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Original Research

Age estimation using tooth cementum annulations: bias and sources of inaccuracy

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Abstract

Background: Counting the tooth cementum annulations (TCA) is a method for estimating the age at death of adults by sections of their tooth root. The objective of this study was to assess the precision of counting the cementum incremental lines and the congruence between known age and age estimates. Possible factors affecting the accuracy of the estimate were also analyzed. **Methods**: A sample of 67 permanent teeth extracted from individuals with known age (18–84 years) and sex was analyzed to calculate the dental age. **Results**: Results demonstrate an excellent inter- and intra-observer reliability of annuli counting, with dissimilarities within the limits of agreement. A moderate positive correlation was found between chronological age and TCA. Our results showed that age congruence rates differed across age groups (85% congruence in individuals $\leq$30 years; 75% in individuals aged 31–60 years; 60% in the over 60s). Considering the bias, this method showed a clear tendency to underestimate age in specimens from old people. After age 43, the TCA estimate is highly inaccurate exceeding the underestimation of 10 years, on average, in comparison to the chronological age. Both chronological age and dental arch seem to influence the accuracy of estimates, unlike sex and the tooth root number. **Conclusions**: TCA analysis is characterized by high precision and low accuracy, decreasing with age. Therefore, its applicability is limited in elderly subjects. The choice of methods for age estimation in adult skeletal remains should take into account the particular age range of individuals. We recommend using different age estimation methods to verify the reliability of the performed assessments.

Keywords: age estimation; forensic anthropology; cementochronology; tooth cementum annulations

1. Introduction

Tracing the biological profile of an individual from skeletal remains is a major issue in forensic science, as well as in the study of ancient human populations. With particular reference to the forensic context, the anthropological analysis will allow for personal identification even when genetic analysis is not possible. While some analyses may lead to a fixed discrete classification (e.g., male or female, when a sex determination can be achieved), others, such as the methods traditionally used to estimate the age at death in adults [1–3], give a fairly wide range within which the age of the individual examined is presumably included. The difficulty in narrowing this range in adults depends on the fact that the methods used are generally based on the degree of bone remodeling or degenerative processes, which are strongly affected by biological variability or environmental factors. As a rule, the greater the age of the individual examined, the wider the age range; therefore, in some cases, only generic indications can be provided (e.g., age >50 years using dental wear [4], maxillary suture obliteration [5], pubic symphysial surface [6] and sternal rib end [7]). Given this, the possibility of achieving a more defined age of death is an important methodological objective in the identification process.

Analyzing tooth cementum annulation (TCA) is a histological dental method routinely used on non-human mammals [8] that has been demonstrated to have important applications on humans. The method is based on the principle that dental root cementum is subjected to continuous growth throughout life, resulting in the formation of annual incremental lines. These lines appear under the microscope as pairs of light and dark layers: each pair would represent annual growth. Adding the incremental line count to the age of eruption of the tooth yields an age estimation of the individual [9].

Although the prospect of determining the age at death in a more precise manner is very tempting, TCA analysis can hardly supplant traditional anthropological methods because it is not yet sufficiently standardized and tested in humans. Despite previous research in humans (see among others [10–13]), the results shown in the literature studies reported heterogeneous accuracy with correlation coefficients ranging from 0.42 to 0.97 between chronological and biological age [14]. A progressive decrease in accuracy and bias has been shown with aging, leading to an underestimation of age especially in individuals over 40 years of age [11]. This has certainly increased the uncertainty in the application of the method by researchers [15]. From a technical point of view, despite numerous published protocol proposals (among others [12,14,15]) starting with Stott *et al.* [10], there are still difficulties in reading incremental bands [16,17]. In addition to the destructive nature of this analysis, the current low diffusion of this method depends on several technical and theoretical reasons, starting from which is the most appropriate technique up to the interpretation of the biological nature of annuli [18]. Another highly debated issue concerns the use of tooth types. While some studies claim that only mono-rooted teeth are appropriate for TCA estimates [15], others consider all tooth types to be adequate [19].

Ideally, TCA analysis could be useful in supporting identification also in poorly preserved dead bodies if the protocols were better defined, validated, relatively simple, and using tools commonly found in a forensic histology laboratory. The main aim of this study is, therefore, to verify the applicability and reliability of TCA-estimated age in a sample of the Italian population with known age and sex by evaluating inter- and intra-observer effect on annulation counts, looking for procedures that do not require specific equipment (low-speed diamond saw) and verifying whether all tooth types are suitable. In addition to the validation of the annuli counting, this study aims to test the method's effectiveness and limitations in estimating age with regard to chronological age, sex, root type, and dental arch.

2. Materials and methods

2.1 Sample

The study sample consists of 89 permanent teeth extracted from 89 individuals. The teeth, from all types and quadrants, were collected from several dentists and dental clinics of Bologna and Ferrara (north Italy). All tooth extractions were performed for therapeutic purposes during the period 2018–2019. The age and sex of individuals were noted, but the person's anonymity has been preserved. The main reasons for extractions were periodontal disease and caries. The age of individuals ranged from 18 to 84 years. Of this sample, 67 teeth from 24 men and 43 women were viable for testing, as 22 could not be counted and were removed from the sample at an initial stage, either because of the poor quality of the tissue after preparation or because of irregular histology in the cementum incremental lines, as suggested in such cases [12]. Moreover, according to Kagerer and Grupe [19], only teeth with no or only marginal periodontal diseases were selected for this study. Teeth with root caries were excluded. Any additional tooth extracted from the same person was excluded. Fifty teeth of the sample were extracted from the upper dental arch and 17 from the lower arch, including a total of 6.0% incisors, 3.0% canines, 11.9% premolars, and 79.1% molars. No preference was accorded as to the tooth type, using both multi-rooted and mono-radicular teeth. Each tooth was assigned a numeric code and was photographed before sectioning.

2.2 Preparation technique

For the histological analysis, the preference was accorded to decalcified sections. The procedure followed was consistent with the protocol shown by Foster [20]. Crowns were removed with a Dremel® 3000 Rotary tool, to shorten the time required for decalcification; roots were then submerged in Osteomoll® decalcifying solution (CH_2O 4%, HCl 10%) for approximately 36–48 hours, with some of the hardest teeth requiring even 72 hours before reaching the required level of decalcification. Each root was further cut transversally to its middle third and embedded in paraffin (Leica ASP300 processor, Histoline TEC2900 incorporator). The middle third area was then cut into 10 μm sections starting from its occlusal end (Leica HistoCore auto cut microtome), for at least 3 for each sample.

The staining was performed using hematoxylin and eosin, after each section was deparaffinized in xylene for 10' and rehydrated in a descending ethanol series (10' in 100%, 5' in 90% ethanol, 5' in 70% ethanol) and rinsed in deionized water. The sections were then dipped in hematoxylin for 1.5', put in tap water for 2' and then in eosin for 1.5', then dehydrated in an ascending ethanol series (50%, 70%, and 100%) and cleared in xylene for 10' before mounting coverslips.

Fig. 1. Cementum annulation count in a section of 10 μm with hematoxylin and eosin stain. The original color, contrast, brightness, and saturation of the image have been modified to enhance the annuli readability.

2.3 Microscopic analysis

The sections were examined using Optika Microscope B-500Ti at 10× and 40× magnification and real-time image Motic Images Plus software (version 3.0.11.36; developed by Motic China Group Co., China) for digital image enhancement (Fig. 1). This allowed the observation of the complete cementum band to assess the best counting points, in which four different images were taken. In the case of multi-radicular teeth, when different roots were within the middle third area, we considered all the available and readable roots. The images were enhanced by contrast improvement and adjusted either through the grey-scale gradation or embossing procedures, thus obtaining distinct alternating dark and light annulation lines that were initially counted manually at the monitor by the same operator (A) by marking them individually on digital images with ImageJ© software (Version 1.8.0_172; developed by Wayne Rasband and contributors, National Institutes of Health, Bethesda, MD, USA) to reduce the risk of error. When a difference in the annuli count was found among the 4 images, the one with the highest number of annuli was chosen. Six months after the first survey, operator A repeated the counting on a randomly selected subsample, which was also independently examined by another operator (B).

To obtain an estimation of a person's age, cementum lines were added to the age at which the root is formed: to this purpose AlQahtani *et al*. [21] "Atlas of Human tooth Development and Eruption" was used as a reference.

It was observed that sometimes, even if cementum is well preserved, the lines are almost indistinguishable.

2.4 Statistical analysis

To test the reliability of counting methods we calculated the intra-observer and inter-observer error using Interclass Correlation Coefficient (ICC). For this analysis, we compared the number of lines observed by two different operators, the first one performed by the same operator six months apart (A1 vs A2), and the latter by a different one (A vs B).

Further statistical analyses were performed on the whole sample and by tooth type (mono- and multi-rooted), dental arch (upper, lower), sex, age groups. The defined age groups were as follows: young ($\leq$30 years), middle (31–59 years), old ($\geq$60 years) adults.

We computed the Bias (Δ) of estimated age as the difference between the estimated age and the chronological age in the whole sample and sub-samples, as Δ = Age^{est}– Age^{chron}. The % Inaccuracy (%Δ) of estimated age was evaluated as the difference in absolute value between the estimated age and the chronological age divided by chronological age (in percent), as %Δ = (|Age^{est}– Age^{chron}|/ Age^{chron}) $\times$ 100. We carried out scatterplots to delineate the relationship between chronological age and Bias using the regression line.

Comparisons between subsample means were performed using the *t*-test or Mann-Whitney U when the sample was $<$30 individuals. One-way ANOVA on ranks (Kruskal–Wallis H test) has been used for comparisons between more than two groups. Multiple regression analysis was performed to assess possible predictors of inaccuracy in the age estimation from annuli counts. The regression model included a variable in the continuous scale (chronological age), while sex (0 = females; 1 = males), tooth type (0 = multi-rooted; 1 = mono-rooted), and the dental arch (0 = lower; 1 = upper) from which the tooth was extracted were included as binary variables with females, multi-radiculated teeth, and lower arch as the reference groups. Multicollinearity of the data was evaluated by variance inflation factors (VIF), with VIF values $<$10 assumed to be acceptable [22,23].

All tests were performed with the *p*-value set at 0.05 for significance using Statistica software version 11.0 (StatSoft Srl, Tulsa, OK, USA) and MedCalc Statistical Software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium).

3. Results

3.1 Intra- and interobserver consistency in cementum annuli counts

Six months after the initial count, a recount in a subsample of 25 randomly selected teeth was performed. Intra-observer reliability computed between two independent repetitions of annuli counting by the same operator (A1–A2) showed an ICC of 0.98 (95% CI 0.9409–0.9892; n = 25/67) to be considered excellent. The mean difference between count repetitions was less than one annulus (mean difference = 0.8, and 95% CI –3.0 to +4.5) (Fig. 2a). Inter-observer reliability computed between two different observers (A–B) indicated an ICC value of 0.98 (95% CI 0.9098–0.9927; n = 25/67). Also in this case the reliability indicated by ICC is excellent [24]. The Bland-Altman plot (Fig. 2b) shows the degree of disagreement between the two surveys based on the annuli counts. These dissimilarities are within agreement limits, except for two values. The mean count difference between observers was less than two annuli (mean difference = 1.6, and 95% CI –2.6 to +5.8).

3.2 Chronological age and TCA-estimated age

In our study, we included 89 teeth. On the whole sample, 75% of teeth (67/89) enable annuli counts, with a failure rate of 25% due to poor readability or irregularities in the cementum incremental lines.

If each annulation represents one year of life, the sum of the number of annuli and age of dental eruption should provide an estimate of chronological age. The distribution plot of chronological and TCA-estimated age data is shown in Fig. 3. A broad dispersion of the data with age was found. Pearson's correlation coefficient indicated a significant correlation between age estimate and chronological age (r = 0.565, $p < 0.0001$, n = 67; r-squared = 0.319).

A detailed examination of the three age groups showed that the correlation decreases from the youngest age group characterized by the strongest correlation to the oldest one displaying a non-significant negative correlation ($\leq$30 years: r = 0.753 $p < 0.0001$; 31–59 years: r = 0.397 p = 0.049; $\geq$60 years: r = –0.219 $p > 0.05$).

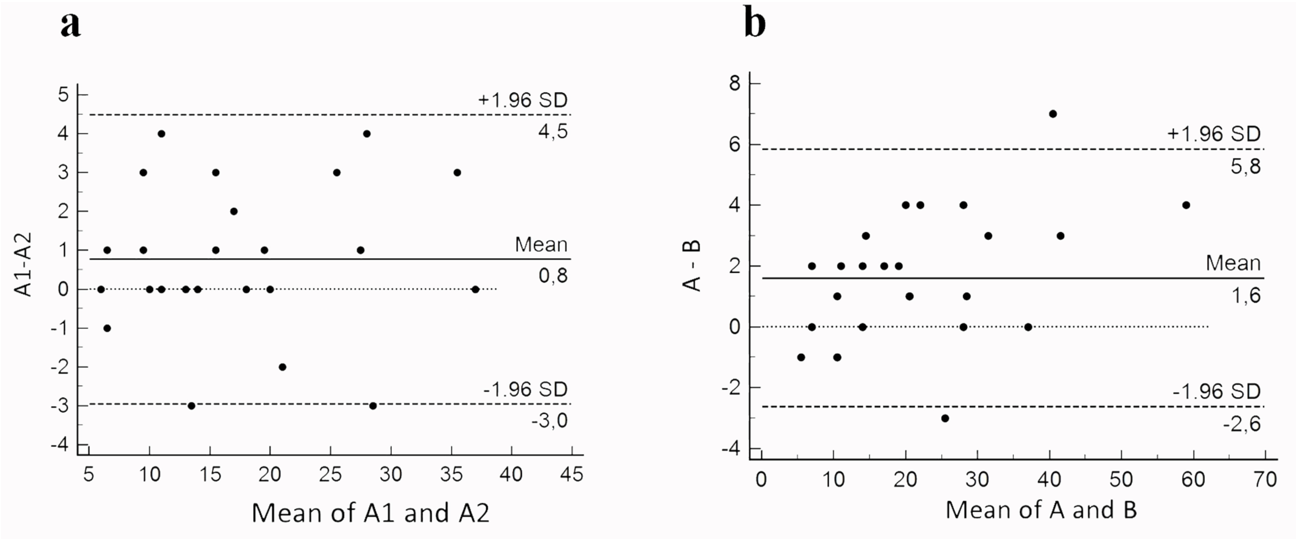

Fig. 2. Bland-Altman plot evaluating (a) the intra-observer variation between first annuli counts (A1) and repeated counts 6 months apart (A2), (b) the inter-observer variation in annuli counts. The solid line displays the mean difference, and the long dashed lines indicate the 95% agreement limits. X-axis: average of the two counts; Y-axis: difference between the two counts.

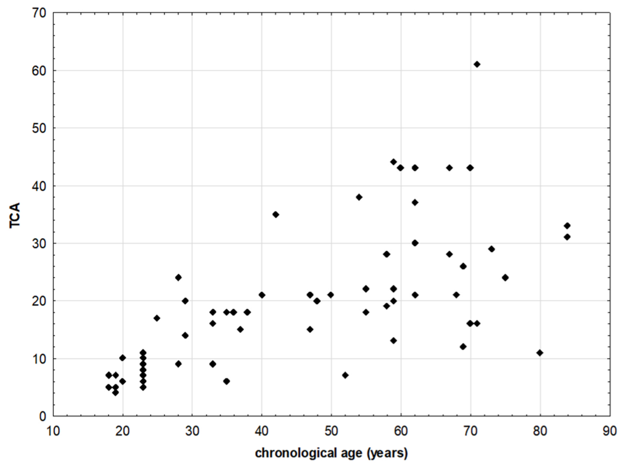

Fig. 3. Overview of sample data showing the relationship between chronological age and age estimate by TCA.

Table 1. Bias (Δ, in years) and Inaccuracy (%Δ) in age predictions according to the number of roots of the tooth, dental arch, sex, and age groups.

Variable	Δ (SD)	%Δ (SD)	*p*
Tooth root			0.351[a]
Single (n = 14)	–20.6 (19.1)	31.6 (24.1)	
Multiple (n = 53)	–10.1 (15.4)	24.8 (19.3)	
Dental arch			**0.020**[a]
Upper (n = 50)	–13.7 (17.6)	29.4 (20.3)	
Lower (n = 17)	–8.0 (13.0)	16.8 (18.3)	
Sex			**0.042**[b]
Males (n = 24)	–21.2 (17.6)	32.9 (23.2)	
Females (n = 43)	–7.3 (13.9)	22.4 (17.9)	
Age groups			**0.0019**[c]
≤30 (n = 22)	1.5 (4.4)	15.2 (2.9)	
31–59 (n = 26)	–12.3 (11.9)	25.7 (11.3)	
≥60 (n = 19)	–28.2 (17.1)	39.6 (16.7)	
Total (n = 67)	*–12.3 (16.6)*	*26.2 (20.4)*	

[a] Mann-Whitney U; [b] Student's *t*-test; [c] Kruskal-Wallis test.

3.3 Accuracy of TCA-estimated age

In the overall sample (n = 67), the average bias was –12.3 years (SD: 16.6), corresponding to an average inaccuracy of 26.2% (SD = 20.4). The comparison between chronological age and estimated age by dependent sample *t*-test indicated a highly significant difference between means ($p < 0.00001$).

We then moved on to consider whether there was a different accuracy in the estimates made on single- and multi-rooted teeth, or in the teeth of the two dental arches, or between sexes (Table 1). Inaccuracy shows higher values in single-rooted teeth, but the differences from multi-rooted teeth are not statistically significant. Estimated age seems closer to chronological age in teeth from the lower arch with statistically significant differences from upper arch teeth (Table 1). A smaller bias between estimated and chronological age and significantly lower percentage inaccuracy were found in the female sex (Table 1). Finally, we analyzed the accuracy of age estimation by dividing the sample into three age groups (Table 1). Chronological age was estimated with a bias of fewer than 2 years, on average, in the youngest group, whereas chronological age was underestimated by more than 25 years in the oldest group. Differences in %Δ between groups were highly significant with an increase in inaccuracy with age.

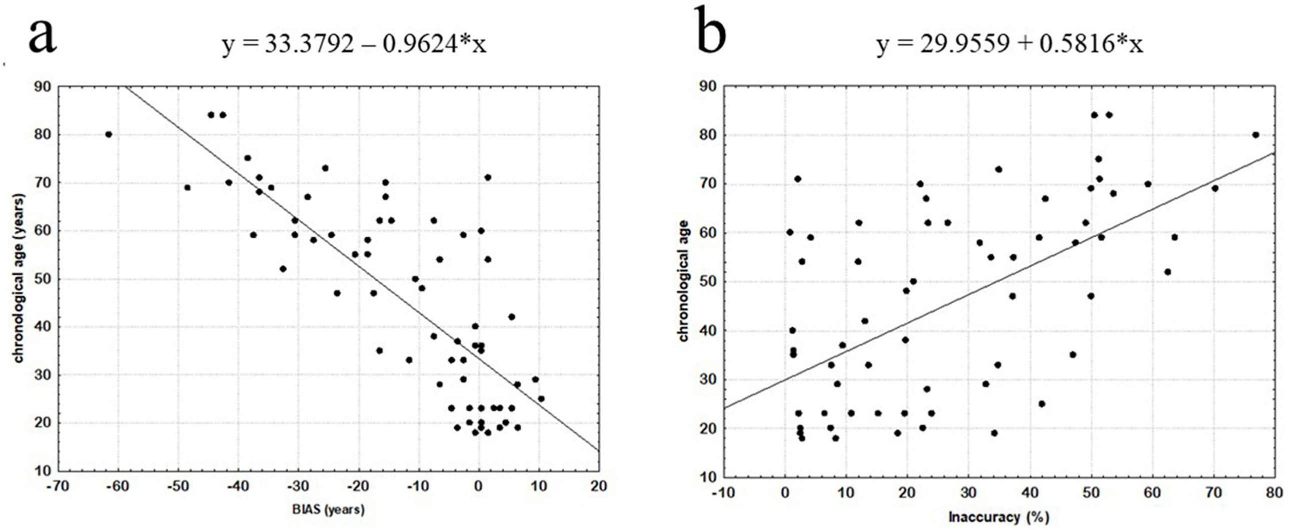

Fig. 4. Scatterplot showing the trend of (a) Bias (Δ) and (b) Inaccuracy (%Δ) in age predictions with chronological age.

Since the age factor seems to strongly affect the accuracy of age estimation, we further analyzed this trend. Scatterplots (Fig. 4), Pearson correlation coefficients and linear regression analysis between inaccuracy (Δ and %Δ) and chronological age (Δ: r = –0.79, $p < 0.0001$, y = 33.38 – 0.96 × x ; %Δ: r = 0.59, $p < 0.0001$, y = 29.96 + 0.58 × x) in the overall sample (n = 67) show that accuracy in age estimation decreases as age increases. While the second diagram (4b) shows a positive linear association between inaccuracy and chronological age, the first diagram (4a) displays a negative linear association between Bias and chronological age with older individuals placed on the upper left side. Therefore, the age of older individuals is underestimated, unlike younger individuals who tend to be close to the x-axis origin.

We performed a multiple linear regression to investigate whether % Inaccuracy (dependent variable) could be explained through some of the examined variables (independent variables), and to determine which of them most predicts the outcome of inaccuracy in chronological age estimation from annuli counts (Table 2).

The model, testing the influence of chronological age, tooth root, sex, and dental arch of the analyzed tooth for the Inaccuracy (%Δ) in age estimation, led to a significant R^2. Inaccuracy was significantly associated with two out of four independent variables: chronological age and dental arch.

In particular, the annulation count performed on the upper dental arch proved to be a significant determinant (negative association) of inaccuracy in comparison to the lower arch. Moreover, inaccuracy increases as the chronological age - the strongest predictor-increases: the unstandardized coefficients of chronological age in the multivariate regression was 0.59 (not reported in the table), implying that one more year of chronological age determines an increase in the inaccuracy of 0.59 percentage points. The total explained variance of the model is 38.5%. Multicollinearity did not arise between the predictors.

Table 2. Predictors of Inaccuracy (%Δ) in age estimation by multiple regression.

Variables	Model			
	β	t	*p*	VIF
Chronological age	0.590	5.381	**<0.00001**	1.289
Tooth root (single)	0.109	1.043	0.301	1.166
Sex (males)	–0.083	–0.805	0.424	1.138
Dental arch (upper)	–0.251	–2.587	**0.012**	1.012
R^2	0.422			
Adjusted R^2	0.385			
p	**<0.00001**			

β, standardized regression coefficient; VIF, variance inflation factor.

4. Discussion

The analysis of teeth can give useful information to establish the age of the individual. This can be of extreme importance in the forensic context concerning both the living and the dead person. In the latter case, the estimation of the age at death is a fundamental element in the identification process. Age estimation can be easily undertaken through dental development and eruption methods in children and adolescents (sub-adults) [25], while these methods are not applicable over the age of 21 [26]. When the third molar is absent, this threshold is set at 14–15 years. Thereafter, other traditional anthropological methods for age determination can be used that are based on degenerative changes, but these show great variability and often do not allow distinguishing between individuals over 50 years. Turning to dental methods again, the Lamendin method yielded very accurate results for middle adults [27], even if it applies only to individuals over 25.5 years of age. In this study, we examined, as a possible alternative method in determining age from teeth in adults, the cementochronology, which was already proposed about 40 years ago to estimate age in humans [10], as in other mammals. The full sequence of cementum annulations provides a record of the individual's life history from the time of tooth eruption to the time of death or to the time of tooth extraction/loss. Being an invasive technique, this is not appropriate for living adults.

Since this promising method for age estimation is currently scarcely adopted in forensic and archaeoanthropological fields, we decided to test the methodological reliability on a sample of teeth extracted from individuals with known sex and age.

The reasons for its low diffusion may lie primarily because, besides being a destructive technique, the need for special and costly equipment restricts its applicability [28]. Consequently, we decided to apply this method by using tools and instruments commonly available in a well-equipped forensic histology laboratory. Starting therefore from the main distinction of the techniques executable on undecalcified sections [10] or decalcified sections [29], we chose to exclude the first ones as they require the use of very expensive and not widely spread devices (low-speed diamond saw), although the other procedures require longer processing times (decalcifying and embedding procedure).

In addition to technical problems that may be an obstacle in the choice of this method, it should be remembered that one of the main reasons for researchers to apply this method with some hesitation is the heterogeneous accuracy of the results [14,17]. In this regard, we first investigated the precision in incremental line counts, and then evaluated the accuracy in age estimation that these counts allowed. The intra-observer and inter-observer reliability of annuli counts made on the same images allowed us to ascertain the precision of the cementum lines counting, achieving excellent results in both cases.

In the whole sample, we observed a moderate correlation between known and estimated age (0.56). Although statistically significant, this value was lower than the average correlation coefficient (0.87) reported in the review by Naji *et al*. [14], and the determination coefficient showed that the annuli counts do not accurately predict chronological age, as only 32% of the variability in chronological age is explained by the estimated age. It is possible that the methodology applied in this study (analysis of TCA on decalcified semi-thin sections of all tooth types) has conditioned this result and that a higher correlation between the variables and, consequently a higher accuracy in estimating age, could have been achieved in this sample if other validated protocols had been used (among others [12,28,30,31]). Sultana *et al*. [32], for example, recently obtained an average difference of just one year between chronological and estimated ages in a sample of 60 teeth extracted from individuals in a narrow age range (20–50 years). Moreover, a strong correlation (r = 0.93) was found in a recent study carried out on 200 teeth of the same type (canines) [15].

The univariate and multivariate analyses conducted revealed that there is no significant difference in the accuracy of age estimation compared to chronological age between single-rooted and multi-rooted teeth. This allows an important possibility of extension of this methodology to dental specimens of any type, in contrast to the technique still prevailing that involves the use of single-rooted teeth only (see among others [11,15]). Our findings have been confirmed by other studies [19,30] but contradict, for example, the study by Sultana *et al*. [32], who observed a greater difference between chronological and estimated age in specimens consisting of third molars -only three third molars were included in their sample- than in the other types of teeth examined.

The different trends of sex comparisons in univariate versus multivariate analyses can be interpreted regarding the distribution of the sample with a reduced presence of males in younger age groups with good accuracy and their increased presence in older age groups characterized by lower accuracy in comparison to females (chi-square = 7.0, df = 2, $p < 0.05$). Indeed, the results of multiple regression analysis showed that there are no differences in accuracy between the sexes, as shown in other studies [15,33].

We also observed a different trend in the accuracy when comparing upper and lower teeth by univariate and multivariate analyses. In this case, we verified that there was a reduced presence of teeth from the upper arch in the younger age group with good accuracy and their increased presence in middle/old age groups, unlike the teeth of the lower arch (chi-square = 7.7, df = 2, $p < 0.025$). Based then on the results of the multiple regression analysis, a significantly greater accuracy resulted for the upper dental arch than for the lower dental arch. This particular aspect, almost completely neglected by studies in the literature, deserves to be better explored and understood. According to Wittwer-Backofen *et al*. [12], comparable reliability resulted for teeth from maxilla or mandible (see also [13,34]). We believe that the comparison between upper and lower teeth from the same individuals could certainly help to verify our findings. Karunakaran *et al.* [35] performed a comparative study between teeth from the two arches of the same individuals (n = 50). Using the database reported in this study, we calculated and compared the %Δ (SD) of maxillary (5.63 $\pm$ 2.43%) and mandibular teeth (7.47 $\pm$ 4.15%) of the same individuals by paired *t*-test with statistically significant results (p = 0.001). This data from the literature, therefore, seems to confirm the different trend in accuracy between dental arches, with better performance in age estimation by maxillary teeth than mandibular teeth.

The comparison between the three different age groups of the sample showed that the age underestimation increases with the increase of chronological age. This result may be due to the greater difficulty in line counting in elderly subjects as the cementum becomes both thicker and denser with thinner lines [36]. We found an accuracy of 85% with a reduced bias (1.5 years on average) up to 30 years of age, while in the later age groups the age estimation worsens with an accuracy that drops to about 75% in the 31–60 years group and about 60% over 60 years of age. As in the forensic field, it is generally believed that an acceptable method of age estimation should not exceed a threshold of ±10 years compared to the chronological age [37], we verified employing the linear regression equations on the entire sample (Fig. 4a) that this threshold falls at 43.4 years of age in our sample. Above age 43, there are underestimates of chronological age greater, on average, than 10 years and increases in %Δ for age prediction that make age estimation problematic using only the TCA analysis according to our protocol. Conversely, Colard *et al*. [28] found the best results in three older individuals compared to younger ones by examining nine anthropological cases by a validated protocol [31]. Consistently with our results, Obertová and Francken [11] found considerable underestimation of age in individuals older than 40 years. Irregularities and a slowdown in the rate of cementum deposition are likely to occur with aging. In addition to the increased risk of error in the elderly because of the greater difficulty in counting thinner and more compressed annuli (less distinctness), the decreased accuracy of TCA-estimated age in the elderly would depend at least in part on changes in the physical properties of cementum with aging due to the reduction in mechanical stress (masticatory forces) with age [38]. Age-related decline in age estimation is a general characteristic of skeletal indicators of age, so much so that it has been suggested that there is no single indicator that can accurately reflect the age at death [39]. According to Martrille *et al*. [27], the best strategy is to use many dental and skeletal indicators to achieve reliable results. In particular, starting from the assumption that no single method of age assessment is adequate for the whole life span since they depend on several factors, such as sex, population, size, and individual characteristics [40,41], it is suggested that the membership of the individual under consideration in a generic young to the old group be initially determined by reliable methods, such as that of the pubic symphysis [42,43] or that of the sternal end of the fourth rib [44]. Thereafter, the age can be estimated using the methods that are more appropriate for that age group. Although these macroscopic methods have some limitations, as they are population-dependent and are based on inverse regression [45], this preliminary age assessment of the skeleton before selecting a specific method of age estimation is also supported by Baccino *et al*. [46] and other authors [47,48], and is referred to as the two-step procedure. Our study indicated that the TCA age estimation can be a reliable method for young adults, but it should be combined with multiple age indicators of age estimation for older adults (age >40 years). Without a doubt, a forensic anthropologist should employ multiple regions of the skeleton and different methodologies of analysis to provide a final age estimation in the process of identifying the deceased.

Another important aspect is cementum composition, which was analyzed by Edinborough *et al*. [49]. They underlined that life-history parameters (e.g., pregnancies, skeletal trauma, and renal disease) for putative cementum deposition periodicity cannot be rigorously calculated, using optical microscopy. Their results reject the possibility of accurate estimation of the distribution of mineralization of tooth cementum using light and scanning electron microscopy alone and conclude that the best analysis for knowledge cementum composition is ToF-SIMS (Time-of-Flight Secondary Ion Mass Spectrometry). Finally, new and important steps have recently been taken in the non-destructive estimation of age at death in adults. In particular, Le Cabec *et al*. [50] have used synchrotron imaging to non-destructively display cementum annulations in known age teeth from 18–19th centuries collection, while Newham *et al*. [51] developed a new method for semi-automatic increment counting using three-dimensional images provided by X-ray propagation-based phase-contrast imaging on fresh dental tissue.

This study has several strengths, which include applying a technique that can be implemented easily without requiring expensive laboratory instruments. Furthermore, we excluded teeth from the same individual, as it may affect the findings [37]. However, the major strength of this study is the analysis of both single- and multi-rooted teeth. This made it possible to exclude that the different tooth types used may condition the inaccuracy of age estimate.

Among the limitations of this study, in addition to the small sample size, there is the use of a sample that consisted exclusively of therapeutically extracted teeth, despite the ongoing debate on this issue [15,52]. Although we cannot exclude that periodontal disease may have affected the apposition of the cementum, we sectioned the middle third of the root according to Broucker *et al*. [53], as only limited effects on the cementum annuli count have been reported in this part of the root. Apart from cases of profound periodontal pathologies, Kagerer and Grupe [19] showed that teeth with only marginal periodontal disease yielded very satisfactory outcomes if the level of section was carefully selected. Other studies also support the slight impact of periodontal diseases on TCA counting [32,54]. No influence was observed by Wittwer-Backofen *et al*. [12] who stated: "the accuracy of the TCA age estimation is independent of periodontal disease".

5. Conclusions

Our results confirm that annuli counting is precise, but the TCA age estimates are poorly accurate when using decalcified, stained, thin sections of all tooth types. Moderate correlations between chronological and estimated ages compared with those in other studies could be explained by methodological differences related to the protocol adopted. In our study, accuracy in the age estimation by TCA is independent of root type and sex, while it mainly depends on the chronological age. Although the influence of dental arch on the accuracy in age estimation still requires further investigation, its relevance as an accuracy predictor emerged from this study. In particular, the analysis of TCA proves to be a very convenient method of estimating age for young adults, but not in older adults (over 40 years old) as cementum lines become increasingly difficult to differentiate with aging. In a forensic context, different approaches to the age at death estimation are always advisable to support the age estimation by TCA, especially in older adults.

Abbreviations

TCA, tooth cementum annulations; ICC, Interclass Correlation Coefficient; VIF, variance inflation factor.

Author contributions

EG-R, NR and MN designed the research study. IS, PF, NR and JM performed the research. EG-R and NR analyzed the data. EG-R was a major contributor in writing the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Data processing complies with the general authorization for scientific research purposes granted by the Italian Data Protection Authority (1 March 2012 as published in Italy's Official Journal no. 72 dated 26 March 2012) since the data do not entail any significant personalized impact on data subjects. Approval by an institutional and/or licensing committee is not required since experimental protocols are not applied in the study.The patients' data were completely anonymized, according to Regulation (EU) 2016/679 of the European Parliament and the Council: "The data protection principles should therefore not apply to anonymous information, i.e., information that does not relate to an identified or identifiable natural person or to personal data made sufficiently anonymous to prevent or no longer allow the identification of the data subject. Therefore, this regulation does not apply to the processing of such anonymous information, including for statistical or research purposes".

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Conflict of interest

The authors declare no conflict of interest.

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