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DOCTORAL THESIS

The Application of Dental Age Estimation Methods:
Comparative Validity and Problems in Practical Implementation

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TABLE OF CONTENTS

THEORETICAL BACKGROUND

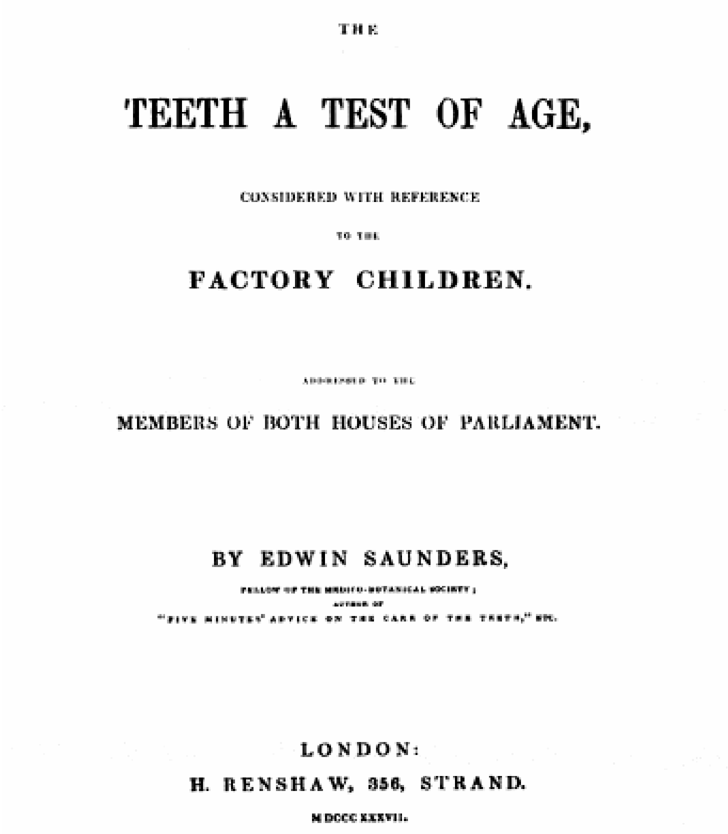
1. WHY DENTAL AGE ESTIMATION?

It is often necessary to estimate an individual's age due to certain questions, related to legal requirements, in palaeodemographic research, or in a forensic context.

Age at death estimation can include different dental aging techniques such as biochemical, morphological, or histological methods. According to the "Recommendations for Age and Sex Diagnoses of Skeletons" (Ferembach et al. 1980), an age evaluation includes the state of dental development, the ossification state of epiphyses, the appearance of ossification centres, the obliteration of the cranial sutures, the state of the pubic symphyseal surface, and the architecture of the femoral head. Rösing et al. (2007) also favour the mineralization and development of teeth in sub-adults and recommend some useful standards for age estimation of skeletons. Furthermore, the authors also refer to more recent techniques such as the racemization of aspartic acid, the layer count in dental cementum, and the evaluation of histological features in bone.

Dental age estimation in the living is mostly based upon non-invasive methods, which evaluate the timing and sequence of defined growth stages of the developing dentition and the sequence or modification of traits in the mature dentition and the surrounding tissues. Therefore, the recommendations for the age estimation of living persons include a dental status and a panoramic radiograph, a general physical examination, and the X-ray examination of the hand (Schmeling et al. 2004).

The use of teeth for determining someone's age has its origin 170 years ago when tooth eruption was first used for dental age estimation in connection with child labour (**Fig. 1**). In response to the need for age estimation of factory children who were not allowed to be employed under the age of nine and with a restricted working time between 9 and 12 years of age Saunders presented tables (Saunders 1837).



THE

TEETH A TEST OF AGE,

CONSIDERED WITH REFERENCE

TO THE

FACTORY CHILDREN.

MEMBERS OF BOTH HOUSES OF PARLIAMENT.

BY EDWIN SAUNDERS,

"FIVE MINUTES' ADVICE ON THE CARE OF THE TEETH," ETC.

LONDON:

H. RENSHAW, 356, STRAND.

MDCCCXXXVII.

Fig. 1 Saunders' cover page. Reproduced from Miles, A.E.W. Dentition in the Estimation of Age, *Journal of Dental Research* 1863a;42:225-263.

Saunders's proposal raises the question if gingival emergence and tooth development constitute stable traits that legitimate the use in dental age estimation. In the era of dental age assessment, the problem of separating possible effects of the environment and hereditary is a controversial issue. Consistently low correlations indicate the lack of a clear association between tooth formation and parameters like the social status, nutritional effects, and somatic development (Garn 1965, Lauterstein 1961). The findings of Demirjian and colleagues suggest that dental development shows no significant relationships with maturity indicators such as the menarche, peak height velocity, or skeletal maturity (Demirjian et al. 1985). These results imply that the mechanisms controlling dental growth and development are independent of general growth mechanisms but closely approximate chronological age. Also, tooth formation is less affected by malnutrition, endocrinopathies, and other pathological insults than other tissues. This was shown by studies on adolescents with severe abnormalities affecting sexual maturation and bone age that were found to have relatively small deviations in timing of dental development (Cattell 1928, Kuhns et al. 1972, Taft 1941). Cameriere et al. (2007c) conducted a study with 2 subsamples of Peruvian schoolchildren (undernourished, well-nourished) and found that nutrition does not affect the process of tooth growth. One reason for this circumstance could be that teeth consist of bradytrophic tissues and do not undergo continuous remodelling processes.

On the other hand, bone age is known to be influenced by the nutritional state (De Simone et al. 1995, Little et al. 2007). However, the skeletal age of immature individuals, mostly derived from the appearance of bony epiphyses and bone size, serves as a valuable marker for alterations and defects of growth when compared to dental and chronological age. Generally, it can be said that the skeleton is useful in determining the physiological age of an individual. Dental age, which is determined from dental hard tissues, is more closely related to someone's chronological age and therefore essential in age estimation.

The timing of tooth development is highly heritable but also population-specific. This was shown by comparison of different populations. Distinct stages in tooth development differ remarkably up to years between different ethnicities (Olze et al. 2004b). Compared to the formation (that includes the formation of organic matrix and its subsequent calcification), the eruption (the emergence of teeth through the gingiva) of teeth seems to be less resistant to external influences. It is known that tooth eruption can be affected by tooth loss, caries and malnutrition (Alvarez and Navia 1989).

Another characteristic besides the stable formation mechanisms makes teeth a valuable source of information also in age at death estimation: the extreme hardness and durability of dental hard tissues. It occasionally happens that all that remains of a corpse are some teeth, or just one tooth. This is because teeth are less affected by factors such as scavenging animals, humidity, microbial activities, mechanical forces, and high temperature than soft tissue and bone. It has successfully been demonstrated, that even cremated teeth provide sufficiently conserved substance for a reliable age estimation procedure (Grosskopf and Hummel 1992). Furthermore, a variety of methods is applicable even to single teeth. This offers the chance to estimate an individual's chronological age with only minimal destructive interferences.

2. THE PRINCIPLES OF DENTAL AGE ESTIMATION

Literature on dental age estimation provides broad and extensive information about various methods, their technical implementation, and underlying mechanisms. Dental age can be assessed according to developmental traits such as mineralization, gingival emergence, the quantification of cementum layers or the narrowing of the pulpal space. Degenerative changes such as dental attrition, or periodontal recession also show a correlation with chronological age. Furthermore, a variety of parameters such as the fluorescence intensity and density of dentin, the racemization of aspartic acid or dentin sclerosis help to evaluate the age-related conversion of dental tissues that can be used in human age estimation. The major areas of dental age estimation, but also some related age-dependent changes in human teeth will be outlined below in reference to the literature where further details are available.

2.1. Degree of tooth formation

Tooth formation is a complex process that begins with a gradual reorganization and a shift in the phenotype of embryonic cells. Deciduous teeth start to develop in the 6th to 8th gestational week; permanent teeth in about the 20th week of gestation (Ten Cate 1998). In age estimation the term "tooth formation" usually refers to the mineralization of dental hard tissues and does not necessarily consider unmineralized formation stages of the tooth germ. A reason for this may be that mineralized tissue can be easily evaluated via radiographic methods and remains after death and decomposition of the organism.

Data on the timing of human dental development is based on radiographic, histological, and morphological studies. Calonius and colleagues (1970) published a set of **histological criteria** which allow estimating an individual's age from the 7th week of gestation up to 3 years of age. These criteria include characteristics of bone, salivary glands and tooth formation. Based on the measurement of the developing tooth, Stack (1960) established the correlation between the **dry weights** of the mineralized tooth germs with gestational age in foetuses. Gestational age and the **height of deciduous tooth germs** were also found to be correlated (Hillson, 1996). The prenatal development was also studied histological by Krauss and Jordan (1965) and Sunderland et al. (1987). First data on postnatal calcification has been available since the 19th century published by authors such as Magitot (Legros and Magitot, 1873) and Black (Black, 1883). Standards for deciduous teeth were developed by Liversidge (1993).

The tables of Schour and Massler (1941) have become a classic example of the frequently used **atlas approach**. Observations on whole jaw sections made by Logan and Kronfeld (1933) enabled the authors to prepare diagrams which are each associated with a particular age. This atlas-based system does not evaluate root formation stages but deals with the sequence and timing of emergence of tooth crowns in the oral cavity. Schour and Massler's data represent 22 stages of dental development starting from 5 months *in utero* until 35 years of age (**Fig. 2**). Critical voices noted that the work was based on a small sample of terminally ill children. Kahl and Schwarze (1988) updated the Schour and Massler data and showed a delay in the development of the permanent dentition of their Cologne study group in comparison to the subjects of 1941.

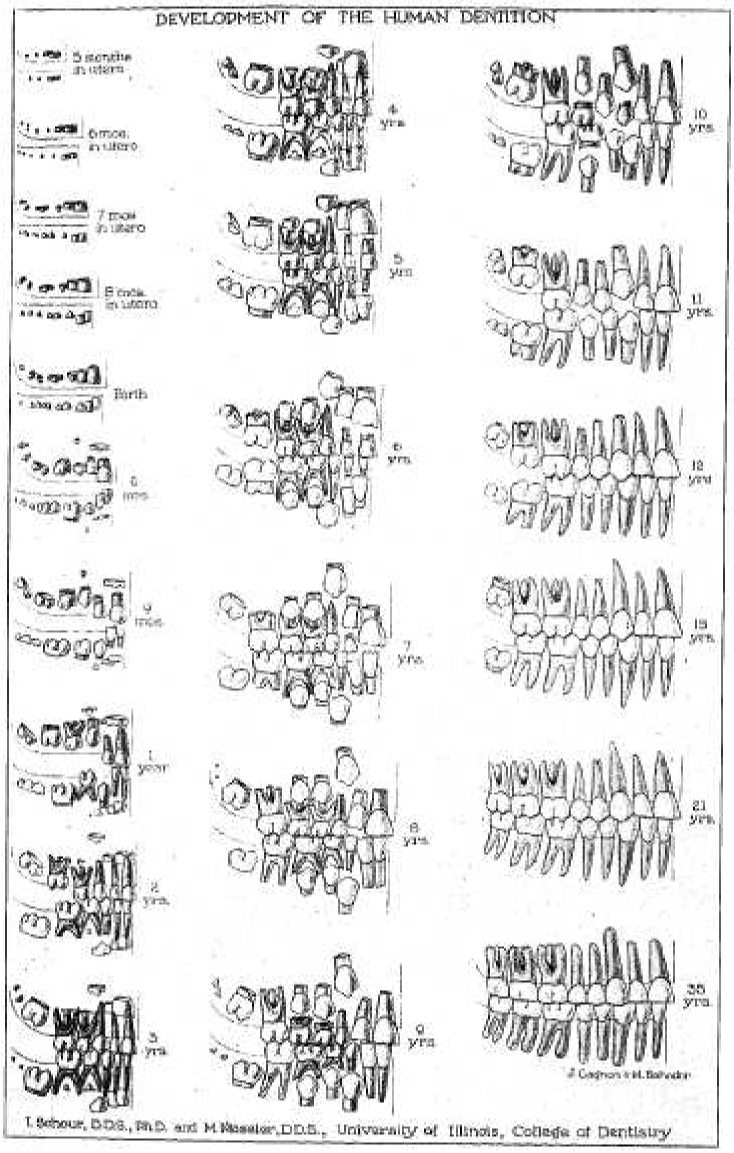


Fig. 2 Schour and Massler's original dental development diagram. Reproduced from Schour, I. and Massler, M. The development of the human dentition, *Journal of the American Dental Association* 1941;28:1153-1160.

Another major revision of the Schour and Massler dental development diagram was done by Ubelaker (1978) who developed charts based on data from Native Americans (**Fig. 3**). These charts were included in the "Recommendations for Age and Sex Diagnoses of Skeletons" of 1980 (Ferembach et al. 1980).

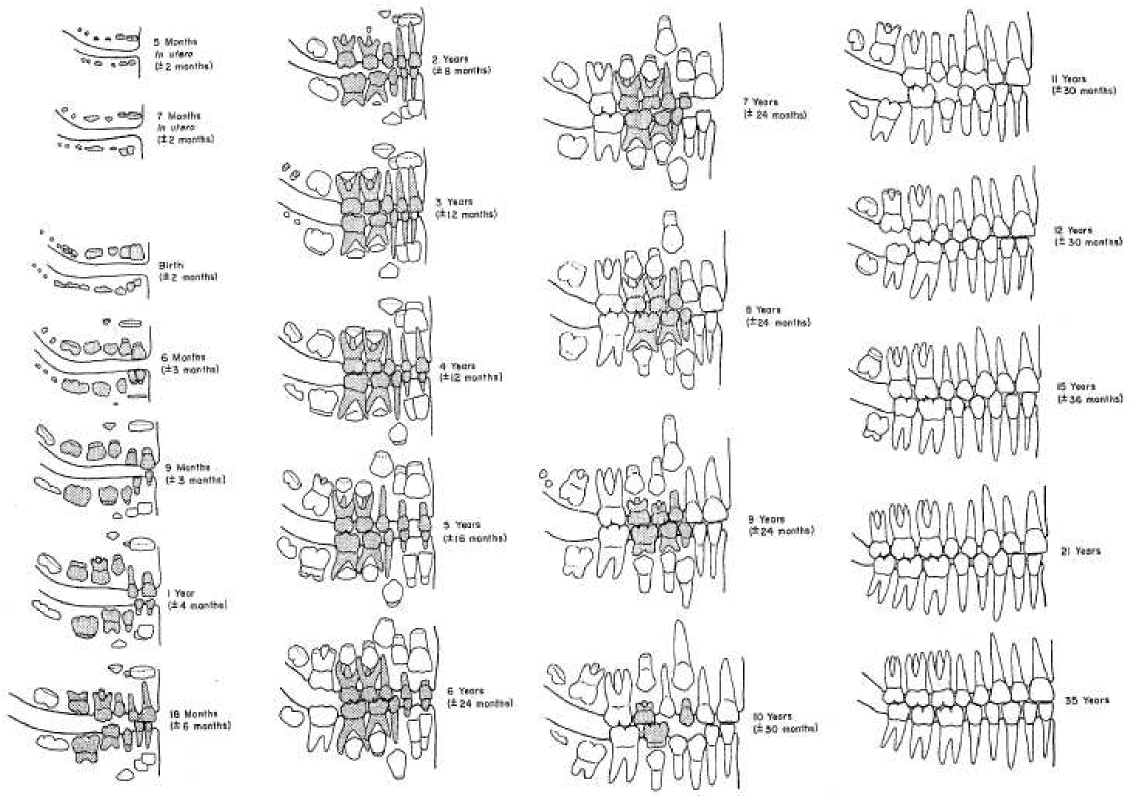


Fig. 3 Ubelaker's revised dental development diagram. Reproduced from Ferembach, D., Schwidetzky, I. and Stloukal, M. Recommendations for age and sex diagnoses of skeletons, *Journal of Human Evolution* 1980;9:517-549.

Gleiser and Hunt (1955) described detailed stages of the calcification of the mandibular first molar based on radiographs in the first follow-up study design in this field (**Fig. 4**).

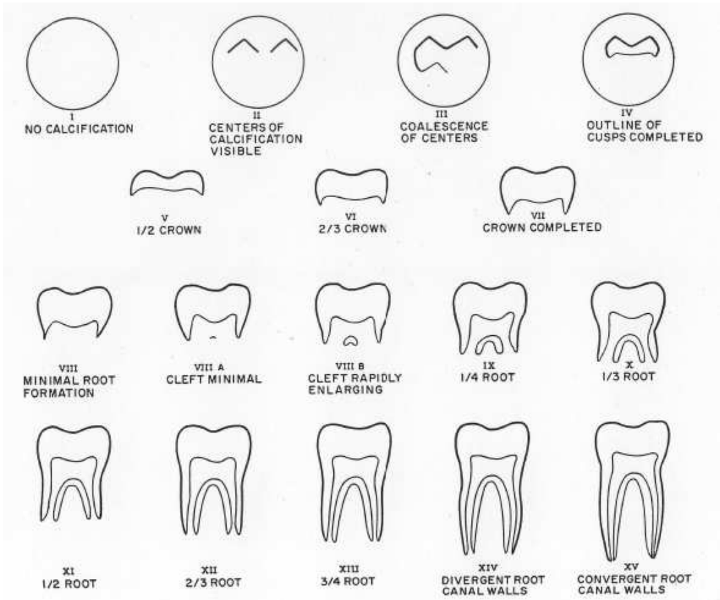


Fig. 4 Gleiser and Hunt's original stages of calcification diagrams of the permanent mandibular first molar. Reproduced from Gleiser, I. and Hunt, E.E. The permanent mandibular first molar: Its calcification, eruption and decay. *American Journal of Physical Anthropology* 1955;13:253-283.

In 1956, Demisch and Wartman presented data on the mandibular third molar. Garn and co-workers published complete data on all permanent mandibular molars and premolars using lateral oblique jaw X-rays (Garn et al., 1959). Moorrees and colleagues used data from the famous Fels Longitudinal Study in combination with their incisisor radiographs. The authors derived the age of attainment for 14 developmental stages from "initial cusp formation" to "apical closure complete" (Moorrees et al., 1963) for the maxillary incisisors and all eight mandibular teeth. These tables were updated by Anderson for all teeth including the wisdom teeth (Anderson et al., 1976) (**Fig. 6**). The stage system of Moorrees et al. was also used by Haavikko who undertook a cross-sectional study to establish the median ages at attainment that mainly confirmed the results of the original paper (Haavikko 1970). Another example for an atlas approach are the charts of Gustafson and Koch (1974) (**Fig. 5, 6**) which are based on the commencement of mineralization, the completion of the crown, the eruption of the respective tooth, and the completion of the root(s).

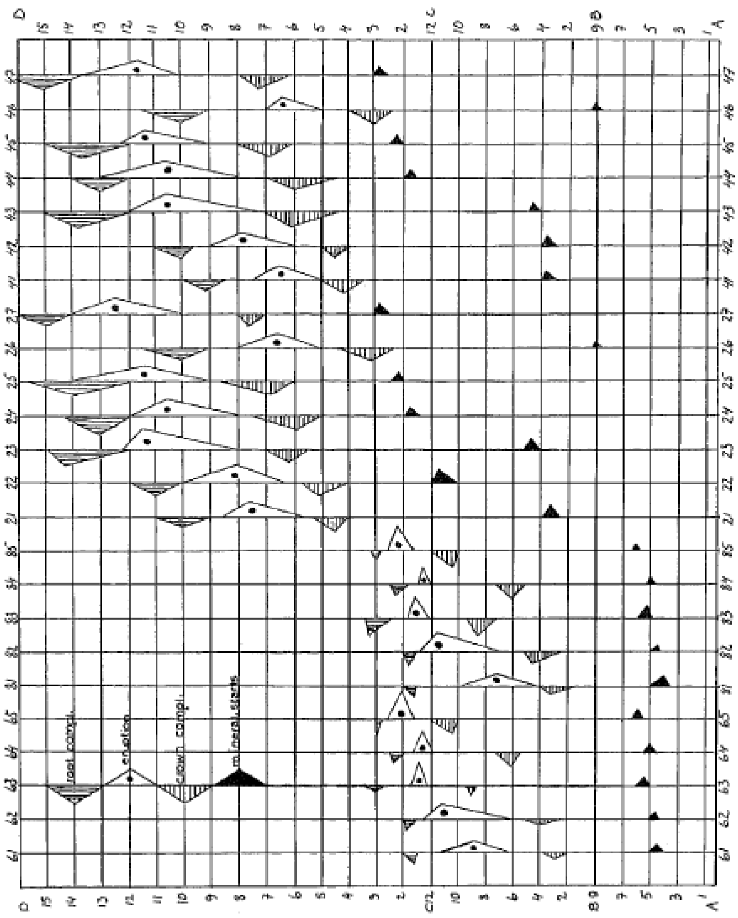


Fig. 5 Gustafson and Koch's original dental development diagram. A-B: intrauterine life, B-C: first year of life, C-D: 2-16 years of age. The base of the triangle represents range and the peak mean age. Reproduced from Gustafson, G. and Koch, G. Age estimation up to 16 years of age based on dental development. *Odontologisk Revy* 1974;25:297-306.

Demirjian at al. (1973, 1976) defined 4 developmental crown and 4 developmental root stages which were based on the appearance of the radiological visible tooth germ. In this approach a **scoring system** was used for the formation of 7 (or 4) left mandibular teeth. Every stage was assigned to a certain dental score. Adding up all scores gave the Dental Maturity Score which constitutes an indicator for an individual's dental maturity. The authors provided percentile distributions where the dental maturity score can be converted into an estimate of the subject's chronological age. **Table 1** lists some more recommended literature for dental age estimation of children.

Table 1 Recommended literature for age estimation in children

Deciduous teeth	Permanent teeth
Gustafson und Koch 1974	Haavikko 1970
Liversidge 1993	Demirjian et al. 1976
	Kahl and Schwarze 1988

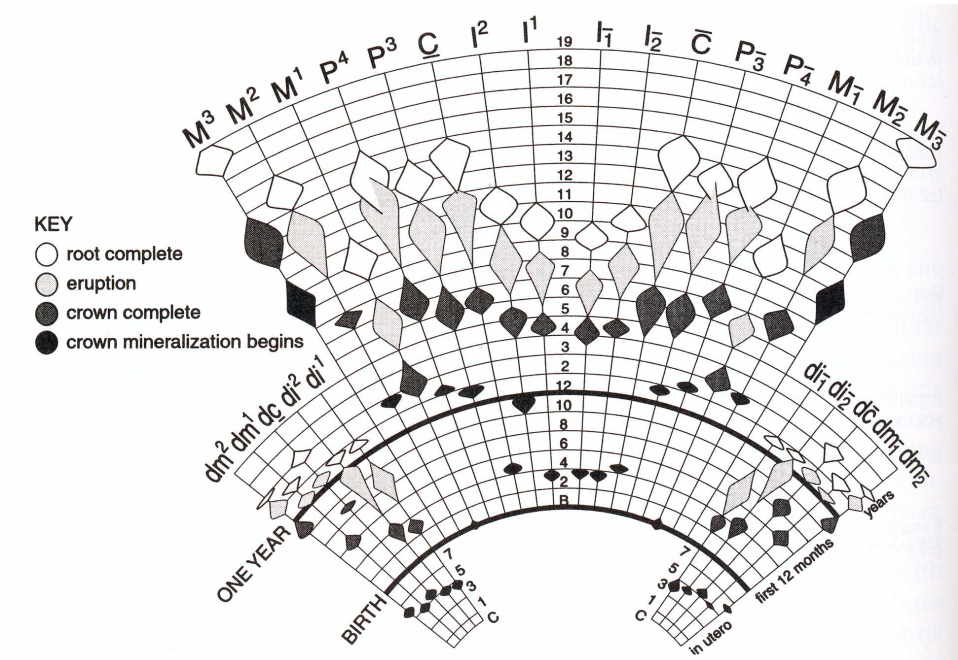


Fig. 6 Variation in the timing of dental development based on Gustafson and Koch (1974), with third molar data from Anderson et al. (1976). Range values are plus or minus one SD for the third molars. Reproduced from White, T.D. and Folkens, P.A.(2005). *The Human Bone Manual*. Burlington: Academic Press. p. 367.

Third molars are the teeth with the highest variability concerning anatomy, agenesis and age of eruption. Age estimation for medicolegal purposes by means of third molar development is used in the age between 14-21 years when all other permanent teeth have finished their formation. According to the recommendations of the interdisciplinary Study Group on Forensic Age Diagnostics, it is suggested to use Demirjian's developmental stages for third molar mineralization analysis (Schmeling et al. 2004). Demirjian's stages have the advantage of being clearly defined with radiographs, diagrams, and written criteria. This was also confirmed by a direct comparison of 5 common classification systems on orthopantomograms of females aged 12 to 25 years (Olze et al. 2005). The use of Demirjian's stages showed the best inter-observer agreement and was found to have the strongest correlation between the age estimate and true age followed by the classification of Kullman et al. (1992).

A well-known study on third molar development was published by the American Board of Forensic Odontologists (Mincer et al. 1993) who used Demirjian's developmental stages to assess the mean ages of attainment. Unfortunately the study is weakened by the diversity of its sample which suggests that this data should not be used in practice. A detailed study on ethnical differences in third molar mineralization was published by Olze et al. (2004b) who evaluated more than 3.000 conventional orthopantomograms. It was shown that Japanese, German, and South African individuals differ up to several years in reaching the respective developmental stage according to Demirjian. Therefore it is recommended to use populations-specific data in age estimation to guarantee for optimal reproducibility and feasibility of the evaluation.

2.2. Rate of formation of incremental structures in the tooth crown

The regular time dependency of some dental features of the tooth crown, examined by histological techniques, allow the assessment of development to estimate chronological age. Boyde (1963, 1989) suggested that age could be estimated from **prism cross striation counts**. Prisms are bundles of hydroxyapatite crystallites which form mature enamel. Crystallites in mature enamel are embedded in a gel-like organic matrix which amounts about 1-2% of the total volume. The prisms do not run straight through the enamel (Boyde 1989). Enamel prisms exhibit series of regular swellings and constrictions that result from the circadian rhythm of enamel secretion. These periodic structures, visible in transmitted light microscopy of ground sections or fractured enamel, are known as prism cross striations. There is evidence that the intervals between cross-striations of enamel prisms represent the amount of prisms formed in a 24-hour period (Hillson 1996). Another dental feature visible in crown enamel are the **Retzius lines** (also: brown striae of Retzius) which run from the enamel-dentin junction (EDJ) in occlusal direction. Every line is thought to be caused by an enamel discontinuity which reflects a certain state of (hypo-) mineralization (Gustafson and Gustafson 1967) during amelogenesis.

Retzius lines can be found in permanent teeth as well as in the deciduous dentition, but only in postnatal enamel (Pantke 1985). The **neonatal line,** which constitutes a borderline between pre- and postnatal formed enamel (and dentin) (**Fig. 7**), is frequently used for age at death estimation. This trait is the first prominent brown stria of Retzius which can be found in deciduous teeth and the permanent first molar and is deemed as the result of a metabolic disturbance during the course of the birth (Schour and Kronfeld 1938).

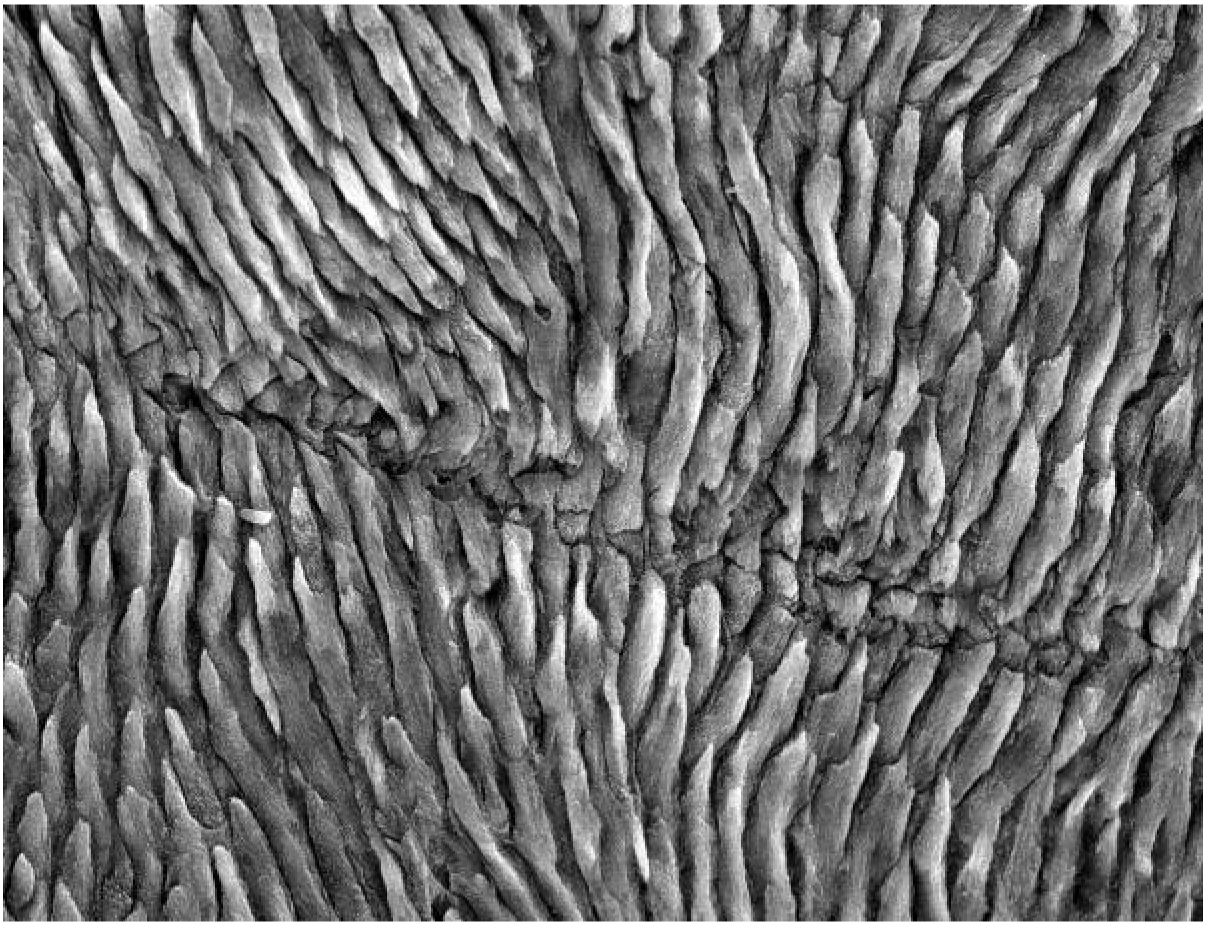


Fig. 7 Neonatal line (600x). Reproduced from Kühnisch, J., Dietz, W., Heinrich-Weltzien, R., Noren, J.G. and Stößer, L. Rasterelektronenmikroskopische Untersuchung zur Neonatallinie. *International Poster Journal of Dentistry and Oral Medicine* 2003;5(1):157.

This feature, combined with the total number of cross-striations, gives an estimate in days for the age at death. **Perikymata**, small visible lines on the enamel surface (**Fig. 8**), constitute the surface manifestations of the Retzius lines at the circumference of the tooth. It is assumed that perikymata are formed at the end of a cicaseptan interval that amounts about 7-10 day in humans and 2-8 in other primate species (Fitzgerald and Rose 2000). Dean and Beynon (1991) used brown striae of Retzius and perikymata groove counts to estimate the age at death. Another study on dental age estimation is based on the perikymata counts alone (Bromage and

Dean 1985) where the authors used cicaseptan intervals of modern humans to estimate early hominid teeth.

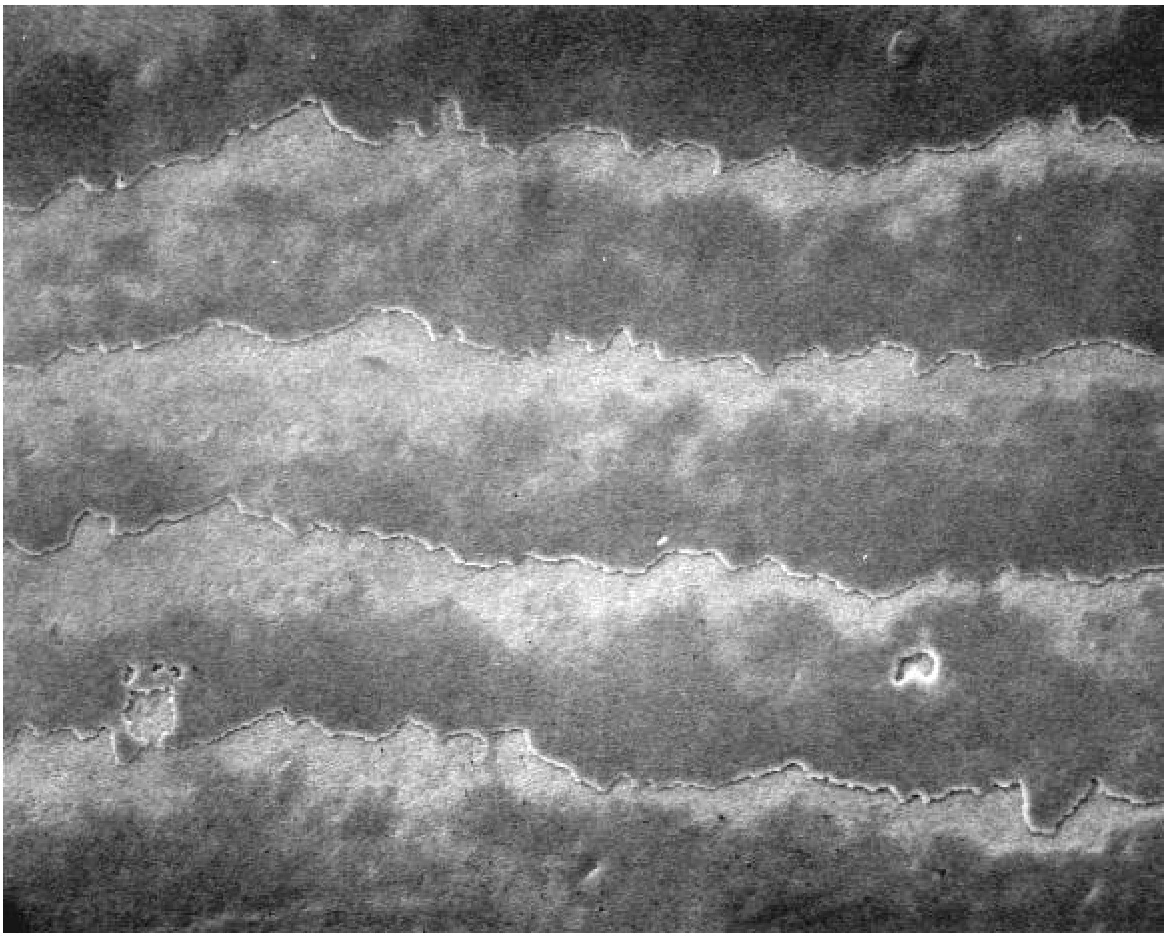


Fig. 8 Perikymata. Scanning Electron Microscopy (SEM) of an enamel surface. Reproduced from Mathias Nordvi, *www.iob.uio.no/studier/undervisning/histologi* (12.08.2007)

2.3. Age related changes of the pulpo-dentinal complex

Dentin, contrary to enamel, continuously forms appositional tissue. This **secondary dentin** is built physiologically and is formed slowly by cells lining the pulp chamber. Secondary dentin formation is initiated subsequent to dentinogenesis (Costa 1986). There is only little difference between primary and secondary dentin, which can sometimes be detected in stained preparations, via microradiography or in polarising microscopy (Hillson 1996). The continuous formation of secondary dentin is thought to be the biological response to masticatory stress and fluctuation of temperature (Rösing and Kvaal 1998). Philippas and Applebaum (1967, 1968) reported varying patterns of secondary dentin formation for different tooth types. The authors found the greatest amount of secondary dentin deposits in molars on the floor of the pulp chamber whereas the greatest dentin deposition occurs in maxillary anterior teeth on the palatal wall of the pulp chamber. In 1925 Bodecker was the first to establish that the apposition of secondary dentin is correlated with chronological age (Bodecker 1925). The apposition of secondary dentin leads to a gradual reduction in size of the pulp chamber and can effect the obliteration of the root canal. There is evidence that secondary dentin apposition occurs faster in male than in female individuals (Kvaal et al. 1995, Zilberman and Smith 2001). Gustafson (1950) introduced linear measurement of secondary dentin for age estimation as one of his proposed 6 criteria. Measurements quantify the amount of secondary dentin indirectly via the assessment of the decrease in size of the pulp cavity. The reduction in size can be quantified by linear assessments directly on tooth sections and on thin slices (Rösing and Kvaal 1998). Woods and colleagues conducted a study on the age-progressive size reduction of the pulp via linear measurements of the maximum pulp width, and root length. An r^2 ranging from 0.32 to 0.41 was found for the coefficient of determination of age with the pulpal width. The additionally measured tooth length gave no results (Woods et al. 1990). The pulp area can also be measured relative to the crown area on sections or radiographs (Rösing and Kvaal 1998). Kvaal and Solheim (1994) reported a method where the pulp width and length is calculated in relation to tooth width and length. A subsequent study (Kvaal et al. 1995) tested the method on periapical radiographs of dental patients resulting in an r^2 ranging from 0.56 to 0.76. The applicability of this method to conventional orthopantomograms (OPGs) was verified by Paewinsky et al. (2005). It was shown that the width ratios of the pulp cavity exhibited a significant negative correlation with age although the authors used a rather small sample between the ages of 14 and 81 years. Cameriere and co-workers focused on canines to study the pulp/tooth area ratio on orthopantomograms (Cameriere et al. 2004) and periapical X-rays (Cameriere et al. 2007a). Another approach employed a combined analysis of labio-lingual and mesial peri-apical X-rays of lower and upper canines from skeletons (Cameriere et al. 2007b). The authors achieved an R^2 ranging from 0.897 to 0.909 and a ME between 2.8 years and 3.89 years depending on the respective regression formula.

Interestingly, the progressive diminution of the pulp cavity has gained less scientific attention than other dental aging methods like attrition. This could be due to the quantification of secondary dentin which requires measurements and does not allow a visual assessment.

Dentin sclerosis (root transparency) (**Fig. 9, 11**) is another feature of the pulpo-dentinal complex which was first described by Tomes in 1861 (Tomes 1861). This trait undergoes a progressive change with age but is also a defensive reaction to caries, attrition, and drug treatment. During a person’s life dentin gradually becomes more calcified which is associated with sclerosis of the dentinal tubules and decreased fracture resistance. Root dentin sclerosis results in reduction of the diameter of dentinal tubules. The refraction index of the intratubular substance becomes the same as that of peritubular dentine. This process leads to a milk-glass like consistency of the dentin, which starts in the late adolescence at the apex of the tooth root and progresses towards the EDJ (Hillson 1996, Rösing and Kvaal 1998). Porter and colleagues examined the effect of aging on the mineral phase of dentin via high-resolution transmission electron microscopy. It was found that the mineral crystallites are smaller in transparent dentin and the tubule lumen appeared to be filled with coarse minerals made of hydroxyapatite. Notably differences in nanostructure between intra- and inter-tubular dentin in transparent teeth were observed. Although the nature of the age-related change is not yet known, the authors suggest a "dissolution-reprecipitation" mechanism for the formation of transparent dentin (Porter et al. 2005).

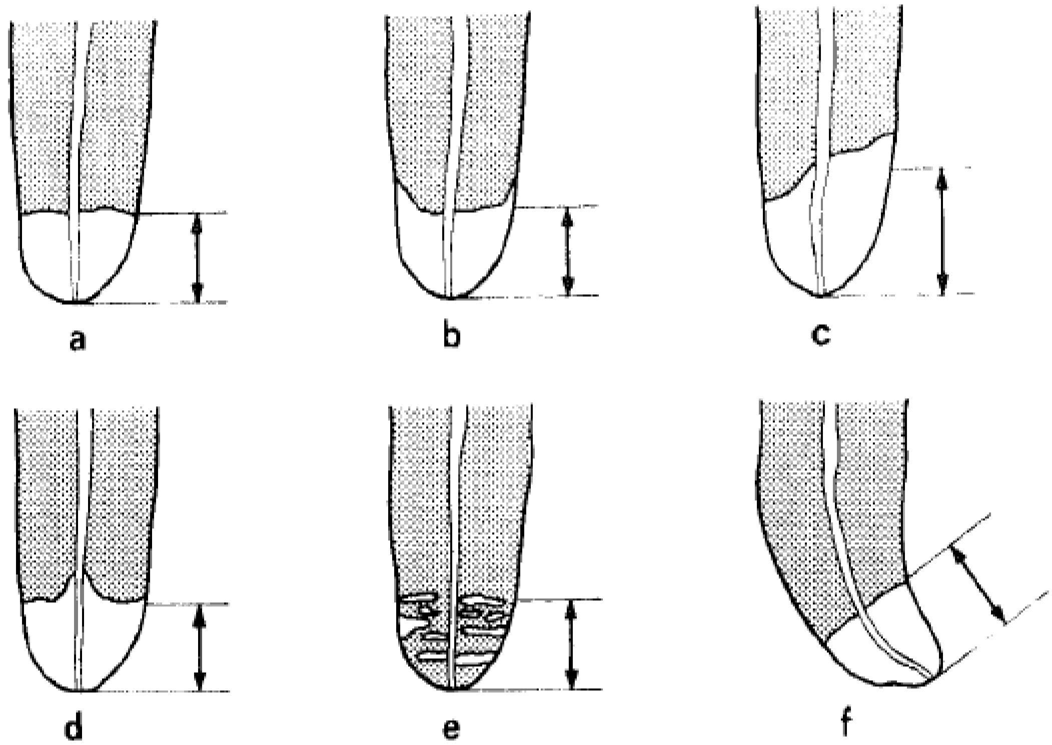


Fig. 9 Types of tooth root transparency and its measurement. Reproduced from Wegener R., Albrecht H. Zur Schätzung des Alters anhand der Zahnwurzeltransparenz. *Zeitschrift für Rechtsmedizin* 1980;86:29-34.

Root dentin transparency was first introduced in 1950 for age estimation by Gustafson as one of his proposed 6 criteria. Kamann (1998) evaluated the width of dentinal tubules via stained frozen sections. The authors found tubules with a diameter of 3-4 µm in under-aged individuals decreasing to 2 µm with advancing age. With a correlation of 0.28 Johnson (1968) failed to establish an index which increases the accuracy of age estimations based on the area of transparent dentin of tooth root sections. Bang and Ramm (1970) documented the association of age and the length of the dentin transparency and concluded that feasible results can be obtained for sectioned and unsectioned teeth specimens of up to 75 years of age. Bang and Ramm's technique was re-evaluated on a sample of histological sections derived from an osteological collection (Drusini et al. 1990). 21.13% of the cases showed <± 5 years error of estimation, almost 27% ± 5-10 years error and more than 15% were calculated to have more than ± 20 years error of estimation. The authors concluded that it might be disadvantageous to carry out Bang and Ramm's technique on sectioned teeth because of its destructive character, and due to the fact that sections might not reflect the real limit of the transparent dentin (especially in multi-rooted teeth). Drusini et al. (1991) made an attempt to evaluate the extent of root dentin transparency via computerized densitometric analysis to avoid the typical problems in establishing the boundary between opaque and transparent dentin in both, histological sections and intact teeth. The results showed no superiority of the image analysis system as compared to the caliper. Difficulties in estimating chronological age of skeletal material using the extent of root dentin transparency were reported by Sengupta et al. (1999). The authors applied a protocol for the assessment of root dentine transparency to modern material and to a historic Spitalsfield's sample. The modern sample turned out to correlate well with chronological age whereas the majority of the archaeological individuals were not measurable due to post mortem changes. Another study tested root dentin transparency for age at death estimation in 33 cases from the Charité University Hospital. A correct age estimate was obtained in 18 cases, 14 cases lay within ± 10 years of the true age (Olze et al. 2004a). Mandojana and colleagues (2001) analyzed morphological age-related differences between freshly extracted teeth and teeth derived from skeletal material. Higher values of dental colour, transparency length, attrition, cementum apposition, and secondary dentin were found in skeletal material when compared to the freshly extracted specimens. These results suggest that the post mortem interval could affect age-related morphological changes and requires caution regarding age estimation.

Another technique that uses root dentin transparency for age estimation is the method after **Lamendin** et al. (1992). Lamendin and colleagues presented an approach for age determination for single teeth that used periodontosis height and root transparency as parameters for age prediction (Lamendin et al. 1992) (**Fig. 10, 11**). Lamendin's mean error was found to be between 8.4 years in the control sample and 10 years in the working sample. The use of gingival regression as a variable has been criticized because the attachment level can easily be influenced by factors such as bad dental hygiene, irritation, systemic diseases, and drug treatment (Foti et al. 2001). The Lamendin two criteria method was also applied to a skeletal collection (Prince et al. 2002) of non-French origin. It was shown that the method also worked well with this material although the statistical analysis suggested that sex may be a relevant factor when employing the Lamendin method. The authors proposed to evaluate potential sex differences concerning the variables "periodontosis" and "root transparency" in further research. González-Colmenares et al. (2007) tested the sex-specific formulas developed by Prince et al. (2002) and obtained more accurate age estimations in a Spanish population than with Lamendin's original equation (1992). It was reported that the application of the Lamendin technique can be problematic in case of historic skeletal material (Megyesi et al. 2006). The mean error was found to be higher than in the original paper; approximately 35% of the evaluated teeth did not show any root transparency. The authors concluded that the presence of root transparency and the preservation of the teeth had a significant influence on the accuracy of the age estimate. Martrille and colleagues evaluated skeletal individuals from the Terry collection and found a quite similar mean error (ME) of 11.3 years for the Lamendin method when compared to the original results. The lowest mean error and standard deviation (SD) that was found was 6.1 years and ± 4.7 years in the age group of the 41 to 60 year old individuals (Martrille et al. 2007).

Fig. 10 Tooth 44 of a 27 years old male, view from distal. No visible signs of transparent dentin or periodontal regression.

Fig. 11 Tooth 34 of a 66 year old male, view from distal. Pronounced root transparency and periodontal regression.

Although the Lamendin method contains a certain degree of subjectivity due to the diffuse limit between transparent and opaque dentin, it offers the chance of dental age estimation in the field situation (Slaus et al. 2007).

2.4. The quantification of age related changes in cementum

Cementum is a dental hard tissue with mineralized organic matrix forming attachment sites for the periodontal fibres that link the tooth to the alveolar bone. Human tooth root cementum does not undergo continuous physiological remodelling processes like bone does. It is formed as primary acellular cementum and secondary cellular cementum that is arranged in layers (lines) (**Fig. 12, 13**) surrounding the human tooth root dentin. The layered appearance is due to structural differences in the mineral phase, an optical phenomenon that is possibly related to altered mineral crystal orientations (Cool et al. 2002) and reflects a cyclic annual formation pattern. One pair of alternating light and dark lines should therefore correspond to one year in an individual's life. If the count of cementum annulations is added to the tooth specific eruption age, the result is an estimate of the chronological age. A possible explanation for the layered organization of human cementum is the fact that growth hormone affects cementogenesis (Becks et al. 1948, Clayden et al. 1994, Li et al. 2001) which could be influenced by environmental factors such as the seasonal change (Bellastella et al. 1998, Holdaway 1997, Rudolf 1991).

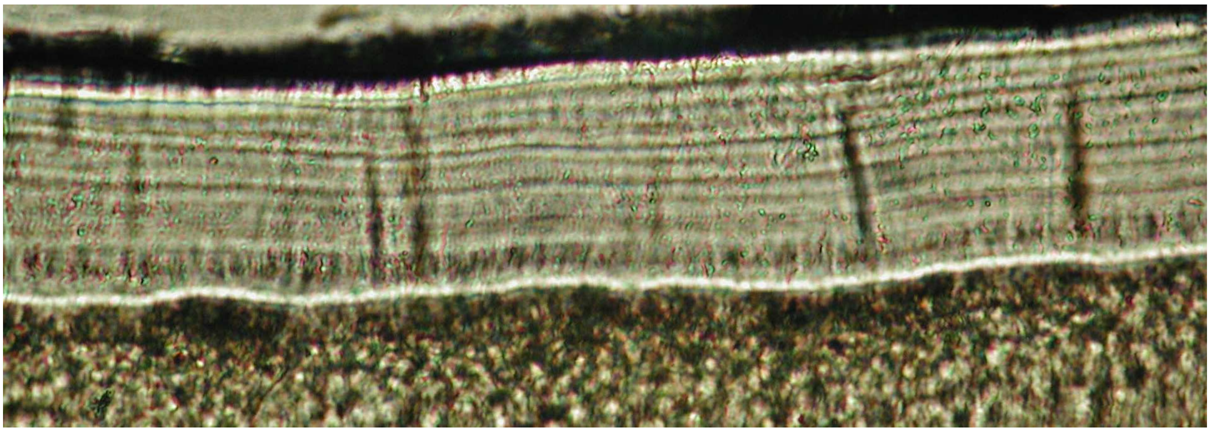


Fig. 12 Tooth cementum annulations of a 23-year-old male, tooth 32.

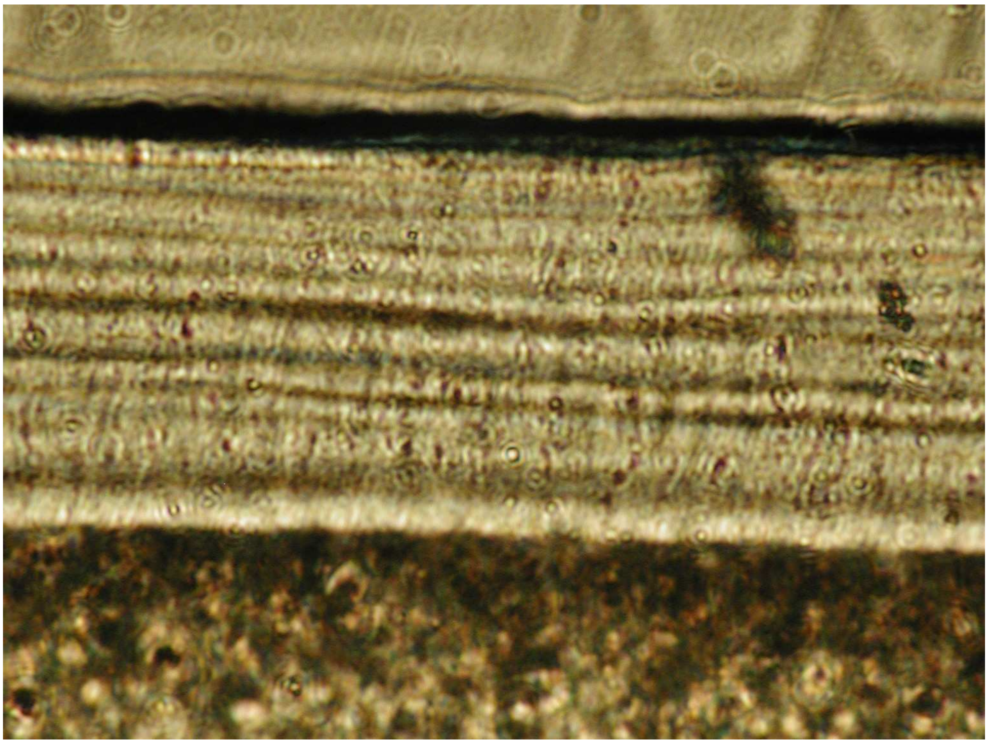


Fig. 13 Tooth cementum annulations of a 27-year-old male, tooth 44.

Gustafson introduced cementum for human age estimation (Gustafson 1950); Zander and Hurzeler were the first to discover a linear relationship between the growth of cementum and chronological age (Zander and Hurzeler 1958). **The quantification of layers in human tooth cement** as a technique for age at death estimation has it origins in wildlife biology (Klevezal and Kleinenberg 1967, Klevezal 1996) where the cement layering was routinely used as an aging method. Stott and co-workers were the first to publish a study on the use of cementum layer counting for age estimation in humans (Stott et al. 1982) (**Fig. 14**).

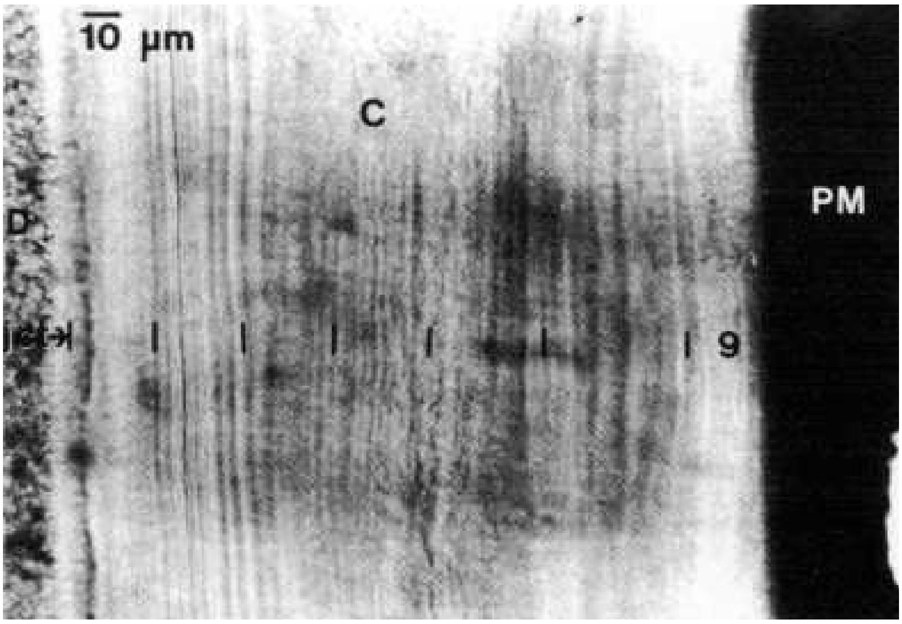


Fig. 14 One of Stott's original figures representing the cross-section the lateral lower right incisisor from a 76-year-old male. Reproduced from Stott G.G., Sis R.F., Levy B.M. Cemental Annulation as an Age Criterion in Forensic Dentistry. *Journal of Dental Research* 1982;61:814-817.

Some studies did not reveal a relationship between chronological age and the count of tooth cementum annulations (Lipsinic et al. 1986, Lucas and Loh 1986, Miller et al. 1988). Lipsinic and co-workers found an increasing underestimation with advancing age (Lipsinic et al. 1986). Miller et al. (1988) worked with thick resin sections, a factor that might have impeded a proper observation of tooth cementum annulations. Others reported a well correlated connection between cementum layering and chronological age (e.g. Charles et al. 1986, Condon et al. 1986, Maat et al. 2006, Nageshkumar and Nirmala 1998, Wittwer-Backofen et al. 2004) although a greater divergence between estimated and true age in older persons has been found (Pilloud 2004). Another approach aimed to verify the correlation of the thickness of radicular cementum and chronological age (Pinchi et al. 2007). Unfortunately the authors decided to use conventional paraffin histology with previous decalcification. This procedure leads to shrinkage, morphological changes, and artefacts in tissues that are high in calcium and phosphate. Therefore, decalcification steps of any kind lead to erroneous results and should be avoided in the analysis of cementum layering. A very recent paper further evaluates the seasonal apposition of cementum and its layered appearance (Wedel 2007). It was found that a marked transition between translucent (growth) and opaque (dormant) lines occurred in late September, while a less obvious transition occurred between March and April. Moreover, a significant relationship between the band width and growth season was stated.

The quantification of cementum layers is currently ranked as "second line" method in dental age estimation after the more precise racemization of aspartic acid (Rösing et al. 2007). Due to the fact that the nature of the mechanism that forms the cementum layers is not fully understood yet, it would lead to a significant improvement if further research provides more information about the cause and chronology of cementum layering.

2.5. Age-related change of tooth colour

It is generally known that teeth become more yellowish and brownish with age. The progressive **discolouration** is due to the degradation of organic components in the dental hard tissues, the deposition of external substances in enamel and dentine, mineralization, and associated alterations of the refractive index (Bang 1989). De Jonge (1950) and Brudevold (1957) recognized that tooth discoloration and staining must be associated with age but they did not pursue this observation further. Biedow (1963) suggested that tooth colour should be added to Gustafson's six criteria system for age estimation (1950) instead of the root resorption parameter. Ten Cate et al. (1977) presented a study where chronological age was estimated by tooth colour via visual assessment of untrained individuals, colour densitometry, and the visual assessment of trained individuals. The authors found a clear relationship between root colour and chronological age and best results when trained personnel evaluated the specimens. Solheim (1988a) used a dental shade guide to estimate the crown colour; root colour was evaluated by densitometry and by visual ranking according to a 5 degree scale. The weakest correlation was found for the dental shade guide in association with age. The densitometric measurements and the visual assessment each resulted in an improved Pearson's correlation. Lackovic and Wood (2000) used a flat-bed digital colour scanner to measure the CMYK-values at four different points of each root. Values between 0.81 and 0.94 were calculated for the correlation between the different colours and age, subdivided by sex. Another attempt to quantify the deepening of tooth colour was made by spectroradiometry (Martin-de las Heras et al. 2003). The authors performed the measurements as suggested by the International Commission on Illumination and obtained chromaticity coordinates, luminance, whiteness index, and yellowness index. The calculated correlation coefficient of the linear regression models ranged between 0.53 and 0.75; the average deviation amounted 13.7 years. Additionally, the influence of the post mortem interval on the colour measurements was also studied. The correlation between chronological age and colour turned out to be weaker in teeth obtained from skeletal remains than in freshly extracted teeth.

All these attempts to employ tooth colour as a measure for chronological age are still not satisfying. The problem of quantifying colour properly still remains. Moreover it needs to be determined if age-related root discolouration is a gradual process that proceeds mostly unaffected by external influences.

2.6. Change in chemical composition of teeth with age

A chemical component that changes with age is the **nitrogen** content which was found to increase with advancing age. This is thought to be correlated with the increasing amount and intensity of pigment that causes the change in colour of older teeth (Bhussry and Emmel 1955). No increase in **phosphate content** was found with advancing age but **metal ions (Fe, Cu, Sn, and Pb)** accumulate in older individuals (Brudevold 1957) by entering the outer enamel surface from the saliva. Bang and Monsen evaluated the **calcium content** of teeth and found a higher concentration coronal than apical. In older individuals the calcium content of teeth increases and is associated with an equalization of the different calcium concentrations (Bang and Monsen 1968). Energy-dispersive X-ray analysis shows that spherical structures in dentin comprise of calcium phosphate. The **fusion state of calcospherites** is correlated to chronological age in all layers of dentin (Atsu et al. 1998).

Another age related change in the chemical composition of dental tissues, useful for age estimation, is the **racemization of aspartic acid**. In the living body, proteins are normally composed of the L-form of amino acids, turning polarised light towards the left. Only some exceptional molecules are physiologically synthesized using D-form amino acids that have the optical property to turn the light towards the right. A gradual transformation of the L-form into the D-forms (racemization) of amino acids occurs throughout lifetime as well as after death. The racemization is related to factors such as temperature, pH, humidity and others. This process can be detected in metabolic inactive or bradytrophic tissues such as teeth. The racemization of aspartic acid was also used to investigate tissue turnover as a secondary phenomenon (Gineyts et al. 2000, Maroudas et al. 1998). It was first introduced to assess age from enamel (Helfman and Bada 1975) and was later applied to the analysis of dentin and cementum (Helfman and Bada 1976, Ohtani et al. 1995). Different racemization rates were reported for the acid soluble and the acid insoluble protein fractions of dentin (Ohtani and Yamamoto 1990). Using gas chromatography (GC) a number of researchers have confirmed the correlation of chronological age and aspartic acid racemization as the inevitable result of the natural aging of proteins. Fewer recent studies employed high performance liquid chromatography (HPLC) with UV detection (Gillard et al. 1990, Sajdok et al. 2006) or fluorescence detection (Benesova et al. 2004, Carolan et al. 1997, Fu et al. 1995, Mornstad et al. 1994). Due to the use of different analytical methods, sample preparation techniques, inconsistencies in the reported racemization ratios and the resulting age estimates were recognized. Therefore, recommendations have been published in order to establish an internationally accepted standard procedure (Waite et al. 1999).

An as yet uncommon technique in dental age estimation is the analysis of the **fluoride concentration** in dentin. Fluorides, which are contained in drinking water, food, and dietary supplements, are resorbed in the gastrointestinal tract and accumulated in dental hard tissues and bone. Dentin shows a higher capacity for fluoride uptake than enamel due to its tubular structure, collagen matrix, and water content. The concentration of fluorides in dentin is dependent on the fluoride administration and occurs most notably in areas near the pulp (Weatherell and Hargreaves 1966). Steinecke et al. 1995 (cited in: Schramm 2002) evaluated the age dependent fluoride content in enamel and dentin and found a correlation of r=0.31 for enamel and r=0.8 for dentin. Schramm (2002) compared dentin transparency and fluoride concentration in dentin in regard to their validity for dental age estimation. It was found that 69% of the cases were estimated right with ± 5 years deviation using the fluoride method. Age estimation via dentin transparency resulted in 61% of specimens that were estimated with ± 5 years deviation. Both methods exhibited deviations larger than 10 years when applied to >75 year old specimens, although the fluoride method proved to be more accurate in this case. Overall, the standard deviations of ±6.4 and ±6.6 for transparency and the fluoride method were comparable.

However, the analyzed sample was composed of selected teeth deriving from individuals who had lived in regions with a fluoride concentration in the drinking water not exceeding 0.3 ppm F^{-}. Long-term consumption of water with fluoride above this level as well as regular administration of dietary supplements could lead to an overestimation of age by the fluoride measurement method.

2.7. Fluorescence of dental hard tissues and its relation to age

A very interesting approach was presented by Kvaal and Solheim in 1989 who evaluated the **fluorescence of dentin** and cementum in 100 human second premolars. The authors chose red fluorescence from green excitation which was analyzed by a photometer. The tooth roots were sectioned longitudinally and scanned in a transverse direction at midroot area. The obtained plotter diagrams showed initial and final peaks representing the intensity of fluorescence recorded for cementum. Deep troughs in between were interpreted to represent the intensity of the fluorescence of dentin. The statistical analysis revealed a stronger fluorescence from cementum than from dentin and no statistically significant contra-lateral or sex-specific differences were found. Regression analysis of age versus fluorescence yielded a correlation coefficient of 0.73 for dentin and of 0.77 for cementum. The intensity of the fluorescence was found to be slightly stronger in teeth removed from human remains than for teeth from living patients. Tetracycline incorporation exhibited only slightly stronger red fluorescence during green excitation. Although the authors used an arbitrary scale for their measurements, which does not allow using the results for dental age estimation, they give an idea of the promising relationship between fluorescence in dentin, cementum, and age. Partial correlation coefficients demonstrated that an increase in fluorescence intensity is strongly associated with the deepening of dentin colour even after correction for age and extraction reason.

However, the fluorophores responsible for the respective emission peaks in the visible spectrum as well as the underlying cause for the deepening of dentin colour with age still remain to be identified.

2.8. Density of teeth in relation to age

Few studies investigated the altered **density of human teeth** in relation to age. Shikano found that the density in incisisors increased with age (Shikano 1956). It was assumed that a result of 2.28 (unit not specified) corresponds to an age of 50 years (± 4 years), a value of 2.32 was attributed to a 60-year old person (± 4 years).

Heuschkel and colleagues (1979) evaluated the density via a pycnometer in 130 teeth. Residual moisture was not removed but the pulp cavity was drilled out and the tooth sectioned into crown, root, and a middle piece. Unfortunately, the analysis did not consider the different tooth pieces. A standard deviation ranging from 0.123 to 0.131 was found, a significant relationship with age was not shown. Dufkova and Branik (1983) evaluated a total of 63 single rooted teeth with a pycnometer after an empirically determined drying time at 105°C to guarantee a stable weight at time of analysis. The authors could also show that a buoyancy method, carried out by a hydrostatical weighting instrument, produced a larger error due to the method itself. Although the measured standard deviation was ranging from 0.008 to 0.022, age estimates would produce deviations up to 20 years which makes the use of a density measurement in practice questionable. However, the density did increase with age, therefore it would be interesting if improvements of the introduced method could lead to more reliable results. Rheinwald (1966) evaluated the relative density of enamel with the pycnometer method. Although the crowns were manually separated from the root by drilling, the results clearly show that density increases with age. This trend, which is observable in dentin and enamel, is an indirect product of the age-related changes in mineral composition and hard tissue structure.

2.9. Epidemiologic criteria in dental age estimation

An example of an epidemiologic criterion for age estimation is the **DMFT-Index** (also: CER-Index or EKF-Index). The DMFT-index is a measure for the severity of caries and its effects in a population. It is expressed as the mean of carious (D=decayed), missing (M=missing), and filled (F=filled) permanent teeth (T=teeth) per person. The DFT-index expresses the mean of carious and filled teeth. Friedrich et al. (2003) evaluated orthopantomograms of patients aged 14 to 24 years and tried to answer the question whether fillings of wisdom teeth allow dental age estimation. The values “decayed”, “filled”, “decayed and filled” or “not decayed and not filled” were registered and subjected to statistical analysis. The majority of wisdom teeth in this sample showed neither caries nor fillings. Regardless these facts, the authors found an association between filled wisdom teeth and an age of at least 18 years. Another study examined if the DMFT index or the third molar DFT index could be used to determine whether a person has reached the age of 21 or not. The DMFT index showed to be variable and therefore unsuitable for age determination in this study. An indicative value was attributed to a third molar **DFT-index** of 4, because all examined subjects with an DFT of 4 were at least 21 years old (Andreas et al. 2004). A continuative study of the same authors evaluated the predictive value of the DMFT index of all permanent teeth, all permanent teeth excluding the third molars, the DFT index of third molars projecting beyond the occlusal plane, the eruption of third molars, and the periodontal recession of second molars. The probability of correct classification (of being 21 years of age or not) was 69.7% for males and 71.4% for females. Friedrich and colleagues evaluated panoramic radiographs of more than 1000 patients in 2005. The authors tried to determine whether prosthetic restorations, root canal fillings, and periodontal bone loss are correlated with chronological age, especially the 18^{th} birthday. A high correlation between the number of prosthetically restored teeth and chronological age was observed although these findings tended to be rarely present in the observed age group (14-24 years). A predictive value was also attributed to periodontal bone loss (Friedrich et al. 2005b). Another study out of the same sample showed that the values "decayed", "missing", and "filled" correlated weakly with chronological age (Friedrich et al. 2005a).

The DMFT-index can be used for an orientation but does not have strong significance in human age estimation by reason of high interindividual variation and the influence of dietary habits and caries prophylactic measures. A rough estimation becomes possible if the caries rates in the specific age groups (decades) are known (Endris 1979).

2.10. Attrition as a marker for age estimation

Attrition describes the most obvious evidence of age-related influence on the human dentition: The loss of tooth substance at incisal edges and occlusal surfaces. Attrition is not uniquely caused by the prolonged exposure to masticatory forces, but is also correlated with dental hygiene, dysgnathy, bruxism, and dietary habits. In modern people, the attrition rate is too slow for age estimation purposes so that attrition can only be used in an anthropological context for historical material. Attrition is evaluated by examining incisal and occlusal tooth surfaces according to the respective method in order to allocate an age to the specimen. Miles presented a method which calibrated the rate and pattern of attrition in molars with eruption. The author developed a wear diagram of the three molars with an age scale by selecting a baseline group of the working sample from an Anglo-Saxon burial site where at least one of the permanent molars was still erupting. (Miles 1962, Miles 1963a, 1963b) (**Fig. 15**). Murphy (1959) suggested a standard diagram graded from A to H that illustrates the gradual dentin exposure. A very similar system is that of Smith where a certain score is assigned to the exposed dentine pattern (Smith 1984). Also quite analog is the method by Molnar (1971) which records the exposure of dentine, but also the shape and orientation of wear facets. Widely used is a method based on British Neolithic to Medieval skeletal material which was subdivided into four age subclasses (Brothwell 1963, 1989). Brothwell's table represents the expected dentine exposure in the permanent first three molars. The resulting wear pattern also worked in non-British material (Hillson 1979). Another attempt was made to record attrition by measuring the crown height although the results showed only a moderate correlation with the chronological age (Mays at al. 1995, 2002), possibly due to the slow attrition rate of the study sample.

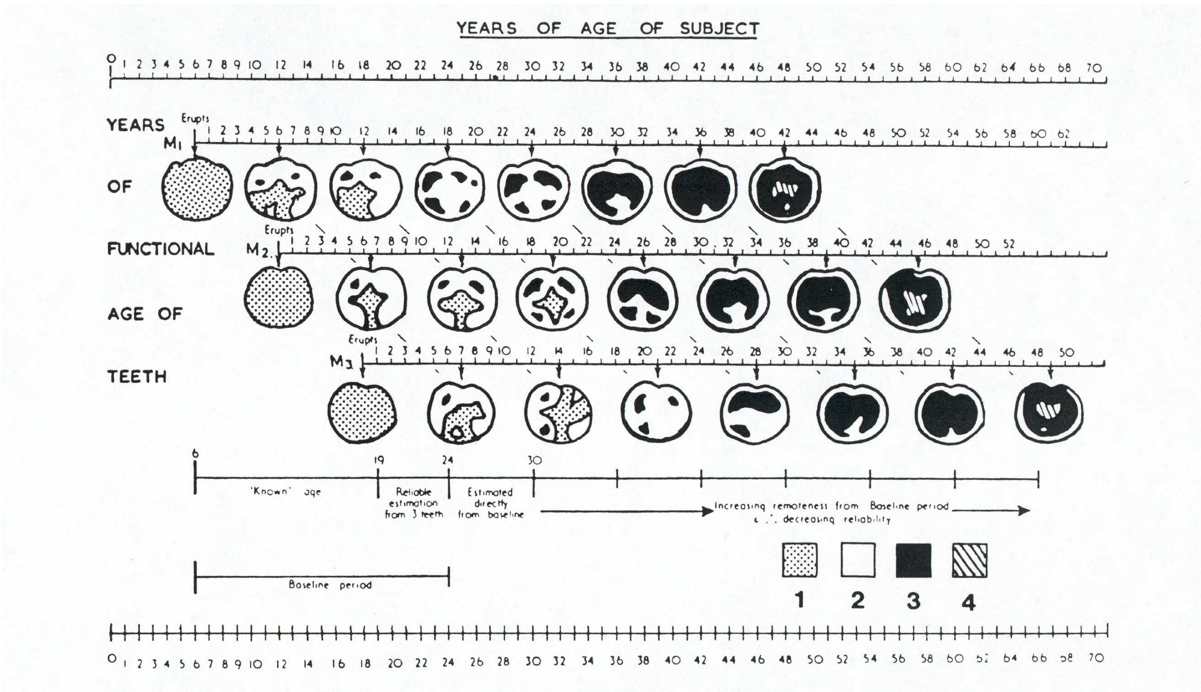


Fig. 15 The attrition scheme devised by Miles (1962) to assist in the estimation of age for individuals from earlier populations (redrawn). Tooth wear symbols: 1 unworn or polished enamel; 2 severely worn enamel surface; 3 progressive dentin exposure; 4 secondary dentin or pulp cavity. Reproduced from Işcan, M.Y. (1998). *Age markers in the human skeleton*. Springfield: Charles C. Thomas.

Solheim (1988b) evaluated the attrition scoring systems of Gustafson (1950), Johanson (1971), and Dalitz (1962) on a sample consisting of 1000 teeth. The highest correlation between age and attrition was found in mandibular second premolars (r=0.68) scored according to Johanson, the weakest correlation exhibited maxillary first incisisors and canines scored according to Gustafson (r=0.22). Johanson's scoring system showed the strongest correlation with age in most types of teeth whereas an influence of sex was also detected. An approach using dental plaster casts, in combination with a computer assisted image analyzer for the assessment of dental attrition, showed that molars tend to have higher positive r values for the correlation of attrition area with age (lateral incisisor: 0.20, second molar: 0.55). A multiple stepwise regression analysis using the variables "attrition area" and "attrition number" revealed correlation coefficients up to 0.93. However, a wide individual variation of the attrition was observed within the tooth types. Furthermore, it was shown that the splitting of attrition into the variables "area of attrition" and "degree of dentin exposure" led to a further improvement of the method (Kambe et al. 1991).

Most of the methods that are used to quantify attrition contain a degree of subjectivity and result in relatively broad age ranges. Moreover it has to be considered that larger teeth show slower attrition than smaller teeth (Hillson 1996). If possible it should be avoided to use attrition as a sole indicator in dental age estimation. It is suggested to use attrition in conjunction with other aging methods to obtain a coherent and reliable age estimate.

2.11. Combined Methods

Gustafson combined 6 age-dependent variables (attrition, periodontal attachment, secondary dentin, cementum apposition, root resorption, root dentin transparency) for age estimation carried out on longitudinally ground sections (Gustafson 1950). Each variable was assigned separately to a score of 0-3. The sum of scores gave the estimated age via Gustafson's linear regression equation which should be applicable to all tooth types. The scores were found to be highly correlated with chronological age (r=0.98) and resulted in a SD of ± 3.63 years. A larger SD of ± 10 years (r=0.80) was found after revision of Gustafson's formula (Burns and Maples 1976). Maples and Rice (1979) recalculated the data and identified statistical errors in the original publication, but failed to demonstrate the original error estimate. These re-calculations provoke several researchers to re-evaluate the Gustafson method again. Lucy and Pollard (1995) published another statistical analysis of Gustafson's data and found also the Maples and Rice paper being erroneous. Miles made an attempt to make the method more objective by measuring the root dentin transparency instead of a visual assessment (Miles 1963a).

Another approach was presented by **Johanson** (1971) who applied a new scoring system to the original Gustafson technique (**Fig. 16**). Furthermore, he developed multiple linear regression equations and found the root dentin transparency to be most strongly correlated to chronological age followed by secondary dentin.

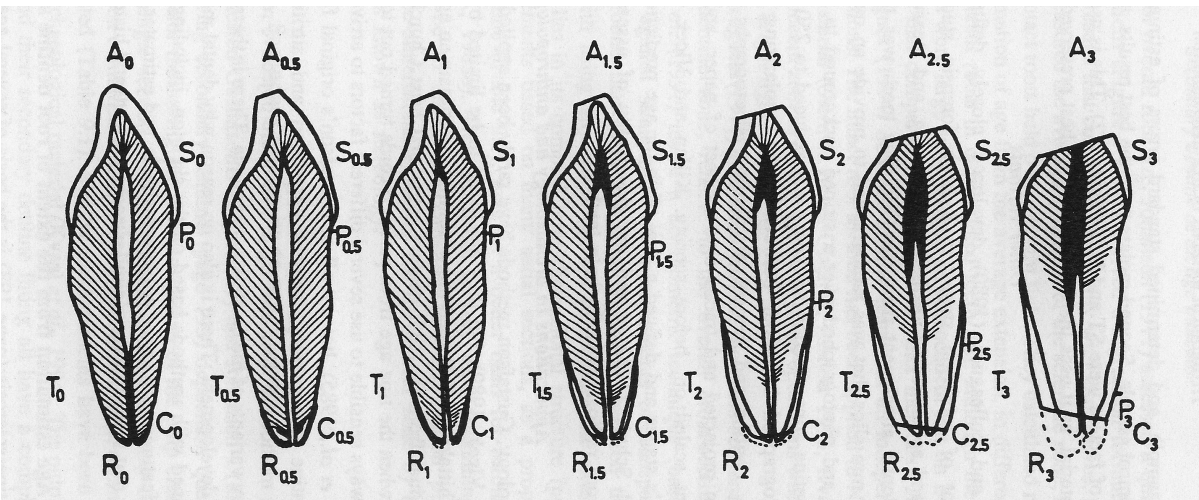


Fig. 16 Johanson's point scoring system. Reproduced from Johanson, G. Age determination from human teeth. *Odontologisk Revy* 1971;22:1-126.

The combination of several, strongly age-dependent dental features constitute a very promising approach for the estimation of dental age. Nevertheless use combined methods often variables of unequal reliability which might not be beneficial for an increase of accuracy.

FORMULATION OF RESEARCH QUESTIONS

The objectives of the present dissertation were to analyze five dental age estimation methods regarding their comparative validity and practical implementation. Furthermore, a purpose of the investigation was to supplement the literature cited above with data on dental age estimation in Austrian individuals.

In detail, the first study was undertaken to establish Austrian reference data on the timing of third molar mineralization evaluated according to the developmental stages proposed by Demirjian et al. (1973). It was an aim of the analysis to determine a possible sexual dimorphism in wisdom teeth mineralization. The prevalence of third molars in both of the lower quadrants was assessed in order to estimate the frequency of third molars. Another purpose of the study was to determine the probability of whether an Austrian individual is at least 18 years old based on Demirjian's stage H.

The aims of the second exploration were to apply Kvaal's method (Kvaal et al. 1995) to digital OPGs of Austrian juveniles and to verify if dental age estimation by means of secondary dentin is accomplishable in young adults. Therefore, it was decided to analyze whether the regression formulae of Kvaal et al. (1995) and Paewinsky et al. (2005) yield feasible and reproducible results that would legitimate their application to forensic age estimation.

The third study compared the potential performance of three dental age estimation methods regarding their objectivity and reproducibility. Due to the lack of a study that examines the quantification of tooth cementum annulations in comparison to macroscopic procedures, it was decided to explore whether the methods after Lamendin et al. (1992), Bang and Ramm (1970), and the quantification of tooth cementum annulations (e.g. Charles et al. 1986, Condon et al. 1986, Stott et al. 1982, Wittwer-Backofen et al. 2004) produce comparable results that can be used for dental age estimation. It was supposed that the methods do not yield equal accuracy, precision, and bias due to the different methodologies. Therefore, each of the named methods was evaluated in the same teeth sample to explore possible differences. Moreover, a goal of the study was to highlight vantages and disadvantages of the methods, but also to critically discuss problems of the practical implementation.

MANUSCRIPT 1: The chronology of third molar mineralization in the Austrian population-a contribution to forensic age estimation

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ABSTRACT

The aim of the present study was to determine the chronology of third molar mineralization and to establish Austrian reference data. Therefore, a cross-sectional study was undertaken by evaluating 610 panoramic radiographs in order to assess the mineralization status of the mandibular third molars of Austrian male and female individuals (275 males and 335 females) between the ages of 12 to 24.

The evaluation was carried out using the eight grade scheme of Demirjian et al.. Mean ages, standard deviations, standard errors and percentile distributions are presented for each stage of development. Significant differences between the left and right mandibular third molars were not found. Males reach the developmental stages earlier than females, statistically significant differences were noted in stages E and F. Both mandibular third molars were observed in the majority of the individuals of the Austrian sample (477 individuals, 78.2%). For medicolegal purposes the likelihood of whether an Austrian individual is older than 18 years or not was determined.

Key words: Forensic odontology, Dental age estimation, Third molar, Mineralization, Orthopantomogram.

INTRODUCTION

In recent years, the research area of forensic age estimation has gained increasing importance as shown by rapidly rising numbers of age assessment procedures in the German language region [1, 2]. A forensic age diagnosis is a combination of methods which can be used for verification of age to determine if legal age for criminal responsibility has been reached or not. It is applicable in cases of persons without valid identification documents.

Austrian law defines the 18th birthday of an individual as the relevant age limit for reaching adult status. According to the recommendations of the interdisciplinary Study Group on Forensic Age Diagnostics, age estimation of living persons involved in criminal proceedings should include a physical examination, an X-ray of the left hand, a recording of the dental status and an evaluation of a panoramic radiograph.

Radiographic assessment of the degree of third molar formation is a major part for forensic age estimation of adolescents and young adults. Due to the fact that all other permanent teeth have finished their development in this age group [3], third molars represent the only teeth still in development. Previous findings showed that the mineralization of the wisdom teeth is a population specific process and does not occur in every ethnic group at the same age [4, 5]. Therefore, it is crucial to use population specific reference data in forensic age estimation of living people.

Third molars are the teeth with the highest variability concerning anatomy, agenesis and age of eruption [3, 6-8] and are therefore its significance as developmental marker has been questioned. Age estimation for medicolegal purposes by means of third molar development is used in absence of other biological parameters during late adolescence and early adulthood. It should be kept in mind, that the results of some studies should be analyzed critically because of heterogeneous samples, small sample sizes or uncalibrated observers.

In the past, a number of methods were used to evaluate dental mineralization [9-12]. A comparison of these methods is difficult because of different design, number of evaluated teeth and stages. The eight grade scheme of Demirjian et al. [6] was developed for dental age estimation of children based on the first seven teeth. The authors proposed a scoring system which has been subject of critical considerations [13-17].

Only a few studies are available on third molar mineralization in late adolescence; Demirjian's stages are for reasons of accuracy [18] and practicability frequently used [4-5, 19-23].

The aim of the present study was to establish Austrian reference data on third molar mineralization evaluated according to the eight stages proposed by Demirjian et al. [6]. In order to estimate the frequency of wisdom teeth, the prevalence of third molars in both of the lower quadrants was assessed. From a medicolegal perspective, we determined the likelihood of whether an Austrian individual is at least 18 years old, which indicates adult status in Austria.

MATERIALS AND METHODS

The cross-sectional sample consisted of 610 orthopantomograms taken between 2002 and 2004 at the Bernhard Gottlieb University Dental Clinic, Vienna. The radiographs of 335 females and 275 males between the ages of 12 and 24 years (**Table 1**) were collected randomly over a period of three months. In the present study, it was focused on analyzing a sample which is representative for the Austrian population. Therefore, individuals with foreign surnames were excluded in order to get a homogenous Austrian sample. The individuals treated the University Dental Clinic are of different social classes and live in various regions of Austria. The anamnesis sheet of each individual was copied, and, if required, parts of the medical history. From the medical data, possible diagnostical findings, medications, orthodontic treatment and oral surgeries were recorded. Because of the satisfying data for each individual, it was decided not to make further exclusions in order to get information about possible outliers.

Table 1 Number of individuals per age group and sex

	Age (years)													Total (n)
	12	13	14	15	16	17	18	19	20	21	22	23	24	
Male	7	12	13	16	15	15	18	22	29	29	34	36	29	275
Female	15	11	14	19	17	23	29	30	34	33	44	39	27	335
Total	22	23	27	35	32	38	47	52	63	62	78	75	56	610

From each orthopantomogram, three digital pictures with different exposure times were made to compensate for the unequal brightness of the radiographs.

The mineralization of both mandibular third molars was evaluated following the eight grade scheme proposed by Demirjian et al. [6]. All ratings were assessed by the same observer (A.M.).

Intra- and inter- observer reliability was tested by reexamining 70 radiographs after several months. The orthopantomograms were chosen at random from the total sample and reevaluated under blinded conditions by the first observer (A.M.). The same radiographs were rated by a second observer (B.M.), who passed and initial training. Statistical comparison was performed using a paired *t*-test assuming a significance level of 5%.

Statistical analysis was performed using SPSS 10.0 and 13.00 for Windows (SPSS Inc., Chicago, IL), Microsoft® Excel 2000 for Windows and InStat for Mac® (GraphPad Software Inc.). Absolute and relative numbers of mandibular third molars in the Austrian population were determined. Mean ages with standard deviation and standard error at each developmental stage were calculated. Percentile distributions for both sexes at each stage were assessed. Additionally, the data was plotted to describe the association between score and age. For the convenience of statistical analysis, the data was subgrouped into one year age classes. The score was calculated by means of correspondence analysis with subsequent rescaling between 1 and 100. To demonstrate mandibular third molar mineralization at each stage, growth curves with the age as a function of attained stage in percent of the stages C-H were created. For this purpose, the following logistic equation was employed: f(x)=1/(1+Exp(a+b*x)). To determine the coefficient of determination (R^2) of each fit, the ratio of the difference between the corrected total sum of squares and the residual sum of squares was calculated. Unpaired t-tests were performed to evaluate developmental differences between both mandibular molars. Sex differences were determined using the Mann-Whitney U test. For each stage, the likelihood that the individual was at least 18 years of age was calculated.

RESULTS

Statistical analysis did not reveal significant intra-observer or inter-observer differences by repeated scoring of a subsample of 70 orthopantomograms ($P > 0.05$). Inter-examiner agreement was found in 85.0%, with no difference between two ratings exceeding more than one developmental stage.

The mean age of the 610 individuals of the analyzed sample was 19.84 ± 3.45 years, ranging from 12.01 to 24.96 years (**Table 2**).

Table 2 Age of individuals by sex

	Males	Females	Total
Number of individuals	275	335	610
Minimum age	12.08	12.01	12.01
Maximum age	24.95	24.96	24.96
Mean age	19.98	19.73	19.84
95% C.I.	19.56; 20.39	19.36; 20.10	19.57; 20.12
S.D.	3.48	3.42	3.45

Figure 1 details the absolute and relative numbers of mandibular third molars in the Austrian sample. Both sexes were pooled for this analysis, because it was not possible to exclude individuals with previous extractions. 477 (78.2%) out of 610 individuals possessed both lower molars. One mandibular third molar was recorded in 69 (11.3%) individuals; no lower molars were recorded in 64 (10.5%) cases.

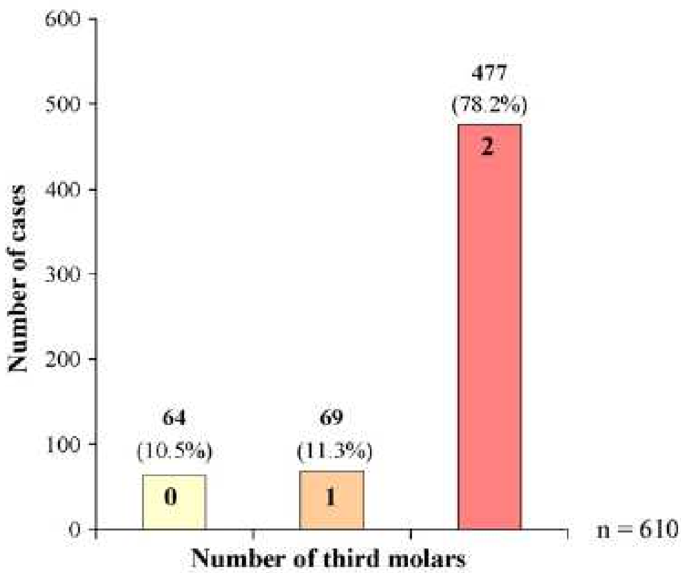

Fig. 1 Number of mandibular third molars

Table 3 and 4 chart mean ages, standard deviation and standard error of each developmental stage for the teeth 38 and 48, which are subdivided by gender.

Table 3 Mean ages, S.D. and S.E. at each stage for tooth 38

	Female (mean)	S.D.	S.E.	Male (mean)	S.D.	S.E.
A	12.6	–	–	–	–	–
B	12.3	0.3	0.1	12.7	0.4	0.2
C	14.6	1.6	0.4	13.7	1.0	0.3
D	15.4	1.8	0.4	16.1	2.3	0.6
E	17.8	2.5	0.5	15.6	1.8	0.4
F	18.4	2.4	0.4	17.5	2.6	0.6
G	20.3	2.1	0.2	20.1	2.1	0.3
H	22.9	1.3	0.2	22.4	1.8	0.2

Table 4 Mean ages, S.D. and S.E. at each stage for tooth 48

	Female (mean)	S.D.	S.E.	Male (mean)	S.D.	S.E.
A	12.3	0.5	0.3	–	–	–
B	12.4	0.4	0.2	12.7	0.4	0.2
C	13.8	1.3	0.4	13.5	1.0	0.3
D	15.5	2.0	0.5	15.3	1.6	0.4
E	17.2	2.8	0.6	15.1	1.6	0.4
F	18.5	2.3	0.3	17.6	2.0	0.4
G	20.5	2.2	0.2	20.1	1.9	0.3
H	22.8	1.4	0.2	22.5	1.8	0.2

The correlation between chronological age and Demirjian's stages is expressed in **Figures 2 and 3**.

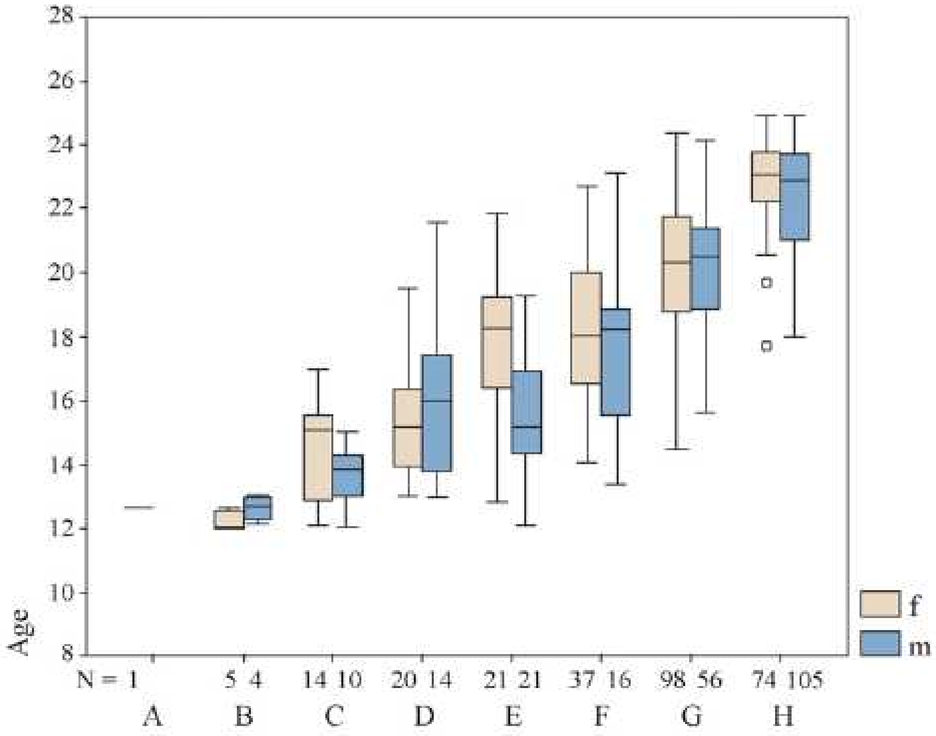

Fig. 2 Box plots of the correlation between chronological age and Demirjian's stages for tooth 38. Outliers are marked with °.

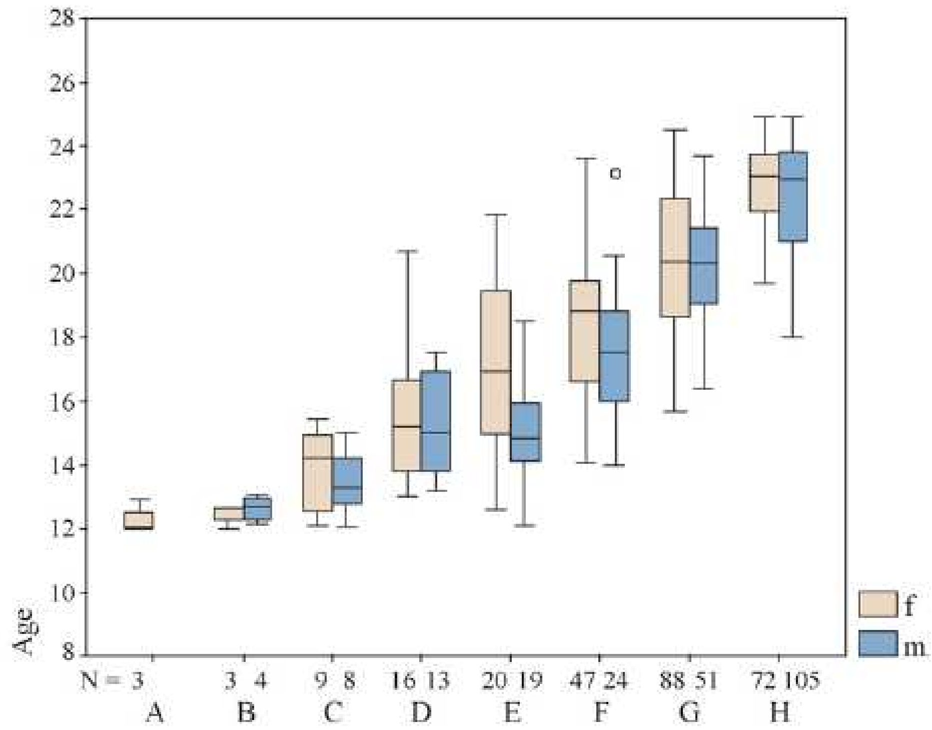

Fig. 3 Box plots of the correlation between chronological age and Demirjian's stages for tooth 48. Outliers are marked with °.

There is a trend for earlier third molar formation in females which becomes apparent at the early crown formations stages which show a slight advanced development in females than in males. At the developmental stage B, girls reach the indicators for this stage about 0.4 years earlier than boys. But there is a faster rate of formation in the male individuals that becomes strongly evident at stage E with boys reaching the corresponding criteria more than 2 years earlier. This result is consistent in the following stages with males being approximately 6 months ahead of the Austrian females (**Figure 4**).

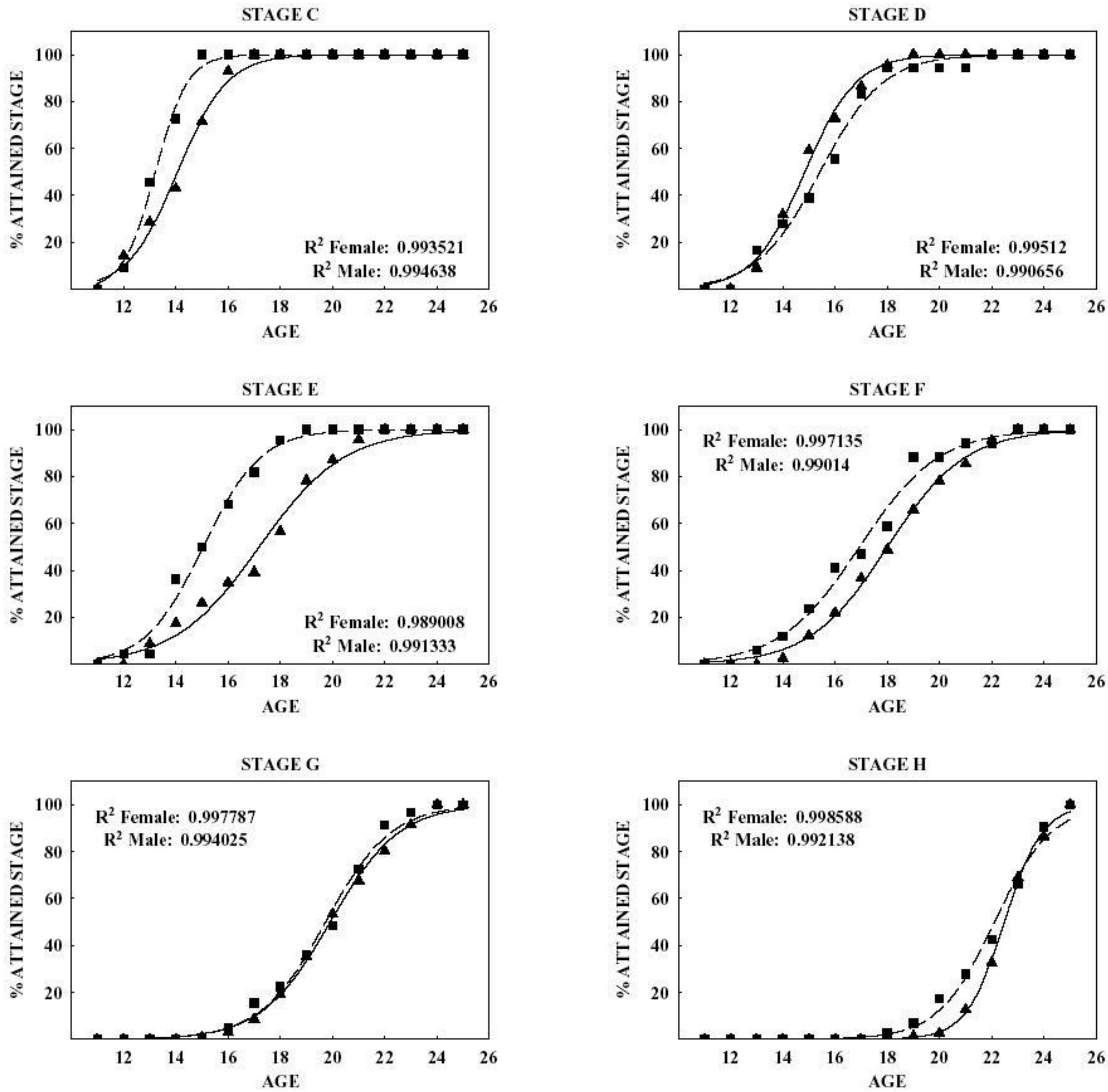

Fig. 4 Age as a function of attained stage in Austrian males (■) and females (▲) for Demirjian's stages C-H, including the coefficient of determination (R^2) of each fit.

The score as a function of age is presented in **Figure 5**. The score used here does not correspond to the maturity score originally used by Demirjian but gives an idea about the mineralization rate in each age group. According to the slope of the graph, third molar mineralization seems to occur faster in young individuals and decreases when the formation is almost finished. It can also be seen that the female mineralization, which is faster at the beginning of tooth formation, gradually decreases and stays finally at a lower level than the male one.

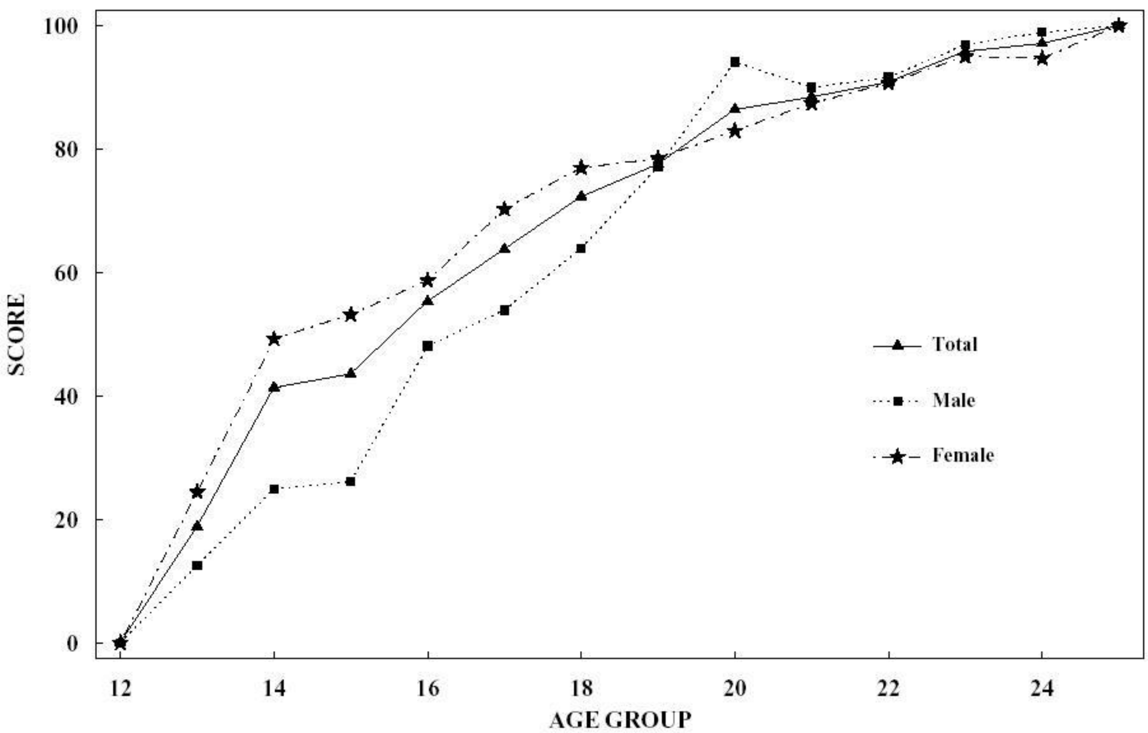

Fig. 5 Age as a function of score in Austrian males, females and total values of fourteen age groups using values derived from Correspondence Analysis. Rescaled.

A statistically significant sexual dimorphism was noted for stages E ($p < 0.01$) and F ($p < 0.05$). The data is presented in **Table 5 and 6** as percentile distribution to point out the faster rate of third molar mineralization in male Austrians.

Table 5 Age distribution expressed in percentiles for tooth 38

	10	25	50	75	90
H, male	19.7	21.0	22.9	23.8	24.5
H, female	21.2	22.2	23.1	23.8	24.6
G, male	16.8	18.8	20.5	21.5	22.6
G, female	17.6	18.7	20.4	21.7	23.5
F, male	13.8	15.5	18.2	18.9	21.3
F, female	15.1	16.5	18.0	20.0	22.1
E, male	14.1	14.3	15.2	17.0	18.4
E, female	13.9	15.7	18.2	19.4	20.9
D, male	13.1	13.7	16.0	17.5	19.8
D, female	13.2	13.9	15.2	16.4	18.4
C, male	12.1	12.9	13.8	14.5	15.0
C, female	12.2	12.8	15.1	15.7	16.6

Table 6 Age distribution expressed in percentiles for tooth 48

	10	25	50	75	90
H, male	20.0	21.0	22.9	23.9	24.5
H, female	20.8	21.9	23.0	23.8	24.7
G, male	17.0	19.0	20.3	21.6	22.4
G, female	17.5	18.6	20.4	22.4	23.3
F, male	15.3	15.9	17.5	18.8	19.9
F, female	15.1	16.6	18.8	20.0	21.3
E, male	13.0	14.1	14.9	16.1	18.3
E, female	12.9	14.9	17.0	19.5	21.5
D, male	13.3	13.7	15.0	17.0	17.3
D, female	13.1	13.8	15.2	16.9	18.7
C, male	12.1	12.6	13.3	14.2	15.1
C, female	12.1	12.5	14.2	15.1	15.5

With completed mineralization of the mandibular third molars at stage H, 90% of the individuals are 18 years of age or older. 75% of male and female individuals are already 16 years old when they reach the developmental stage D.

The development of the right and left side mandibular third molars was compared using unpaired t-tests. Statistically significant differences between the two sides were not found.

Based on Demirjian's stage H, **Table 7** expresses the likelihood of an Austrian being at least 18 years of age. The likelihood of an individual to have reached adult status with fully developed mandibular wisdom teeth amounts 99.5% for males and 99.3% for females. Additionally, one individual of the male subsample (out of 105 cases) and one individual of the female subsample (out of 74 cases) were found to present complete root formation of tooth 38 before their 18th birthday. Respectively, all orthopantomograms showed a completed root formation of tooth 48 at 18 years of age or more (105 males, 72 females).

Table 7 Likelihood of an Austrian individual being over 18 years of age based on Demirjian's stage H

	Male	Female
48	100.0	100.0
38	99.1	98.7
Mean	99.5	99.3

DISCUSSION

Demirjian et al. [6] distinguished four crown stages and four root stages in their mineralization scheme. They used figures (A-H) instead of numbers to prevent the impression of an equal duration of each stage. Moreover, no absolute measurements have to be taken and estimations about future root lengths are not necessary. Hence, the use of Demirjian's stages in forensic age estimation is easy to implement and is practical. As seen in the literature [4-5, 19-24] Demirjian's stages are used to assess third molar mineralization in adolescents and young adults.

Prieto et al. [23] analyzed 1054 orthopantomograms of a Spanish population between 14 and 21 years of age. In comparison to this study, stage C is reached by individuals of the Austrian sample about 0.8-1.3 years earlier. This slight developmental advance of Austrian subjects continuously diminishes in the following stages. At stage H (apex closure), Spanish individuals complete the wisdom teeth mineralization 2.8-3.2 years earlier than Austrians (sample size and age range of the Spanish population suggest critical observation). This finding is consistent with the study of Mincer et al. [21] and Arany et al. [19]. In relation to American individuals, as well as to Japanese subjects, Austrian individuals enter only the early stages at a younger age. Stages F to H are reached later, with an advancement of 2.0 years when compared to American subjects and 0.9-1.1 years when compared to Japanese adolescents at stage H. This finding is inconsistent when compared to data from Japanese individuals of a study performed by Olze et al. in 2004, which revealed an obvious heterogeneity when compared to the results of others [19]. This might be due to sample selection or differentially calibrated observers. Compared to individuals of South African origin [5, 24], gender differences seem to become apparent. Austrian male individuals reach stages F to H up to 1.1 years earlier; women of the Austrian sample met the criteria for the same developmental stages 0.5-1.1 years later than their South African counterparts. The results of studies performed by Olze et al. [22, 24] suggest a slightly faster mineralization rate in Austrian than in German individuals. The individuals evaluated by these authors were up to 1.5 years older when they reached the stages than the Austrian individuals. Only stage E was passed by female subjects of the German population about half a year earlier.

The male and female data were pooled to evaluate the frequency of mandibular third molars, because sufficient data about possible previous extractions of the individuals was not available. Therefore, it was focused on the determination of the number of individuals who can be evaluated by the presence of their mandibular wisdom teeth. Thus, 477 individuals or 78.2% of the Austrian population can be evaluated by 2 radiological observable mandibular third molars. 69 individuals (11.31%) showed only one, 64 (10.5%) no third molar. Although the sample may include individuals with extractions, these results are in good accordance with previously reported data [25].

No significant side differences of mandibular third molar mineralization in both genders were found. This trend was confirmed by other studies [19, 21-23, 25-28]. Differences observed in practice may be due to coincidence and usually do not amount to more than one stage.

The results of this study show a faster development of third molars in Austrian males than females and did not differ from findings of previous studies [3, 8, 10, 19, 21, 23, 25, 27]. This is a unique trait of third molars which expresses the sexually dimorphic character of tooth formation. Levesque et al. [25] demonstrated that Franco-Canadian males are reaching Demirjian's stages earlier than girls beginning with stage F. Prieto et al. [23] found a significant sexual dimorphism in stages E to G with males reaching the stages described by Demirjian et al. [6] earlier. In the Japanese Population investigated by Arany et al. [19], males entered the stages earlier than females; a significant gender difference was observed in the stages D, E and G. In line with these findings are reaching male individuals of the Austrian population the root formation stages (E-H) earlier than females. A significant difference was found in the stages E and F.

Many studies exclude individuals with any kind of pathology or irregular tooth development from further investigation. Insufficient space or a mutated axis of the germ do affect the eruption or inhibit this process [23-30]. Impacted third molars are thought to have delayed root formation [10]. However, a total exclusion of individuals with an impaired tooth development from reference studies has practical implications. Thus, odontological age estimation is only applicable to individuals with a regular dental status. Friedrich et al. published a study with 1053 subjects in order to evaluate the root formation of impacted third molars. The authors could demonstrate that retained third molars are not associated with impaired root formation [7].

Schmeling et al. [31] published for the Study Group on Forensic Age Diagnostics recommendations on the estimation of living persons involved in criminal proceedings. As noted by Wedl et al. [32], the authors defined requirements on reference studies which should be the subject of critical reconsideration. Under question are the terms "genetic-geographic origin", "socio-economic status" and "state of health" which must satisfy the recommendations of the AGFAD. Some of the studies referred to by Schmeling et al. [31] do not fulfil these standards. A standardized quality of reference studies has to be attained. But how far are "socio-economic status" and "genetic-geographic origin" reliable in a study? First these parameters must be well-defined, second the ethical aspects of these terms should be taken into account. Another question arises when the state of health must be determined. Besides the inaccurate definition, the opinions about influencing factors on tooth development widely differ.

Factors which do influence tooth formation, development and eruption are hard to detect. Acute systemic illnesses are currently discussed just like chronic diseases such as renal insufficiency, hypocalcaemia, hypothyroidism, but also malnutrition and sexual maturity [33-36]. A different approach was proposed by Pelsmaekers et al. [37] using a longitudinal study design. The authors evaluated the root formation of monozygotic and dizygotic twins and developed an ACE-model (additive genetic, common environmental and specific environmental). Based on this model, less than 8% of variance was attributed to specific factors. Radiation therapy seems to have an impact in the sensitive phases of germ and root formation [38, 39], just as an impairment of the germ by fracture [40]. Within the scope of some genetic diseases like Apert-[41], Williams-[42], Turner- [43-44] and the Fragile X-Syndrome [45], impairment in root formation has been described. The last named two diseases might be of particular importance for age estimation because an advanced root development was demonstrated. As noted by Rösing [46], a high number of affected evaluated individuals would limit the applicability on healthy individuals. On the other a hand, complete exclusion does not reflect reality and inhibits further analysis of possible influence factors.

For these reasons, it was decided to include all Austrian individuals for assessment and to take a closer look at possible outliers for further analysis. Surprisingly, none of the outliers possessed any characteristics associated with an altered growth. On the other hand, individuals with severe diseases who were estimated to be possible outliers showed no precarious findings. According to these results, the assumption is supported that tooth development underlies strong regulation mechanisms which seem hard to affect, even under pathological conditions.

According to the results of previous studies, Demirjian's developmental stage H could serve as a useful developmental marker to answer the question whether an individual is already considered as an adult. This stage marks the easy recognizable, fully mineralized tooth with apex closure. Therefore, the probability for an individual being older than 18 years was determined (Table 7). The calculated percentages indicate the degree of confidence whether a person has reached the age of 18 years. In the present sample, only two cases being younger than 18 years of age at the Demirjian's stage H were detected. The analysis showed, that the finished third molar mineralization indicates that the probability that an Austrian individual is at least 18 years old, is 99 % certain.

Conclusion

The present investigation provides representative data on mandibular third molar mineralization in the Austrian population. It can be concluded, that in the case where two mandibular molars are present, the probability for an Austrian individual to be at least 18 years is 99.5 or 99.3% for males and females, respectively in the case were tooth mineralization is completed (Demirjian's stage H). These results indicate that Demirjian's stage H might constitute a helpful marker in age estimation for medicolegal purposes; nonetheless wisdom tooth mineralization can always only be seen as just one detail in the interplay of structural chances during human growth. One should keep in mind, that age estimation by means of third molar mineralization is always limited due to biological variance. The obtained data may not be valid in other populations; therefore future research of third molar mineralization is necessary in order to make population specific reference data accessible for practical use. Moreover, an evaluation of the combination from the AGFAD recommended three methods is essential to demonstrate the accuracy of the procedure and to provide standardized quality in forensic age estimation. Although Schmeling et al. [2] published a verification of age estimation in living persons at the hospital Charité, which was performed in line with the recommendations of the AGFAD, it is not verified yet if this method increases the accuracy of age estimation as expected.

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MANUSCRIPT 2: On the Applicability of Secondary Dentin Formation to Radiological Age Estimation in Young Adults

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ABSTRACT

Literature provides linear regression formulas for dental age estimation which is based on radiological two-dimensional measurements of the pulp size. The aim of the present study was to explore if the previously presented regression formulas could lead to statistically sound results and to appropriate repeatability when applied on young individuals.

Orthopantomograms of forty four Austrian individuals, aged between 13 and 24 years, were selected at random. In accordance to the reported method, 6 teeth on each orthopantomogram were chosen to carry out the measurements. Statistical analysis was performed in order to assess the difference between the estimated and the true chronological age.

The regression formulas reported by Kvaal et al. (1995) led to a consistent underestimation; the regression formulas reported by Paewinsky et al. (2005) resulted in a constant overestimation of age. The statistical analysis of intra-observer and inter-observer variation revealed a variation width below 2%, respectively.

KEYWORDS: forensic science, forensic odontology, dental age estimation, secondary dentin, orthopantomogram

INTRODUCTION

Accuracy and precision of dental age estimation do depend on the age of the examined individual. Best results can be achieved, when the individual growth is fast and there are a number of teeth in development. Dental age estimation of individuals who are older than 14 years of age constitutes a great challenge. All permanent teeth, except the third molars (if present), have finished their development in this age group (1).

Up to now, a multiplicity of methods have been applied on this problem. Best results were provided by the analysis of tooth cementum annulations (2) as well as the determination of the degree of aspartic acid racemization, (3, 4) which can be correlated with chronological age. These methods are invasive; hence they cannot be used in living individuals and in cases where it is not acceptable to extract teeth for ethical, cultural or religious reasons.

Kvaal et al. (5) presented a method, which is based on radiological measurements and does not require extraction. The authors were able to demonstrate the negative correlation of a composition of different rations of the two-dimensional pulp size, which depends on the amount of secondary dentin, and chronological age.

Secondary dentin formation is initiated after dentinogenesis (6). The odontoblasts lining the pulp cavity continuously form layers of secondary dentin deposited along the wall of the dental pulp chamber. In 1925 Bodecker established that the apposition of secondary dentin was correlated to age (7). Secondary dentin is build-up throughout life and laid down on the pulpal surface of the primary dentine. This process leads to a continuous decrease in size of the pulp cavity (8-14). As a consequence of this deposition there is a tendency towards pulp obliteration. The pattern for the secondary dentin formation varies among the different tooth types. In maxillary anterior teeth the greatest dentin deposition occurs on the palatal wall of the pulp chamber with subsequent deposition in the incisal tip and the remaining walls. In molars the greatest dentin deposition is on the floor of the pulp chamber; lower amounts are deposited on the occlusal and lateral walls (13, 15,16).

Secondary dentin deposition was introduced for age estimation in the method by Gustafson (10), where secondary dentin is one parameter in addition to attrition, periodontal recession, cementum apposition, apical translucency and external root resorption.

In 1993, Drusini published a study which confirmed the negative correlation between the coronal index after Ikeda et al. (17) and the actual age of individuals using soft X-ray photos of intact adult teeth (18). The author was able to show that the correlation coefficients range from -0.73 (female molars) to -0.89 (female premolars).

The method by Kvaal et al. (5) represents an independent procedure to examine the relationship between pulpal size and chronological age. Based on the investigation of periapical radiographs, from individuals older than 20 years of age, it was shown that Pearson's correlation coefficient between age and the different size ratios for each type of tooth was significant. When six teeth of each individual were included, a coefficient of determination (r^2) for the estimation of 0.76 was calculated.

Paewinsky et al. (19) verified the applicability of this method on orthopantomograms (OPGs). They used a sample consisting of 168 individuals in between the ages of 14 and 81 years. A significant negative correlation between the width ratios of the pulp cavity and chronological age was shown. The authors presented linear regression formulas, although the same study found a higher coefficient of determination in upper lateral incisors when a cubic or logistic regression model was constructed.

The objective of the present study was to apply Kvaal's method to digital OPGs of Austrian juveniles and to evaluate whether the linear regression formulas of Kvaal et al. (5) and Paewinsky et al. (19) could yield feasible and reproducible results, which would legitimate their use in forensic age estimation.

MATERIAL AND METHODS

OPGs from 44 individuals with known age and gender were selected for the study (mean age: 19.2 years; 18 males; 26 females; **Table 1**). OPGs were taken in the period between 2002 and 2004 at the Bernhard Gottlieb University Dental Clinic, Vienna. Individuals with foreign surnames were avoided in order to get a homogenous Austrian sample.

TABLE 1 Age distribution of the individuals studied

Age (Years)	No. of Orthopantomograms (OPGs)
13	2
14	3
15	4
16	7
17	3
18	1
19	3
20	4
21	5
22	4
23	5
24	3
Total	44

OPGs showing pathological processes in the apical bone, rotated or overlying teeth were not chosen. The examined teeth had to be in normal functional occlusion and free from any manifestations of traumatic insults. Furthermore, teeth with fillings, crowns, and carious lesions were excluded from the evaluation.

From each OPG, three digital pictures with different exposure times were made to compensate for the unequal brightness of the radiographs.

The measurements were carried out on 6 teeth as described previously (5): One maxillary central incisor (tooth 11 or 21), one maxillary lateral incisor (tooth 12 or 22), one maxillary second premolar (tooth 15 or 25), one mandibular lateral incisor (tooth 32 or 42), one mandibular canine (tooth 33 or 43) and one mandibular first premolar (tooth 34 or 44). Due to the fact that Kvaal et al. (5) did not find significant differences between teeth from the left and the right side of the jaw, the teeth were selected from the left or the right side, depending on the sharpness and quality of the OPG in the respective region.

The maximum tooth length, the pulp length, the root length on the mesial surface from the enamel-cementum junction (ECJ) to the root apex, the root and pulp width at the levels A, B and C were measured according to Kvaal et al. (5) (**Fig.1**) using Adobe Photoshop 6.0® (Adobe

Systems Incorporated, San Jose, CA, USA). The following ratios were calculated: The ratio between length of pulp and root, the ratio between length of tooth and root, the ratio between length of pulp and tooth, the ratio between width of pulp and root at ECJ (level A), the ratio between width of pulp and root at midpoint between level C and A (level B) and the ratio between width of pulp and root at mid-root level (level C).

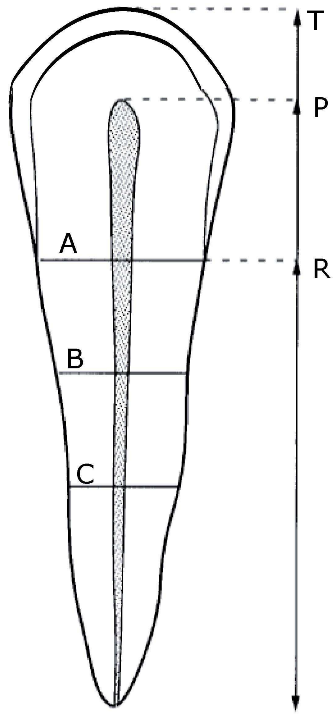

Fig. 1 Diagram showing the measurements made according to Kvaal et al. (5): maximum tooth length (T); root length on the mesial surface (R); maximum pulp length (P); root and pulp width at the enamel–cementum junction (ECJ) (A); root and pulp width midway between measurements levels A and C (B); root and pulp width midway between apex and ECJ (C).

All measurements were made by the same observer (A.M.). Repetitive measurements were performed under blinded conditions after several weeks to evaluate the intra-observer variation. Inter-observer variation was checked by recruiting a second observer (C.F.), who passed an initial training and measured the same OPGs like the first observer.

The previously published regression formulas of Kvaal et al. (5) and Paewinsky et al. (19) were used for age estimation.

Statistical analysis was performed using SAS 9.1 for Windows (SAS statistical software, SAS Institute, Cary, NC, USA) in order to assess the difference between the estimated and the true chronological age

RESULTS

A random sample of ten OPGs was selected and repetitive measurements by the same observer on the ten OPGs were performed. The variation width due to two measurements was below 2%. The intra-observer variation was small and is negligible. Most variation is due to interindividual difference and type of teeth.

Additionally, a second observer made the same measurements on the ten selected OPGs. The variation width of the measurements due to two different observers was below 2%. The inter-observer variation was small and is negligible. Most variation is due to interindividual difference and type of teeth.

SAS 9.1 (PROC VARCOMP) was used to estimate variance components.

Table 2 and **Table 3** detail the results of the calculations carried out using the formulas reported by Kvaal et al. (5) and Paewinsky et al. (19), respectively.

The results were expressed as mean estimated age with standard deviation, minimum and maximum as well as mean difference between the chronological and estimated age with standard deviation, minimum and maximum.

A positive result (of the mean difference between the chronological and estimated age) indicates the number of years that the age was underestimated.

A negative result (of the mean difference between the chronological and estimated age) indicates the number of years that the age was overestimated.

Age estimation performed with the formulas reported by Kvaal et al. (5) for the ratio of single teeth resulted in a mean underestimation of 31.44 years. If age estimation was carried out with the help of the equation for three maxillary teeth, the chronological age was underestimated approximately 38.21 years. The use of the formula for three mandibular teeth led to a mean underestimation of 47.10 years. If the measurements of all six teeth from both jaws were included in age estimation, the calculation resulted in a mean underestimation of 46.04 years.

The regression formulas reported by Paewinsky et al. (19), that use the ratio between the width of the pulp and the root at level A, yielded to a mean overestimation of the real chronological age of 20.88 years. If the age was estimated with the equations which use the ratios at root level B, the overestimation got even higher, namely 22.01 years. The application of the calculation formulas, which uses the ratio between the width of the pulp and the root at level C, resulted in a mean overestimation of the chronological age of 31.92 years.

Table 2 Mean estimated age and mean difference between chronological and estimated age in years based on regression formulas reported by Kvaal et al. (5)

	N	Mean	Std. Dev.	Minimum	Maximum
Estimated age - Single teeth	44	-12.06	13.91	-74.48	16.15
Difference between chronological and estimated age - Single teeth	44	31.44	13.45	8.01	89.78
Estimated age - Three maxillary teeth	44	-18.84	7.27	-34.47	-0.27
Difference between chronological and estimated age - Three maxillary teeth	44	38.21	5.98	24.43	49.77
Estimated age - Three mandibular teeth	44	-27.72	8.84	-48.78	-8.07
Difference between chronological and estimated age - Three mandibular teeth	44	47.10	7.58	32.23	65.17
Estimated age - Six teeth from both jaws	44	-26.67	7.39	-41.48	-6.38
Difference between chronological and estimated age - Six teeth from both jaws	44	46.04	5.84	30.54	56.23

Estimated ages were calculated using the formulas reported by Kvaal et al. (1995)

Table 3 Mean estimated age and mean difference between chronological and estimated age in years based on regression formulas reported by Paewinsky et al. (19)

	N	Mean	Std. Dev.	Minimum	Maximum
Estimated age, Width ratio at root level A	44	40.25	27.05	-207.05	83.37
Difference between chronological and estimated age, Width ratio at root level A	44	-20.88	26.70	-60.40	222.35
Estimated age, Width ratio at root level B	44	41.39	17.57	-7.80	96.88
Difference between chronological and estimated age, Width ratio at root level B	44	-22.01	16.39	-72.27	20.83
Estimated age, Width ratio at root level C	44	51.29	18.08	-24.86	107.65
Difference between chronological and estimated age, Width ratio at root level C	44	-31.92	17.15	-84.49	41.25

Estimated ages were calculated using the formulas reported by Paewinsky et al. (2005)

DISCUSSION

Secondary dentin apposition occurs throughout life and leads to a reduction in size of the pulp cavity. Presently there is no evidence that this process occurs in a linear manner, or that every age group needs the same time span to present itself with a defined amount of secondary dentin. Although linear regression is widely used in forensics to provide the estimate of a measurement, for instance the age at death or the living stature, it should be kept in mind, that human growth is a non-linear process (20). Tooth development in its entirety underlies demonstrable chronological (21-23), environmental, hereditary (24,25) and sexual differences (26). Our results clearly indicate the inapplicability of the regression equations of Kvaal et al. (5) and Paewinsky et al. (19) on a young sample like ours. The age estimations were far away from the real chronological age. The use of the formulas reported by Paewinsky et al. (19) resulted in a consistent overestimation; the equations of Kvaal et al. (5) led to a constant underestimation. Kvaal and colleagues, who developed the original regression formulas for age estimation by means of secondary dentin, did this using a relative small, cross-sectional sample representing a large age span. It could not be ruled out, that any possible influences of chronological or sexual differences were abolished by using a small sample size. Paewinsky et al. (19), who evaluated the applicability of Kvaal's method on OPG's, showed an increase of the coefficient of determination (r^2) of the maxillary lateral incisors when non-linear regression models were employed. The authors did not further explain why it was decided to present linear regression equations for the description of the correlation between chronological age and the width ratios of the pulp cavity. This finding is in accordance with results of Woods et al. (27), who concluded that the timing of secondary dentin formation are more closely fit by a curved than a straight line. Therefore, it could be possible that the formation rate of secondary dentin formation does underlie chronological differences which, in turn, would imply the need for further research to provide sufficient data for age estimation.

Another study, which tested Kvaal's method on OPG's (28), also used a rather small-sized sample and found quite similar results when compared to the original publication. Kvaal *et* al. (5) and Bosmans et al. (28) expressed their results as "standard error of the estimate" (S.E.E.). Due to the fact, that this statistical value does not reflect the error in single cases, but only when applied a great number of times to normally distributed data, one should be aware of misinterpreting the results. As properly discussed in a paper by Snow and Luke (29), the authors faced the problem of estimating the stature of a female by constructing confidence intervals. In this approach, the confidence interval is about as twice as large as the published value of the standard error of the estimate. We do not support the application of the presented regression formulas for age estimation of living persons according to Kvaal and coworkers like others do (19). In our opinion, it should be verified that the presented equations do have comparable accuracy when applied to different age groups or different populations.

From the results of this study it can be concluded that the regression equations reported in Paewinsky et al. (19) and Kvaal et al. (5) can not be applied to a young sample like ours (13.03 – 24.61 years). The usage of these formulas led to age estimations which are far away from the real chronological age. However, only limited conclusions can be drawn from a single study. Further research is required to assess if secondary dentin deposition does underlie chronological or regional differences. The application of the regression formulas reported by Kvaal et al. (5) and Paewinsky et al. (19) on age estimation of living people should only be done when bearing in mind the limitations of this method.

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MANUSCRIPT 3: Comparison of the validity of three dental methods for the estimation of age at death

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ABSTRACT

The aim of the present study was to compare the accuracy, bias, and practicability of two macroscopic and one histological age at death estimation methods on human teeth. The sample was comprised of 67 permanent teeth, obtained from 37 individuals aged 20 to 91 years. Age was predicted according to the methods proposed by Lamendin et al. (LAM), Bang and Ramm (BR), and the quantification of tooth cementum annulations (TCA).

Inter- and intra- observer variability was assessed to evaluate the reproducibility of the methods. The statistical analysis was first performed on the entire sample and then for each age group (20-40 years, 41-60 years, >60 years) separately.

TCA was found to be most accurate in all age groups. Its mean absolute error (MAE) of the estimated age was about half as high as the MAE for both LAM and BR. BR achieved approximately the same MAE as TCA for old adults only.

LAM displayed the highest precision in the young and the old age group whereas TCA was more precise in the middle age group. TCA was found to be the most precise method when the precision was calculated for all ages.

Considering the bias, all methods displayed a tendency to overestimate age in young and to underestimate it in old specimens. The exception to this rule was TCA, which provided unbiasedestimates for young adults.

The higher accuracy and precision recommends favouring TCA over LAM and BR, provided that the required know-how and equipment are available.

Keywords: Forensic odontology; Human teeth; Age at death estimation; Identification; Comparison of methods

INTRODUCTION

Age at death estimation of human remains constitutes an important contribution to the identification process of missing people, and also provides valuable data in the paleodemographical analysis of historical skeletal remains. The dental hard tissues belong to the most durable substances produced by the human body and show the best resistance against post-mortem alterations caused by humidity, high temperature, microbial activities, and mechanical forces.

Moreover, several techniques for dental age estimation are reported to be more accurate than age predictions based on other somatic traits such as the closure of the cranial sutures [1] or the decline in the architecture of the trabeculae of the femoral head [2].

Dental age estimation methods are either based on the well ordered cascade of changes that occur during the formation and eruption of teeth or they rely on continuous processes that alter and diminish the quality of dental tissues even when individual growth is completed. Nevertheless, the validity of age at death estimations in adults using these variables still remains to be evaluated in greater detail and in controlled studies.

Dentin and cementum are hard substances that are, contrary to enamel, continuously synthesized and maintained throughout all stages of a person's adult life. These gradual and lifelong changes make dentin and root cementum ideal substrates for the study of aging processes and it has been demonstrated that they can provide valuable information for the estimation of age at death [3-5].

Age-related transparency of the tooth root is a physiologic trait which appears after the age of 20 [6, 7] and is a result of the deposition of a densely packed matrix with hydroxylapatite crystals within the dentinal tubules. This leads to an equalization of the refractive index between the intra-tubular and inter-tubular dentin, which causes an increased transparency of the affected dentinal regions. The transparency progresses from the root apex towards the cervical region and is visualized when the tooth is placed on a light box [6]. Lamendin and co-workers [6] used the tooth root transparency for age estimation in combination with gingival regression (periodontosis), for which an additional measurement of the tooth length is necessary.

Gingival regression, another age-dependent feature, is a multifactorial process, which was introduced for age estimation by Gustafson [8]. However, pathologic factors may play a major role in the occurrence of gingival regression and it therefore constitutes one of the criteria which have to be evaluated critically. Lamendin et al. [6] established a regression formula, which is suitable for both sexes and ought to be applicable to all types of single rooted teeth. Thus, Lamendin et al.'s method (LAM) is easy to apply and uses only one single rooted tooth that does not require further preparation or specialized technical equipment.

Bang and Ramm (BR) [3] also studied the phenomenon of transparent dentin, but in a more sophisticated way. They presented regression equations for sectioned and unsectioned teeth considering tooth type and the length of root transparency that included multi-rooted teeth. The authors proposed formulae dependent on the transparency as the sole morphological predictor for the estimated age [3].

Thirdly, the evaluation of tooth cementum annulations (TCA) was chosen to compare a popular histological method with the two macroscopic procedures. Cementum is a hard tissue with mineralized organic matrix, which does not undergo continuous remodeling. It is formed as primary acellular cementum and secondary cellular cementum that is arranged in layers (lines) surrounding the human tooth root dentin. This layered appearance is due to structural differences in the mineral phase, an optical phenomenon that is possibly related to altered mineral crystal orientations [9] and reflects a cyclic annual formation pattern. One pair of alternating light and dark lines should therefore correspond to one year in an individual's life. If the count of cementum annulations is added to the tooth-specific eruption age, the result is an estimate of the chronological age. A possible explanation for the layered organization of human cementum is the fact that growth hormone affects cementogenesis [10-12] which could be influenced by environmental factors such as the seasonal change [13-15].

In 1950, cementum was introduced for age estimation [8]; 8 years later Zander and Hurzeler established the linear relationship between an individual's chronological age and the continuous growth of cementum [16]. In the following decades, a number of scientists used the quantification of tooth cementum annulations to estimate the age of a variety of wild animals although the underlying mechanism of its layered nature is not fully understood. Inhomogenous results were obtained from the TCA of human teeth, the observations ranged from an undetectable relationship between the number of incremental lines and age [17-19] to a well-correlated connection [5, 20, 21]. However, some questions regarding the precision and accuracy of TCA analysis in comparison with other dental age estimation methods are still unanswered. Some authors decided to choose the most promising pictures, to exclude teeth with irregularities from TCA analysis and to increase the number of tooth sections if required. This might be a critical point with regards to the comparability of different studies as well as making it more difficult for the scientist to carry through the adequate procedure.

In this paper, we present a study with the goal of evaluating the potential performance of three methods in dental age at death estimation of human remains and to asses their objectivity and reproducibility.

MATERIAL AND METHODS

The sample consisted of 67 permanent teeth from 37 individuals of known age and sex. The age ranged from 20 to 91 years with a mean of 50.7 years old. A detailed distribution of the number of teeth subdivided by tooth type and sex is shown in **Table 1**. All teeth chosen for the analysis had to be free of any manifestations of traumatic insults, carious lesions and visible signs of resorption in the region of the tooth root.

Table 1 Number of evaluated teeth subdivided by tooth type and sex

			Tooth type						
			Central incisor	Lateral incisor	Canine	First premolar	Second premolar	First molar	Total
Upper jaw	Sex	Male	0	3	2	5	3	0	13
		Female	2	1	0	0	2	0	5
	Total		2	4	2	5	5	0	**18**
Lower jaw	Sex	Male	6	8	3	14	6	1	38
		Female	1	1	6	3	0	0	11
	Total		7	9	9	17	6	1	**49**

Of the teeth 37 were obtained from individuals who had donated their bodies to the Centre for Anatomy and Cell Biology, Vienna, for science and medical education. These specimens were fixed in buffered 4 % formalin solution after removing residual soft tissue.

Another 30 teeth were collected from skeletal individuals of the Weisbach Collection, which is held by the Natural History Museum, Vienna. This collection offers the chance to study skeletal individuals of known age and sex, documentation in cases of pathologic findings makes it possible to avoid such cases. After the macroscopic assessments, a silicon replica of each specimen was constructed. The tooth crown was cut off and returned with the replica to the collection in order to fulfill curatorial obligations and to provide morphological information. The tooth root was subjected to histological processing.

As the skeletal material was air-dried, these teeth did not require a fixation procedure and were used for analysis directly.

Table 2 lists the number of anatomical and skeletal specimens subgrouped by sex. The number of teeth in each age group is shown in **Fig. 1**.

Table 2 Tooth number of anatomical and skeletal specimens subgrouped by sex

		Sex		
		Male	Female	Total
	Anatomical	23	14	37
	Skeletal	28	2	30
Total		51	16	**67**

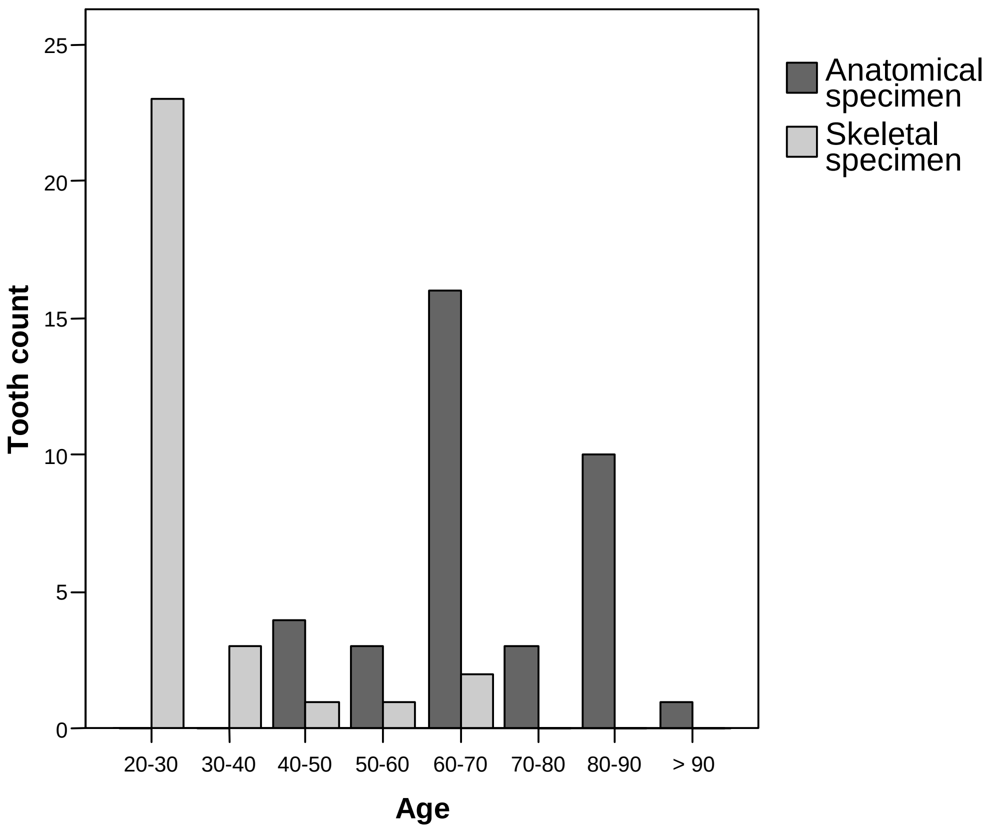

Figure 1 Tooth number per age group for anatomical and skeletal specimens

LAM

Measurements were taken according to the method outlined in Lamendin et al. [6]. This included the assessment of the root height using a sliding calliper (CD-15DC, Mitutoyo, Japan), a millimetre ruler, and a desktop lamp. The root height is defined according to Lamendin et al. as "the distance between the apex of the root and the cemento-enamel junction".

Periodontosis (gingival regression), which is described as "the maximum distance between the cementoenamel junction and the line of soft tissues attachment" [6], was measured at the labial surface of the tooth.

The third dental feature, the transparency of the root, is defined as "from the apex of the root the maximum height of T is measured on the labial surface of the tooth" [6]. Therefore, the transparency was assessed on the labial surface with the help of a light box (slim line 4 cm, Planistar, Himmelstadt, Germany). The estimated age was obtained using the equation $A=0.18 \times P + 0.42 \times T + 25.53$ as proposed by Lamendin and co-workers [6], where A indicates the estimated age, P periodontosis (periodontosis height × 100/root height) and T transparency (Transparency height × 100/root height). Each parameter was measured and recorded three times. The mean of the three measurements was taken for further analysis.

For the assessment of intra-observer and inter-observer variability, 10 teeth were chosen at random. All measurements were taken under blind conditions and at different occasions to avoid potential bias.

BR

Measurements were taken according to the method presented by Bang and Ramm [3] by using a sliding calliper (CD-15DC, Mitutoyo, Japan), a desktop lamp and a light box (slim line 4 cm, Planistar, Himmelstadt, Germany). The total length of the root (RL) was measured "bucally and in the midline from the cemento-enamel junction to the apex". The transparent root dentin was "measured from the apex of the root in coronal direction to the borderline between transparent and opaque dentin". Here a minimal (TL1) and a maximal (TL2) value of the root transparency were recorded, in cases of a horizontal line TL1=TL2. The TM (the mean of TL1 and TL2) value was chosen as the variable for estimation of the chronological age. The estimated age was assessed using the formula $A=B_0 + B_1 \times X + B_2 \times X^2$ (transparent length less than or equal to 9.0 mm) and the formula $A=B_0 + B_1 \times X$ (transparent length more than 9.00 mm) employing the coefficients for intact roots as presented in the original paper [3]. For each measurement, triple-recordings were performed. The mean value of the three measurements was subjected to evaluation.

For the assessment of intra- and inter-observer variability, 10 teeth were chosen randomly. All measurements were taken under blind conditions and at different occasions to avoid potential bias.

TCA

The teeth were subjected to the preparation of undecalcified thin-ground sections as described elsewhere [22, 23]. Briefly, the extracted teeth were fixed in 70% alcohol, dehydrated in ascending grades of alcohol and embedded in light curing resin (Technovit 7200 VLC with BPO, Kulzer, Germany). Further processing was carried out with the Exakt Cutting and Grinding Equipment (Exakt Apparatebau, Norderstedt, Germany). The specimens were cut perpendicular to the root axis at the middle third of the root and three subsequent thin-ground sections of 90-100 µm were prepared. For the analysis of tooth cementum annulations, 3 digital photographs of each section were taken (Nikon Coolpix 990, Nikon, Tokyo, Japan). The digital camera was mounted on a Nikon Eclipse 50i microscope (Nikon, Tokyo, Japan), resulting in pictures in which 1 mm measured 7220 pixels (1 pixel=0.139µm). The tooth cementum annulations were interactively highlighted and counted using Adobe Photoshop® (Adobe, San Jose CA, USA). This process resulted in nine counts per tooth (three sections, three photographs per section) which were conducted by one observer. To obtain the age at death estimate, the mean of the line counts was added to the sex-specific eruption age of each tooth [24, 25]. For the assessment of intra-observer and inter-observer variability, 50 photographs were chosen at random. The photographs were recounted under blind conditions and at different occasions to avoid potential bias.

Statistical analysis

For the assessment of inter-observer and intra-observer variability, the intraclass correlation coefficient (ICC) was used.

The three methods of age at death estimation were numerically compared concerning bias, precision, and accuracy. Bias leads to a systematic underestimation or overestimation of the true chronological age. The mean error was computed [$ME=\sum(\text{estimated age} - \text{true age})/n$] to measure the amount of bias. Precision is defined as the variability of an estimate and is therefore independent of the true value. As a measure of precision of the age at death estimation methods, the variance and the standard deviation of the estimated ages were calculated. Bias and precision combine to define the accuracy of an estimator. The more biased and the less precise an estimator is, the worse its overall ability to make an accurate point estimation. The mean squared error [$MSE=\sum(\text{estimated age} - \text{true age})^2/n$], the root mean squared error [$RMSE=\text{Sqrt}[\sum(\text{estimated age} - \text{true age})^2/n]$] and the mean absolute error

[MAE=∑Abs(estimated age – true age)/n] were used as measures for the age estimation accuracy. All definitions and mathematical formulations were taken from Walther and Moore [26].

In addition to the numerical comparisons of the three methods, the estimated age at death and their residuals (estimated age - real age) were plotted against the real age. Aykroyd and colleagues [27] provide a formula for the slope of the regression of residuals on true age, which is R^2-1, where R^2 is the square of Pearson's correlation coefficient. The regression line intersects the x-axis at the mean age of the reference population, being 58 years for LAM and 46 years for BR. In the case of LAM, R^2 was reported to be 0.33, so the slope of the regression of the residuals on age should be approximately -0.66 if the individuals were sampled from the same population as the reference sample of LAM. BR provides several polynomial regression equations, for each tooth type separately, therefore the slope of the residuals was only estimated roughly by computation of the mean of several correlation indices (0.767) and by the square of the resulting value (0.588). The slopes and intercepts according to Aykroyd et al. [27] were used to plot the regression lines in **Fig. 3**. Teeth from samples of the same population as the reference sample would scatter around these lines.

The statistical comparison of the predictive power of the measured variables (transparency according to LAM, transparency according to BR, periodontosis, cementum layer count) was performed by fitting several linear regression models with age as the dependent variable to the dataset and comparing the fit of the models with a hypothesis test for model comparison. The null hypothesis of the test states that one or more coefficients of the model equal zero. The formula of the test statistic can be found in Faraway [28].

Another regression analysis was performed to evaluate potential differences between Lamendin's original equation and the present sample. Therefore, a multiple regression model was employed for a subsequent comparison of the resulting regression coefficients. All statistical calculations were carried out by using the statistical software R 2.5.0 [29].

RESULTS

Intra-observer and inter-observer variability of LAM, BR and TCA

LAM and BR

A random sample of 10 teeth was selected and repetitive measurements were performed by the same observer. For LAM the variation width due to the repetition of measurements was 36.0% for P, 13.8% for R, and 4.0% for T. For BR the variation width due to the repetition of measurements was 14.2% for R, and 3.1% for TM.

Additionally, a second observer made the same measurements on the 10 selected teeth. For LAM the variation width of the measurements due to two different observers was 37.2% for P, 13.9% for T, and 3.3% for R. For BR the variation width of the measurements due to two different observers was 13.7% for R, and 5.1% for TM of the re-tested specimens.

TCA

A random sample of 50 pictures was selected and repetitive assessments by the same observer on the photographs were performed. The variation width due to the repetition of measurements was 8.1% for the line count of the re-evaluated pictures (**Fig. 2a**). Most of the variation was due to interindividual difference and type of teeth.

Additionally, a second observer made the same measurements on the 50 selected pictures. The variation width of the measurements due to two different observers was 4.7% for the re-tested photographs (**Fig. 2b**). Most of the variation was due to interindividual difference and type of teeth.

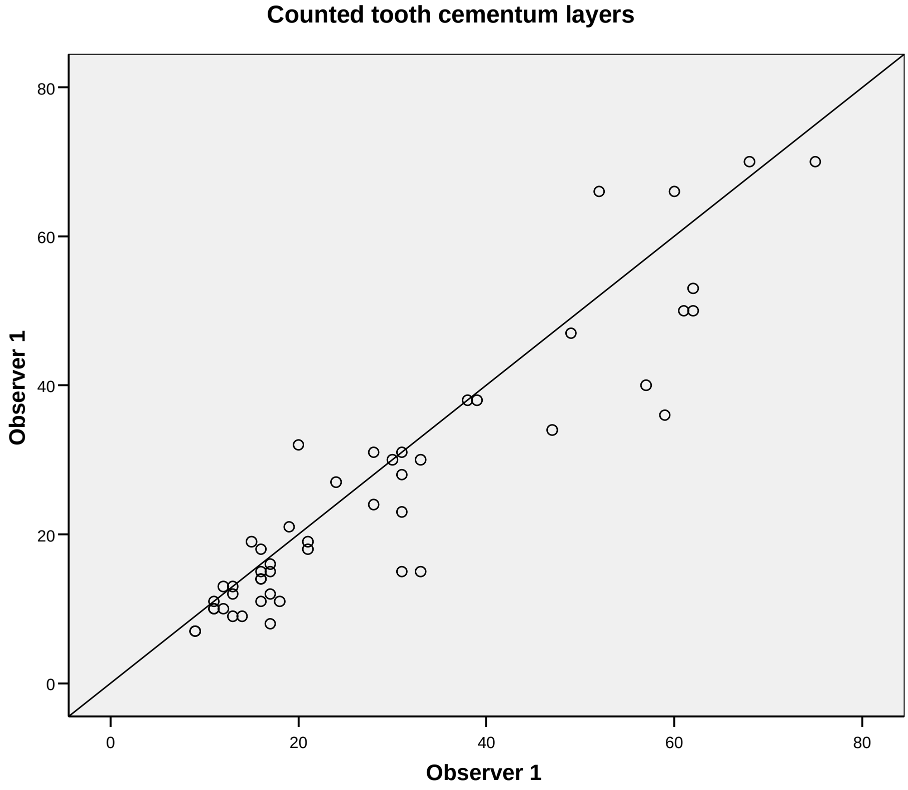

Figure 2a Tooth cementum layer count of observer 1 plotted against the tooth cementum layer count of observer 1

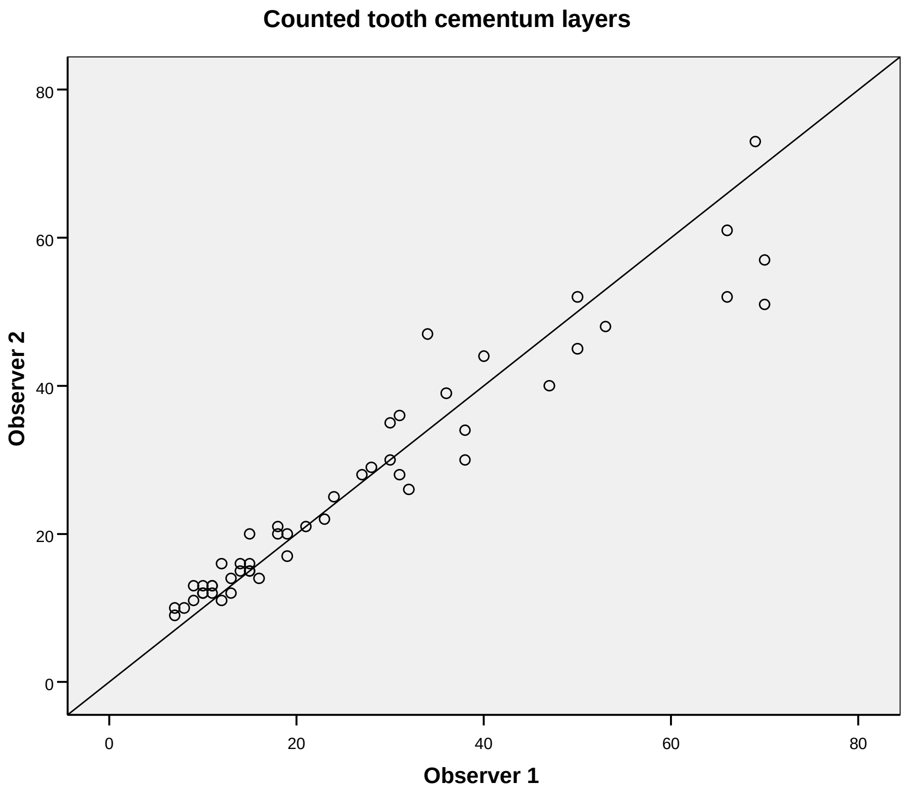

Figure 2b Tooth cementum layer count of observer 2 plotted against the tooth cementum layer count of observer 1

Comparison of LAM, BR and TCA regarding performance in age at death estimation

Fig. 3 shows a graphic presentation of the data where the real age is plotted on the x-axis and the estimated age (or its difference to the real age) is plotted on the y-axis. The black line through the origin represents the line on which the data points would plot if the estimation of age yielded an estimate perfectly equal to the real age. The regression line refers to the residuals on the true age in the reference sample (LAM or BR). This line serves as a tool to discover differences in the regression coefficients of the original sample and the present sample.

The plots show that by means of LAM an overestimation of age in the young age group (20-40 years) was observed while the majority of specimens of both other age groups, middle (41-60 years) and old (>60 years), exhibited an increasing underestimation of age (**3a, 3d**). It can also be seen, that specimens derived from the original sample would exhibit a more pronounced overestimation in the case of the young, but a lower underestimation in the case of the middle and old age groups.

BR tended to overestimate the age of specimens from the young age group while the method was more accurate in case of teeth of the middle age group, but also underestimated specimens derived from the old age group (**3b, 3e**). The line of the original regression seems to mostly fit the predicted age of the specimens from the present sample.

The TCA method revealed remarkably better accuracy and precision in case of young and middle age group specimens which resulted in age estimates relatively close to the real age but underestimated old age group teeth (**3c, 3f**).

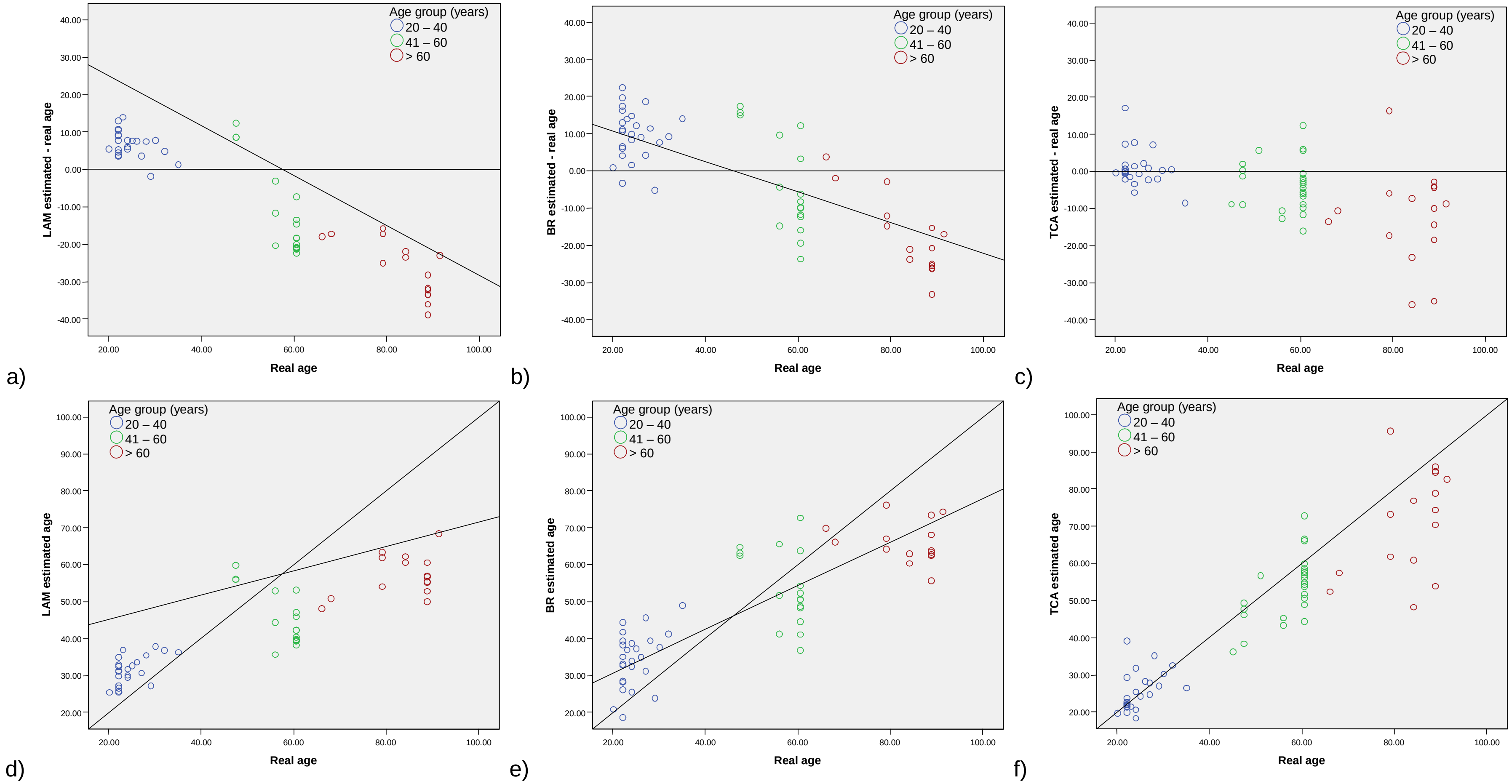

Figure 3 Estimated age minus real age plotted against real age (3a-3c) and estimated age plotted against real age (3d-3f) for LAM (3a, 3d), BR (3b, 3e), TCA (3c, 3f)

Bias of LAM, BR and TCA

The mean error (ME) is presented in **Table 3**. The ME is a bias measure that indicates the mean of all differences between the estimated age and the true, chronological age. The results showed that young age group specimens were overestimated by means of LAM with a ME of 6.80 years, whereas middle age group and old age group teeth were underestimated with a ME of -11.92 and -26.28, respectively.

The application of BR to the present sample produced a mean overestimation of 9.87 years in the young age group specimens, a ME of -3.65 years in the middle and -17.39 years in the old age groups (**Table 3**).

Table 3 Bias of LAM, BR, TCA expressed as mean error (ME) for each age group

BIAS			ME
Age group	20-40	LAM	6.80
		BR	9.87
		TCA	0.83
	41-60	LAM	-11.92
		BR	-3.65
		TCA	-3.73
	>60	LAM	-26.28
		BR	-17.39
		TCA	-12.12

TCA yielded a mean error of 0.83 years in young age group teeth, and a ME of -3.73 and -12.12 years in middle and old age group material, respectively. These results showed that the application of TCA to the present sample found the lowest ME in young und middle aged specimens, whereas BR was able to produce age at death estimates with a ME slightly closer to zero in teeth of the middle age group.

The assessment of the ME also revealed that the methods, except for TCA, with a ME of only 0.83 years, produced an overestimation of age in the young age group, whereas all of the techniques underestimated middle and old age group specimens.

The ME in the sample analyzed as a whole (**Table 4**) amounted to -8.01 years underestimation for LAM, whereas the bias of the whole sample was calculated with a ME of -1.14 years for the BR method which was also less than the ME of -3.97 for TCA.

Table 4 Bias of LAM, BR, TCA expressed as mean error (ME)

BIAS	ME
LAM	-8.01
BR	-1.14
TCA	-3.97

Precision of LAM, BR and TCA

Table 5 details standard deviation (SD) and variance (Var) which served as measures for the precision of the compared methods. According to the calculations LAM revealed the highest precision of the 3 techniques with a SD of 3.60 and a Var of 12.96 years in the age group of the young. Increasing values were found for the middle and old age groups.

BR showed its best precision in the young age group with an SD of 6.80 and a Var of 46.20. BR worked less precisely in the middle group and in the old age group teeth.

Also TCA worked most precisely in the middle age group with an SD of 6.85 with a Var of 46.90. According to the results, LAM featured the lowest SD and Var in the young and old specimens of the present sample compared with the two other named techniques. The highest precision of an age at death estimate in the middle age group was achieved with the TCA method.

Table 5 Precision of LAM, BR, TCA expressed as standard deviation (SD) and variance (Var) for each age group

PRECISION			SD	Var
Age group	20-40	LAM	3.60	12.96
		BR	6.80	46.20
		TCA	4.84	23.42
	41-60	LAM	11.73	137.59
		BR	13.27	175.98
		TCA	6.85	46.90
	>60	LAM	7.61	57.93
		BR	10.46	109.38
		TCA	12.62	159.29

Analyzing all ages (**Table 6**), a SD of 9.38 years and a Var of 87.95 years for the application of TCA was found which constitutes the lowest variability of the estimate regarding the whole sample.

Table 6 Precision of LAM, BR, TCA expressed as standard deviation (SD) and variance (Var)

PRECISION	SD	Var
LAM	15.87	251.97
BR	14.94	223.21
TCA	9.38	87.95

The accuracy of LAM, BR and TCA

The accuracy is given by the mean square error (MSE), the root mean square error (RMSE), and the mean absolute error (MAE) (**Table 7**). These values are distinct measures for the accuracy of the evaluated method, or how close the age at death estimates are to the true chronological age.

The calculations demonstrated that LAM worked most accurately in the young age group. The error of the age estimation increased in the middle age group specimens and even more in the old age group.

A similar trend is given for the accuracy of BR, which is given in **Table 7** for the young, middle and old age groups.

By the assessment of the MSE, RMSE and MAE, TCA was found to be the most accurate age at death estimation method in the young, middle, and old age groups.

Table 7 Accuracy of LAM, BR, TCA expressed as mean square error (MSE), root mean square error (RMSE), and mean absolute error (MAE) for each age group

ACCURACY			MSE	RMSE	MAE
Age groups	20-40	LAM	58.61	7.66	6.95
		BR	141.85	11.91	10.51
		TCA	23.22	4.82	2.90
	41-60	LAM	271.54	16.48	15.43
		BR	178.92	13.38	12.32
		TCA	58.89	7.67	6.43
	>60	LAM	744.57	27.29	26.28
		BR	404.59	20.11	17.91
		TCA	296.14	17.21	14.18

Table 8 shows the results for the accuracy evaluation calculated for all ages which shows that LAM was the least accurate method when compared to BR and TCA.

The present data also show that the TCA method yielded the lowest error of the three evaluated methods regarding all ages.

Table 8 Accuracy of LAM, BR, TCA expressed as mean square error (MSE), root mean square error (RMSE), and mean absolute error (MAE)

ACCURACY	MSE	RMSE	MAE
LAM	311.50	17.65	14.84
BR	220.66	14.85	12.96
TCA	102.35	10.12	6.92

The predictive power of age at death estimation variables

To evaluate the usefulness of each measured variable separately, several regression models were used and the R^2 values were compared. First, the variables of LAM were compared with the variable of BR. The comparison of the models showed no significant improvement of the R^2 after the variable root transparency (TM) as measured by BR was added to the regression model with root transparency (T) and periodontosis (P) of LAM ($p=0.1259$). Thus, no additional information about the age could be gathered when applying the root transparency measurement according to BR. On the other hand, when the age estimation according to TCA was added to the two variables, a highly significant effect could be observed ($p<0.001$) indicating that the cementum layer count adds to the predictive power of LAM. A multiple regression analysis showed that every single variable (root height, gingival regression and cementum layer count) adds significantly to the explained variation of age, independently of the two other variables (**Table 9**). The multiple R^2 of 0.915 is substantially higher than any R^2 of the other models (LAM: $R^2=0.768$, BR: $R^2=0.665$, TCA: $R^2=0.848$).

Table 9 Regression model comparison

Comparison	Model formula	R^2	Sig.
Model 1	true age ~ T + P	0.768	
Model 2	true age ~ T + P + TM	0.778	**0.1259**
Model 1	true age ~ T + P	0.768	
Model 2	true age ~ T + P + TCA	0.915	**< 0.001**

Coefficients	true age ~ T + P + TCA			
	Coefficients	**Std. Error**	**t**	**Sig.**
(Constant)	2.284	2.519	0.907	0.369
P	0.366	0.119	3.064	0.004
T	0.265	0.068	3.885	0.000
TCA	0.708	0.076	9.325	0.000

Differences between Lamendin et al.'s original equation and the equation of the present sample

The plots of **Fig. 3** show that if age is estimated by Lamendin et al.'s method, a deviation of the point scatter from the regression line can be observed. It was suspected that such a large deviation may be the effect of a different relationship between the true age and the age indicators (T and P) in the present sample compared to the reference sample of LAM. To clarify how much the regression coefficients differ, the same multiple regression as used by Lamendin and colleagues was performed on our sample of teeth. The multiple regression analysis of the original publication of Lamendin and co-workers [6] provided the following equation for the age at death estimation: Age (years) = 0.18 x P + 0.42 x T + 25.53. Interestingly, both our regression coefficients (T: 0.61, P: 0.69) are higher than the coefficients of LAM, but only the value of P seems to be significantly higher. The confidence intervals of our regression coefficients (T: 0.42 – 0.80, P: 0.32 – 1.06) include Lamendin's coefficient of T (0.42), but exclude the coefficient of P (0.18).

DISCUSSION

The major advantage of dental age at death estimation is the fact that human dental hard tissues do not undergo remodelling processes like bone. This makes teeth less susceptible to pathological impaction and impaired growth. Moreover, due to their extreme hardness even cremated teeth were found to feature structures still useful for age at death estimation analysis [30]. A number of methods have been employed for dental age at death estimation. Unfortunately, there are only few comparisons of these methods available, especially an analysis which compares the quantification of tooth cementum annulations with commonly used macroscopic techniques is still lacking. It was the goal of the present study to evaluate the performance of three dental age at death estimation methods, LAM, BR, and TCA, but not to analyze any underlying mechanism. The present sample offers complete data about sex and chronological age and therefore fulfils all necessities for age at death estimation analyses.

Reproducibility is a fundamental requirement for any method used in science. Therefore, the reproducibility of LAM, BR and TCA was subject to intra-observer and inter-observer variability assessment.

The results demonstrate that both LAM and BR, suffer from a high variability regarding the tooth root height and the periodontosis assessment. Thus, the respective variable for the transparency of dentin is not the primary source of variability caused by measurement error.

As can be seen in **Fig. 2a** and **2b**, TCA was the most reproducible method with comparatively high intraclass correlation coefficients. This leads to the assumption that the counting of cementum annulations is useful in age at death estimation, especially by reason that not the 3x3 pictures approach, but single pictures were used for variability evaluation.

In **Tables 3** and **4**, the bias of each method is presented for each age group and for all ages. It can be seen clearly that all three methods tended to overestimate the young age group (20-40 years), except the TCA method which showed a very low mean error of 0.83 years. Specimens of the middle (41-60 years) and old (>60 years) age groups were underestimated by all methods. Here BR and TCA showed an almost equal result in the middle, but TCA a far better performance in the old age group teeth.

Previous studies on the quantification of tooth cementum annulations reported difficulties about a correct age prediction of pathologically impacted teeth but also in case of old individuals. Kagerer and Grupe [31] conducted a study on the validity of the age at death estimate carried out on pathologically impacted material and found a mean age deviation between 2.9 and 13.5 years. The amount of the mean deviation was related to the type of pathological impaction involved.

Condon and colleagues [5], who used demineralized longitudinal cementum sections, reported a bias ranging from 3.0-5.8 years overestimation for the age of individuals who were younger than 40 years old. An underestimation of 3.0-7.1 years was shown for individuals who were 40-59 years old. Renz and Radlanski [32] published a very critical study about the analysis of tooth cementum annulations in 2006 although only 8 premolars were included in their evaluations. However, this article recapitulates the difficulties of the quantification of tooth cementum annulations on a well researched basis. The results of the present study regarding LAM are in accordance with the findings of Foti at al. [33], who calculated an underestimation of 7.53 years for the male and 10.97 years for the female individuals in their sample. These authors also demonstrated an increase of underestimation with increasing age. Martrille and colleagues [34] also found an overestimation of young individuals, and reported an increasing underestimation with age by means of LAM. Megyesi et al. [35] tested LAM on historical skeletal material and noted a mean error ranging from 9.3 for the youngest to 46.8 for the oldest individuals. This yielded a higher error than an evaluation of the Terry collection [36], where 32.6 years was the highest mean error which was computed for the age group 90-99 years old. The authors also reported the overestimation in age of young individuals and the underestimation of the older individuals. Soomer and co-workers included Bang and Ramms's method in their comparative study on the validity of dental ageing methods for adults [37]. They computed a mean overestimation of 6.6 years for the whole sample and 3.5 years for the application of LAM to their specimens.

Unfortunately, previous studies on age at death estimation differ significantly concerning the sample, the implementation of the respective method, and the statistical procedures. Therefore, the published results can only be compared very roughly and in consideration of the used study design.

The overestimation of the young age group specimens and the consistent underestimation of the middle and old age group teeth by means of LAM, which can also be seen in **Fig. 3a** and **3d**, is partly due to the application of ordinary regression for arriving at point estimates of the true age in the original papers, and may not only be an effect of the sample. Aykroyd et al. [27] point out that at least part of this error is the inevitable consequence of the statistical procedure which was used to extract an estimate of age from age indicators, namely the ordinary regression with age as dependent variable. When using LAM as the age at death estimation method, all specimens were consistently estimated lower than specimens of the original population would have been. This is indicated by the regression line in **Fig. 3a** and **3d** and suggests populational differences. Due to a multiple regression of age on transparency and periodontosis, it can be speculated that this difference was mostly caused by a lower amount of gingival regression (or periodontosis) than in the original population, but not by a differing formation rate of the root dentin transparency, as this was also confirmed by the fact that this effect can not be observed with BR's original regression (**Fig. 3b, 3e**). BR used only root dentin transparency (and root length) as predictor variable, but not gingival regression. The validity of this finding is certainly reduced by the fact that the present sample consists of mainly two groups, one of which is adult and male, the other of which is older and female. Any relationship found between age and age indicators may partly be a result of this heterogeneity in the sample, although the difference between the regression coefficients of periodontosis is quite large (see **Table 9**).

The regression lines computed according to the formulae of Aykroyd and colleagues [27] may be a valid tool for visualizing the relationship between the residuals and the true age which is solely due to the methodology of achieving a point estimate of the true age by means of ordinary regression. Any interpretation of this relationship in terms of biological reasons would be completely misleading. In comparisons of age at death methods the cluster of residuals against true age may not scatter around this line and strong deviations of the point scatter from the respective regression line may indicate a different relationship between age and indicator variables in the reference sample compared to the test sample, but this has to be interpreted carefully. We advocate confirming this suspicion by actually computing the regression coefficients of the test sample and comparing them to the regression equation of the age determination method.

Precision can be defined as a measure of "the statistical variance of an estimation procedure" [38]. Thus, **Tables 5 and 6** present the standard deviation and variance as measures for the precision for the three methods. Here LAM was the most precise technique in young and old age group, whereas TCA was found to have a lower SD and Var in the middle age class. One possible explanation for the imprecision of the TCA method might be found in measurement errors particularly in the old age group teeth. The very low standard deviation and variance of LAM in the young age group might be due to the reason that this age class usually exhibits only little or no visible signs for gingival regression.

Following to the formulae reported by Walther and Moore [26], the mean square error, the root mean square error and the mean absolute error were computed as measures of accuracy (**Tables 7 and 8**). The results clearly indicate that TCA is the method with the highest accuracy in all age groups, and also regarding all ages as compared to LAM and BR. This finding is in line with the fact that both other methods are based on regression equations, and confirms the present procedure of TCA. Nevertheless, it cannot be ruled out that an approach with a greater number of sections will achieve results that are even more accurate. An automated image analysis could also improve the accuracy of the age at death estimate [39], although it needs to be evaluated if this is also holds true for teeth from very old individuals.

TCA is an expensive, tedious but comparatively precise technique regarding the fact that the measurement depends principally on the absolute count of lines. Unfortunately, no standardized protocol of how to prepare the sections and how to count them exists which may lead to errors in the assessment of the line count. The majority of researchers seem to recognize a pair of light and dark bands as one "line", although its annual deposition in humans has not as yet been proved. In practice, the quality of the line count suffers from superimposing structures, cementocytes and an optical "thinning" in older individuals. Concerning the preparation process it can be said that the kind of embedding medium might not be crucial, but the thickness and the cutting angle could contribute to the quality of the section. The number of sections and the line count itself do produce an effect on the result; therefore, one should carefully choose the approach. Excluding sections with poor visibility is a subjective form of sample selection, which impairs the age assessment because it depends on the individual judgement of the observer. This reduces the reproducibility and applicability of the method, as no specific rules are given for the selection of "poor" sections. Due to this reason it was decided to subject all sections to further analysis, which revealed that 14 sections of the whole sample could not be evaluated, mostly due to reasons such as superimposition.

The approach with 3 subsequent sections and 3 pictures per section was chosen to guarantee a standardized procedure. It could be speculated that the age at death estimate becomes more accurate, as more sections and pictures are evaluated. The effect that especially teeth of the old are underestimated by TCA could be largely due to measurement error. The cementum of specimens of the old age group appears to be not only thicker, but also much more densely packed with optically thinner lines, a phenomenon which significantly complicates the line counting.

Furthermore, the costs of a method also affect the effective implementation. The costs for a single thin-ground section for TCA amount to about 200 € and special equipment is required, a factor which might restrict the applicability of TCA.

LAM and BR are both macroscopic methods that are inexpensive, quick, but less accurate. The fact that the eye must decide about the dimension of the root transparency or the gingival regression makes it more susceptible to measurement error and bias. Moreover, it is inevitable that an age at death estimation by means of LAM or BR leads to an overestimation of young age group specimens and to an underestimation of middle and old age group teeth. This is mostly a result of the use of regression-based techniques of analysis for converting age indicators into estimated ages [27]. Nonetheless, handling of BR and LAM is uncomplicated and therefore allows the realization of dental age estimation under adverse conditions. An example for this is the use of the latter by the International Criminal Tribunal for the Former Yugoslavia [37], its application to victims from the war in Croatia [40].

CONCLUSION

This study aimed to increase awareness about the differing sensitivity of the dental age evaluated by death estimation methods. It is crucial to choose the suitable method for a reliable diagnosis, the possibility of estimates differing widely from the real age used in research or for medico-legal purposes may be higher than is realized.

The results of the present study indicate that the assessment of tooth cementum annulations should be favoured over the methods of Lamendin et al. and Bang & Ramm, although a standardized procedure would further improve its performance and comparability. Moreover, it could be shown that a combination of tooth cementum annulations with the variables of Lamendin et al. (transparency and periodontosis, each divided by the root length) potentially leads to an increases of the predictive power beyond the capabilities of each method alone. Further research concerning the mechanisms that underlie the formation of tooth cementum annulations but also of transparent root dentin would be a great benefit for human age at death estimation.

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SUMMARY OF THE RESULTS AND OUTLOOK

The study "The chronology of third molar mineralization in the Austrian population—a contribution to forensic age estimation" provides reference data on third molar mineralization in the Austrian population. Within the present cross-sectional evaluation panoramic radiographs of 610 Austrians between 12 and 24 years of age were assessed using the 8 developmental stages of Demirjian et al. (1973).

It was found that 78.2% of the analyzed individuals possessed both mandibular third molars; 11.3% of the subjects were recorded to have one lower third molar. Previous records about an earlier occuring third molar mineralization in females were confirmed. A statistically significant sexual dimorphism was noted in Demirjian's stages E ($p<0.01$) and F ($p<0.05$). A comparison with previous study data suggests ethnic differences in the timing of third molar mineralization. Moreover, the probability of whether an Austrian individual is at least 18 years old was determined. In the case where two mandibular third molars were present, the probability for an Austrian individual to be at least 18 years is 99.5% and 99.3% for males and females, respectively in the case were tooth mineralization is completed (Demirjian's stage H). This indicates that Demirjian's stage H constitutes a reliable aid in dental age estimation. The obtained data is exclusively valid in Austrian individuals and cannot be used for dental age estimation in other populations. According to the recommendations of the interdisciplinary Study Group on Forensic Age Diagnostics, an age analysis of living persons also includes an X-ray of the hand and a physical examination. The author supports a combined technique that helps to avoid incorrect age estimates due to the biological variance of a single trait. Nonetheless, a large study that validates the use of the combination of methods is still lacking although this procedure is already in use.

The second investigation "On the applicability of secondary dentin formation to radiological age estimation in young adults" aimed to explore whether previously published regression formulae for dental age estimation based on radiographic two-dimensional measurements of the pulp size lead to reproducible and statistically sound results. Panoramic radiographs of 44 Austrian individuals, aged between 13 and 24 years, were selected to carry out the measurements proposed by Kvaal et al. (1995). The use of the regression formulae reported by Paewinsky et al. (2005) led to a consistent overestimation, the equations of Kvaal et al. (1995) resulted in a constant underestimation of age. From these results it can be concluded that the formulae of Kvaal et al. (1995) and Paewinsky et al. (2005) are not suitable for dental age estimation in young adults. It is suggested to evaluate secondary dentin formation with a large sample size in the present age group to obtain reliable reference data. Furthermore, it could be helpful to assess the decrease of the pulp size also by additional parameters as proposed by Cameriere et al. (2007b) or via computerized tomography (Yang et al. 2006).

The third study "Comparison of the validity of three dental methods for the estimation of age at death" focused on the comparison of accuracy, bias, and practicability of two macroscopic and one histological dental age at death estimation methods. The study sample comprised of 67 teeth, obtained from 37 individuals aged 20 to 91 years. Chronological age was estimated by means of the methods proposed by Lamendin et al. (1992) (LAM), Bang and Ramm (1970) (BR), and the quantification of tooth cementum annulations (TCA). TCA was found to be the most accurate age at death estimation method in young, middle, and old age group teeth. LAM displayed the highest precision in the young, and the old age group whereas TCA was more precise in the middle age group. TCA showed to be the most precise method when the precision was calculated for all ages. Considering the bias, all methods displayed a tendency to overestimate age in young and to underestimate it in old specimens. The exception to this rule was TCA, which provided unbiased estimates for young adults. The higher accuracy and precision recommends favouring TCA over LAM and BR, provided that the required know-how and equipment are available. Further studies in order to assess the exact mechanism and timing of tooth cementum layering, secondary dentin apposition, and dentin sclerosis would be helpful to improve the accuracy of age estimates. Moreover, a generally accepted procedure for the preparation and evaluation of root cementum annulations would be desirable to establish common standards.

ZUSAMMENFASSUNG AUF DEUTSCH

Die Studie "The chronology of third molar mineralization in the Austrian population—a contribution to forensic age estimation" legt Referenzdaten zur Mineralisation des dritten Molaren in der österreichischen Population vor. Im Rahmen dieser Querschnittsuntersuchung wurden Panoramaröntgenaufnahmen von 610 ÖsterreicherInnen im Alter zwischen 12 und 24 Jahren gemäß dem 8-gradigen Entwicklungsschema nach Demirjian et al. (1973) beurteilt.
Es wurde festgestellt, dass 78,2% der beurteilten Individuen beide mandibulären Molaren besaßen; bei 11,3% der Subjekte wurde ein unterer dritter Molar erfasst. Bisherige Erfahrungen über den zeitigeren Beginn der Weisheitszahnmineralisation bei Frauen wurden bestätigt. Ein statistisch signifikanter Sexualdimorphismus wurde in den Stadien E ($p<0{,}01$) und F ($p<0{,}05$) nach Demirjian beobachtet. Weiters wurde die Wahrscheinlichkeit eines österreichischen Individuums ermittelt wenigstens 18 Jahre alt zu sein. Waren beide mandibulären Molaren vorhanden, so betrug die Wahrscheinlichkeit wenigstens 18 Jahre alt zu sein 99,5 bzw. 99,3 % für Männer bzw. für Frauen, jeweils im Falle der vollendeten Zahnmineralisation (Stage H nach Demirjian). Dies legt nahe, dass Demirjians Stage H eine wertvolle Hilfe in der dentalen Altersschätzung darstellt. Die gewonnenen Daten sind ausschließlich für österreichische Individuen gültig und können nicht zur Altersschätzung in anderen Populationen herangezogen werden. Gemäß den Empfehlungen der interdisziplinären Arbeitsgemeinschaft für Forensische Altersdiagnostik inkludiert eine Altersbestimmung am Lebenden außerdem ein Handröntgen sowie eine körperliche Untersuchung. Die Autorin befürwortet eine kombinierte Technik die dazu beiträgt, inkorrekte Altersschätzungen aufgrund der biologischen Varianz eines einzelnen Merkmals zu vermeiden. Trotzdem fehlt noch immer eine große Studie, welche die Verwendung der Methodenkombination validiert, obwohl sich selbige bereits in Verwendung befindet.
Die zweite Untersuchung "On the applicability of secondary dentin formation to radiological age estimation in young adults" untersuchte, ob bisher publizierte Regressionsgleichungen zur dentalen Altersschätzung, basierend auf radiographischen, zweidimensionalen Messungen der Pulpagröße, zu reproduzierbaren und korrekten Ergebnissen führen. Es wurden Panoramaröntgenaufnahmen von 44 österreichischen Individuen, im Alter zwischen 13 und 24 Jahren, ausgesucht um die Messungen nach Kvaal et al. (1995) vorzunehmen. Die Verwendung der Regressionsgleichung von Paewinsky et al. (2005) führte zu einer konsistenten Überschätzung, die Gleichungen nach Kvaal et al. (1995) resultierten in einer konstanten Unterschätzung des Alters. Aufgrund dieser Ergebnisse kann gefolgert werden, dass die Formeln

nach Kvaal et al. (1995) und Paewinsky et al. (2005) für die dentale Altersschätzung junger Erwachsener nicht geeignet sind. Es wird empfohlen Sekundärdentinbildung anhand einer großen Stichprobe der vorliegenden Altersgruppe zu evaluieren um verlässliche Referenzdaten hierzu erhalten. Weiters könnte es hilfreich sein, die Verminderung der Pulpagröße anhand zusätzlicher Parameter, wie von Cameriere et al. (2007b) vorgeschlagen zu ermitteln, oder aber mittels Computertomographie (Yang et al. 2006).

Die dritte Studie „Comparison of the validity of three dental methods for the estimation of age at death“ konzentrierte sich auf den Vergleich der Genauigkeit, des Bias sowie der Praktikabilität zweier makroskopischer und einer histologischen dentalen Methode zur Schätzung des Sterbealters. Die Stichprobe bestand aus 67 Zähnen von 37 Individuen im Alter zwischen 20 und 91 Jahren. Die Schätzung des chronologischen Alters erfolgte gemäß den Methoden nach Lamendin et al. (1992) (LAM), Bang and Ramm (1970) (BR), sowie der Quantifizierung von Zahnzementannulationen (TCA). Die höchste Genauigkeit des Schätzergebnisses zeigte die TCA- Methode bei Zähnen der jungen, mittleren und alten Altersgruppe. LAM zeigte die höchste Präzision in der jungen und alten Altersgruppe, wohingegen sich in der mittleren Altersgruppe TCA als präziser erwies. TCA war die präziseste Methode, sofern die Altersgruppen nicht getrennt analysiert wurden. Bezüglich des Bias hatten alle Methoden die Tendenz das Alter in jungen Proben zu überschätzen, sowie in alten Proben zu unterschätzen. Die Ausnahme war hier TCA, die junge Erwachsene ohne Bias schätzte. Aufgrund der höheren Genauigkeit und Präzision wird es empfohlen die TCA-Methode LAM und BR vorzuziehen, sofern entsprechendes Wissen und Ausstattung vorhanden sind. Weitere Studien in Bezug auf den genauen Mechanismus und die Chronologie der Zahnzementannulationen, der Sekundärdentinapposition, sowie der Dentintransparenz würden die Genauigkeit von Altersschätzungen verbessern. Weiters wäre eine allgemein anerkannte Prozedur zur Präparation und Evaluation von Zahnzementannulationen wünschenswert um einen gemeinsamen Standard zu etablieren.

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APPENDICES

Appendix 1: Preparation of Tooth Sections by the Cutting-Grinding Technique

A) EMBEDDING

Embedding constitutes alcoholic dehydration, subsequent infiltration and polymerization with light curing resin (Technovit®). This process can be applied to formalin-fixed or macerated teeth for the preparation of high quality specimens within scientific objectives.

The samples are agitated at all stages of embedding.

Formalin-fixed material must be washed in running tap water over night prior to the first dehydration step to prevent formalin precipitations in the Technovit polymer.

Technovit® is a trademark of Heraeus Kulzer, Wehrheim, Germany.

Further information about the equipment for the Cutting-Grinding Technique can be found in Appendix 2.

1. Series of ascending alcohol dilutions (CAS # 64-17-5)

80% alcohol	3 days
96% alcohol	3 days
100% alcohol	4 days

2. Series of ascending Technovit® dilutions

Specimens are kept at 4°-8°C.

Technovit 7200 with Benzoylperoxyde (BPO) and alcohol (30:70)	2 days
Technovit 7200 with BPO and alcohol (50:50)	2 days
Technovit 7200 with BPO and alcohol (70:30)	3 days
Technovit 7200 with BPO and alcohol (100:0)	5 days

3. Polymerization

1. Clean embedding container with alcohol (70%) and allow drying. Expose to vacuum for 20 mins.
2. Put Technovit 7200 VLC (100:0) with BPO (used up to 1x) into the embedding container to cover the floor (2-3 mm).
3. Vacuum for 30 mins (without light), followed by 60 mins vacuum with white light and water cooling.
4. Put the specimens on the polymerized layer so that the region of interest (ROI) is parallel or orthogonal to the later cutting plane.
5. Fill up with Technovit 7200 VLC with BPO so that specimens are covered.
6. Make diagram of the specimens.
7. Vacuum 3-4 hrs (without light).
8. 12 hrs vacuum with white light from below and water cooling
9. 2 hrs white light from above.
10. Control the polymerization.

B) PREPARATION OF THIN GROUND SECTIONS

Technovit® is a trademark of Heraeus Kulzer, Wehrheim, Germany.
All grinding papers, slides, and other consumables can be purchased from EXAKT, Norderstedt, Germany.
Buehler Micropolish II can be purchased from Buehler, Düsseldorf, Germany.
Further information about the equipment for the Cutting-Grinding Technique can be found in Appendix 2.

1. Mark the polymerized Technovit blocks with a millimetric ruler and a permanent marker.
2. Separate the blocks with the EXAKT diamond band saw.
3. X-ray the blocks to determine the appropriate cutting plane (the ROI).
4. Bisect the blocks with the saw at the ROI.
5. Mark the specimen dimensions.
6. Trim the specimen with the Buehler Metaserv Grinder-Polisher. The area opposite to the ROI should be parallel. Smooth the edges.
7. Coplanar adhering: Fix the ROI with double-sided tape reversible to a slide. The mounting plate for the specimen is fixed in the Vacuum Adhesive Press. Technovit 4000 is mixed according to the instructions in appropriate ratio and applied to the bloc area opposed to the ROI. The block on the slide is transferred to the Vacuum Adhesive Press. Bring down the top of the press with the mounting plate onto the specimen which is now covered with adhesive. Remove the slide after hardening of the adhesive.
8. Coplanar grinding: Fix the specimen on the mounting plate in the EXAKT grinding system. Remove material at medium speed with the grinding papers P 320 and P 1200 until the ROI is coplanar. Verify this with a micrometer screw. Differences in thickness should not exceed 5 µm. Measure the thickness of the specimen including the mounting plate and note this value on the mounting plate.
9. Dry the specimen for 15 mins with vacuum and heat in the EXAKT Re-Infiltration Unit.
10. Re-infiltration: Apply Technovit 7200 (without BPO) to the ROI and put it in the Re-Infiltration Unit for 1hr. Break the vacuum every 5 mins. Then activate the blue light for 1 hr (with vacuum).
11. Fix the specimen to the grinding system and grind down with P 320 and P 1200 in order to obtain the same plane like before the re-infiltration. Grind down additional 10 µm. Repeat the re-infiltration process in case of persisting defects in the ROI.
12. Polish the specimen with the polishing paper P 4000.

13. Measure the minimal- and maximal thickness of the specimen including the mounting plate. Note the values on the plate and in the Excel spreadsheet "Kleberstärke".
14. Clean the ROI with petroleum ether (CAS # 64742-49-0) and dry the specimen for 15 mins with vacuum and heat in the Re-Infiltration Unit.
15. Mount a slide onto the ROI. Therefore, measure minimal- and maximal thickness of an appropriate slide and enter the values in the "Kleberstärke". Then, apply Technovit 7210 VLC adhesive to the ROI and put it in the Re-Infiltration Unit. Turn on the vacuum shortly until bubbles are forming. The specimen is transferred to the Adhesive Press where the slide is fixed via vacuum to the upper bar. The lever arm is lowered and presses the ROI with the adhesive on the vacuum-fixed slide. Turn on the blue light for 20 mins to cure the adhesive.
16. Measure minimal- and maximal thickness of the specimen including slide and mounting plate using a micrometer screw. Enter the data in the "Kleberstärke" sheet and note the calculated values of the glue and section thickness on the slide.
17. Fix the specimen via vacuum in the Diamond Band Saw. Adjust the specimen with the hand wheel so that the resulting section on the slide is 300 μm. Saw at speed 8 with water cooling.
18. Grind the specimen down to 250 μm. Therefore, use the measuring system of the Grinding System and the grinding paper P320. The measuring system switches off automatically when the nominal value is reached.
19. Grind the specimen down to the desired section thickness with P 1200. Control the thickness repeatedly with a micrometer screw.
20. Polish the specimen with P 2400 for 1 min.
21. Control the specimen microscopically and with the micrometer screw. Repeat grinding steps if desired.
22. Polish the specimen with P 4000 2 x 2 mins
23. Polish the specimen manually with the Buehler Metaserv Polisher. Use Buehler Micropolish II 0.3 Micron (Deagglomerated Alpha Alumina) powder.

Appendix 2: The Cutting and Grinding Equipment

Fig. 1 Light Polymerisation Unit. The Light Polymerisation Unit polymerizes dehydrated, with resin infiltrated and embedded specimens. EXAKT, Norderstedt, Germany.

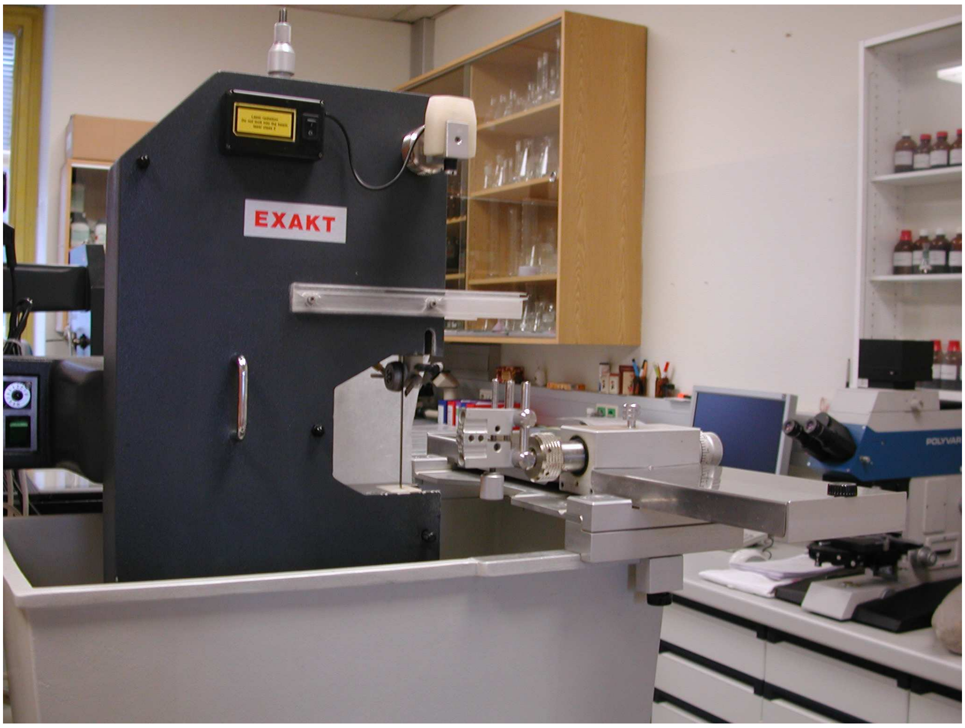

Fig. 2 Diamond Band Saw E 300. The saw is equipped with the EXAKT Contact Point CP Technology which oscillates the specimen during cutting. EXAKT, Norderstedt, Germany.

Fig. 3 Buehler MetaServ Grinder-Polisher. The Grinder-Polisher is used to trim excess resin and to polish sections. Buehler, Düsseldorf, Germany.

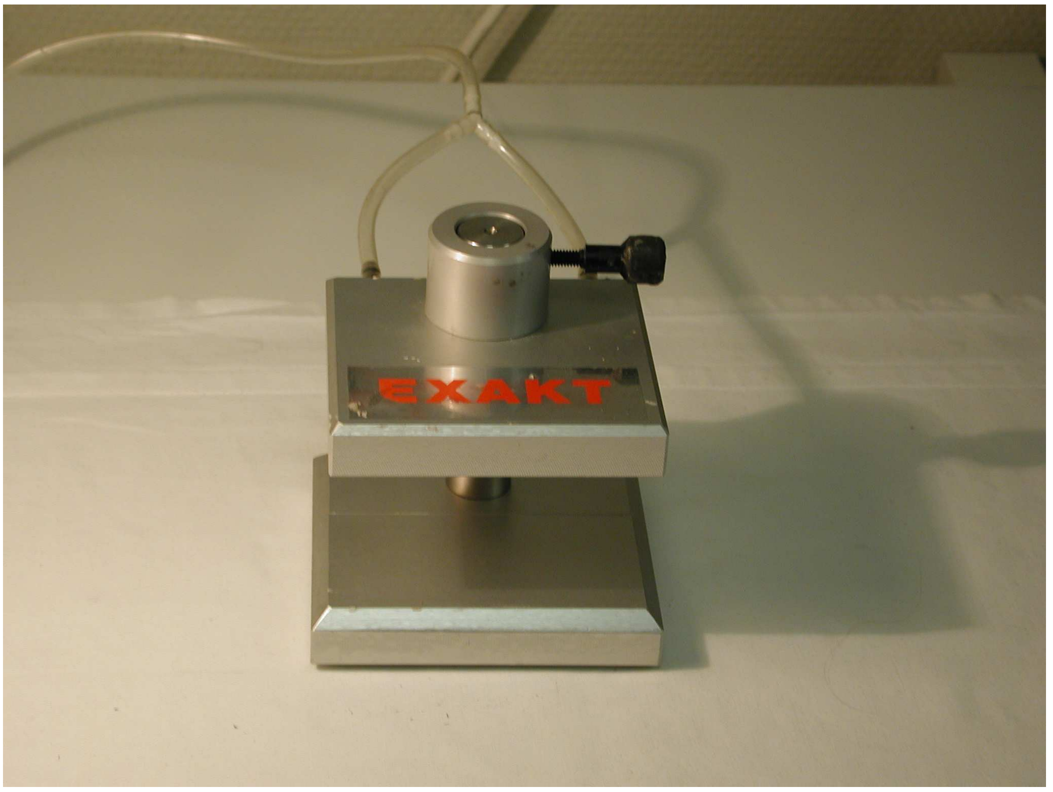

Fig. 4 EXAKT 401 Vacuum Adhesive Press. The Vacuum Adhesive Press is used to mount specimens in specific plane. EXAKT, Norderstedt, Germany.

Fig. 5 EXAKT 530 Re-Infiltration Unit. The Re-Infiltration Unit is used for re-infiltration and drying at several steps of the process. EXAKT, Norderstedt, Germany.

Fig. 6 EXAKT 402 Adhesive Press. The Adhesive Press is used to mount a slide on the specimen via a light curing adhesive. EXAKT, Norderstedt, Germany.

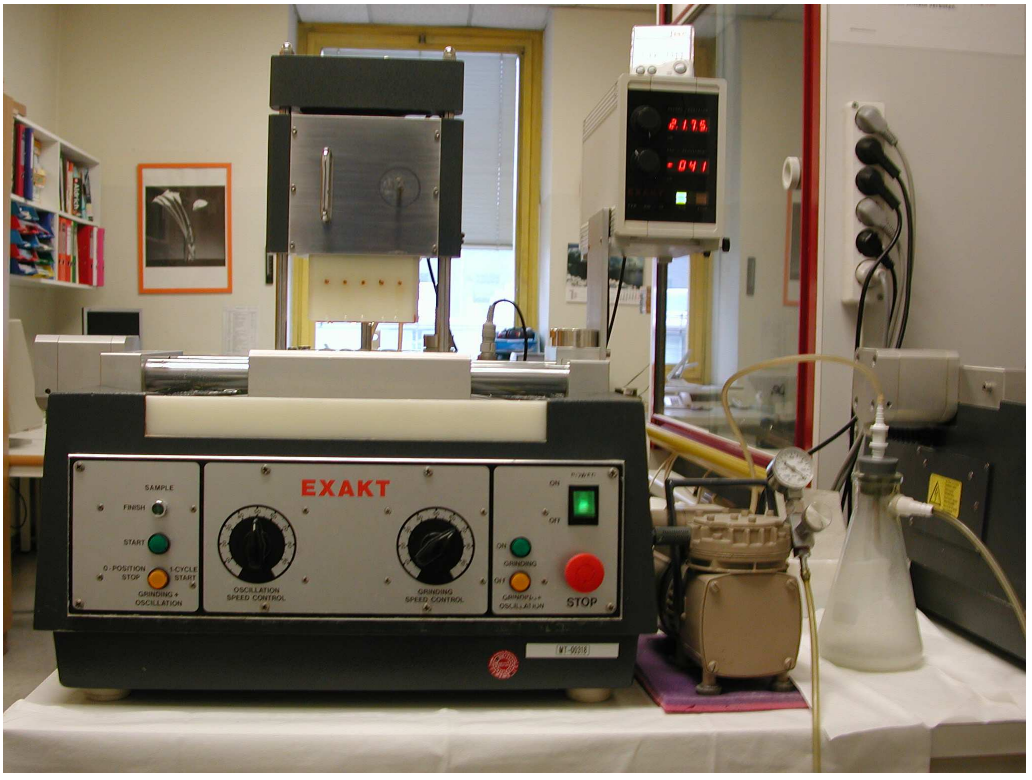

Fig. 7 EXAKT 400 CS Grinding System. The grinding system is used (with special grinding paper) to produce thin ground sections. The deviation of the sample thickness after the grinding process amounts ± 1.5 µm (manufacturer's statement). EXAKT, Norderstedt, Germany.

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne fremde Hilfe verfasst, andere als die angegebenen Quellen und Hilfsmittel nicht benutzt, bzw. die wörtlich oder sinngemäß entnommenen Stellen als solche kenntlich gemacht habe.

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Gehe Deinen eigenen Weg, denn dann verläufst Du Dich nicht.

J. Krämer