick <i>et al.</i> (1995)	5800	Incisors and molars
Reich <i>et al.</i> (1967)	5700	-
Kossof and Sharpe (1966)	4500	Human incisors and molars

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Figure Captions

Fig. 1. Schematic for the location and orientation of the sections.

Fig. 2. The transducer Perspex tip coupled with water to enamel on the pre-marked area. Note the marker colour on the proximal area.

Fig. 3. Representative ultrasonic waveform depicting the enamel surface echo and the amelodentinal junction echo (ADJ)

Fig. 4. Coronal section of a representative specimen at a marked area on enamel. Note that extending the mark to the cut surface was necessary for the histological measurements.

Fig. 5. Bland-Altman plot for enamel thickness measurements with histology and ultrasound using the SOS from each specimen ($n = 30$).

Fig. 6. A histogram showing SOS result across a sample of 30 sections from 15 premolar teeth. SOS values from the literature are also shown.