EVALUATION OF CEMENTOCHRONOLOGY AS AN AGING METHOD FOR INEXPERIENCED RESEARCHERS

by

Emilie Marie Cobb

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Approved by:

____
Gwen Robbins Schug, Ph.D., Thesis Director

____
Laura Ammon, Ph.D., Second Reader

____
Anthony Love, Third Reader

____
Timothy Smith, Ph.D., Departmental Honors Director

____
Ted Zerucha, Ph.D., Interim Director, The Honors College

ABSTRACT

There are numerous different methods of determining the age of an adult human skeleton from the auricular surface or pubic symphysis of the pelvis, attrition of the human dental enamel, and degeneration of other syndesmosis. Age-at-death estimates from cementum annulation counts are one of the most accurate methods available. Cementochronology can provide an estimate for age-at-death despite pathological conditions that affect the bones and teeth; in some cases, where remains are incomplete, fragmented, or damaged post-mortem, a count of cementum annulation**s** might be the only technique possible to obtain an age estimate. This method is of course not without its limitations. Despite its potential accuracy and precision, over the past 20 years, there has been much debate over whether this method should be used for human skeletons, particularly in a forensic context. Concerns are primarily focused on the lack of a standard protocol and validation studies thereof. This thesis will address the question of why counting cementum annulations is potentially so valuable as an age estimation tool and concerns as to whether a recently developed sectioning protocol will make this method more accessible.

There are a variety of different methods to estimate age in human skeletal remains based on joint surface evaluation, observation of dental occlusal surface wear, and sternal rib ends. (Gustafson, 1950; 1962; Işcan et al., 1984; Lamendin et al., 1992; Lovejoy et. al., 1985; Todd, 1920). However, perimortem and postmortem processes can drastically alter skeletal morphology such that teeth are the only elements available for identification and establishing a biological profile. A count of cementum annulations added to the age of eruption for a given tooth is an aging method that is based on a microstructural process that occurs in the permanent teeth throughout the adult lifespan. Cementum annulations are comprised of a material that is inorganic and mostly hydroxyapatite. The annuli accumulate throughout life in layers, unlike bone which remodels continuously, making them useful for aging adult human skeletons (Ten Cate, 1998). This method has a high degree of accuracy and is comparatively precise, with a margin of error of plus or minus 2 years making it one of the most accurate aging methods available for human skeletal remains, if the process is properly performed (Wedel, 2007). Cementochronology is used frequently by archaeologists in estimating the age of teeth from other mammals, but the process by which cementum annulations form in modern humans is not well understood despite recent research interest (Ten Cate, 1998). Despite the uncertainty about underlying biological mechanisms responsible for producing the annulations, validation studies on teeth of known age have demonstrated that the counts can be correlated with known age in 98% of cases (Charles, Condon, Cheverud, Buikstra, 1986). This honor's thesis describes the literature that has hitherto been published on this aging technique, and examines one of the protocols that has been created (ISO-9001) from an undergraduate perspective. This thesis will specifically focus on whether or not cementochronology is a valid and accurate method for estimating age-at-death in adult humans for researchers in the medico-legal context, where evidentiary standards are highest (Wedel, 2007).

LITERATURE REVIEW

The biology of cementum

Composition and formation. Cementum is an extracellular matrix of cementoblasts and it that covers the dentin as a living tissue. This provides attachment for the periodontal ligament (Ten Cate, 1998). The cementoblasts differentiate and begin depositing cementum. During the development of teeth, the cementum occurs as cellular and acellular materials. Acellular cementum develops slowly after tooth eruption until it covers two-thirds of the coronal root surface. At the apex of the tooth root, cementum is thicker and cellular cementum is the most abundant type found. Cementum can also be categorized as fibrillar and afibrillar depending on the presence or lack of collagen fibers. Intermediate cementum is highly mineralized and has a collagen poor-layer. The function of this type of cementum is still debated, although it is thought that it is a precursor for cementogenesis and damage repair (Ten Cate 1998). Acellular afibrillar cementum is composed of glycosaminoglycans (GAGs) and its function is currently unknown. Acellular extrinsic fiber cementum (AEFC or primary cementum) contains type 1 collagen fibers and (GAGs). This type of cementum attaches the root surface to the periodontal ligament. Cellular intrinsic fiber cementum (CIFC or secondary cementum) is composed of cementocytes embedded in intrinsic fibers which are parallel to and cover the root; CFIC is involved in damage repair and post-eruption movement of the tooth. The final type of cementum is cellular mixed stratified cementum. This type of cementum contains alternation layers of AEFC and CIFC, and is covered with a thin layer of AEFC. It involves reshaping the tooth surface to compensate for shifting of the tooth in its socket (Ten Cate 1998).

Cementum is composed of approximately 65% (by weight) inorganic material and 35% organic material. The inorganic material is mostly hydroxyapatite, similar to the composition of bone (Ten Cate, 1998). The organic material of cementum includes: calcified extrinsic collagenous Sharpey's fibers, extrinsic type 1 collagen fibers, glycosaminoglycans, and proteoglycans (Ten Cate, 1998). This material initially is formed *in utero* when the Hertwig epithelial root sheath around the tooth germ (the beginning stage of tooth formation) disintegrates (Ten Cate, 1998).

Structure and layering of cementum. Cementum is an avascular tissue that is softer than dentin because it is less mineralized. Cementum rarely is repaired except when mechanical stress on the root has been removed or if the root is being reabsorbed (Hillson, 1996). Even then, repair of cementum is not guaranteed. This lack of alteration to the cementum that is typical in human beings, and the fact that cementum grows in irregular light and dark bands of acellular cementum makes this material ideal for age estimation. These bands are likely caused by seasonal changes in collagen orientation and mineralization (Lieberman, 1994).

Through polarized Raman spectrometry, a type of spectrometry that can provide information about molecular orientation using polarization that is either parallel or perpendicular to the extinction of the laser, more information regarding the microstructure of the bands has been discovered (Raman FAQs- What is polarised Raman Spectroscopy?, 2017). The proposal that has emerged due to this type of research is that a higher mineral to organic material ratio is found in the darker bands, and that the dark bands also have more organized collagen fibers. Thus, it is assumed that the winter rest period of the year creates dark, small, more organized and more mineralized bands when compared to light bands of cementum, because it would be consistent with evidence found in zooarchaeological work which is that the winter rest period results in slower tissue growth (Colard et al., 2016) Under a light microscope or scanning electron microscope, these bands can be seen and counted in order to determine approximate age at death and season of death (Lieberman, 1994). It is thought that cementum creation and the incremental lines may be impacted by the following different factors: hormonal cycle, season, ecological conditions, biomechanical forces, and nutrition (Czermak A, Czermak Ad, Ernst, Grupe, 2006). Cementum and incremental line creation may be explained by the effects of parathyroid metabolism. Parathyroid metabolism is responsible for the regulation of blood calcium levels by interacting with vitamin D. However, the exact process of cementum synthesis and mineralization, and how annual incremental lines are produced is currently unknown

(Czermak et al., 2006). A pair of the dark and light bands equals one year (annulation) of an individual's life. A positive relationship between known age and known date-of-death, and the cementum bands has been found in studies performed on human teeth (Wedel, 2007).

Cementochronology is currently the only aging method that involves the consideration of a tissue that continuously grows in an individual's life (Naji et al., 2016). It has been found to have about a 95% confidence interval with error margins of about two and a half years. Periodontal disease, intra-individual correlations, and sex differences have, to date, not been found to have a quantitative impact on cementum formation (Wittwer-Backofen, Gampe, and Vaupel, 2003). Although this finding does contradict a study from the 1980's, which states that the margin of error is greater between premolars and canines when the tooth is demineralized, that study has not since been repeated (Charles et al., 1986). This same study found that the margin of error for cementochronology was about six years, which has been lowered by more recent studies (Condon, Charles, Cheverud, Buikstra, 1986).

Cementochronology in archaeology

In the past, counting cementum annulations to estimate age has only been common in archaeological contexts for non-human mammals or ancient humans. Over 72 different species of mammals have successfully been aged by zooarchaeologists using cementochronology (Naji et al., 2016). Examples of age estimation in ancient humans come from a study in Western Europe where two individuals from the Yamnaya culture in the Early Bronze Age, and three individuals of the Katakombnaya culture in the Middle Bronze Age were estimated. The Early Bronze Age remains were from a 20 to 30 year old woman and a 15 year old girl. The Middle Bronze Age remains were from an 18 year old woman, a 60 to 65 year old man, and an 18 month to two year old child (Klevezal and Shishlina, 2000). In this study, the researchers state that although annulations are more visible when decalcified and stained this cannot be done to fossilized teeth because it can destroy the cementum. They found that the ages determined from the cementum layers corresponded with the age already determined through other aging methods. The exception was the older man and the child; the annulations were not present for either of the individuals (Klevezal and Shishlina, 2000). This is consistent with studies on modern humans which have shown that annulations are difficult to read in humans older than about 50 years of age. This could be due to the annulations not forming as frequently or due to postmortem changes in cementum (Klevezal and Shishlina, 2000).

A study using samples from Damdama, an Indian Mesolithic site in the state of Uttar Pradesh, demonstrates the use of cementochronology and macroscopic methods for bioarchaeology. The age of 18 adult human skeletons was estimated using traditional methods and then teeth were chosen based on which ones could be properly removed without damaging the material (Schug, Brandt, and Lukacs, 2012). The teeth were not decalcified and stained, and were sectioned longitudinally due to the fragility of the teeth. Ages for three of the female skeletons were 9 to 10 years below the age determined through macroscopic methods, and two of the females had cementum age estimates that were nine to 10 years over the age previously determined (Schug et al., 2012). Young adults did not have any statistically significant discrepancies between methods. The range for this study was below or above nine years of age and this was within the margin of error for the macroscopic methods (Schug et al., 2012). It is proposed that increased mobility for females of this population caused an overestimated age. This suggests that activity, which may be culturally influenced, could play a factor in determining age for macroscopic aging methods (Schug et al., 2012). Thus, for individuals with extreme wear on their joint surfaces, counting cementum annulations may be a more accurate indicator of age. The younger females, who had lower ages determined by macroscopic methods, may also have been impacted by activity (Schug et al., 2012). This study shows that cementochronology can alter the demographic of a population formed around traditional aging methods, and that it should be used in tandem with other methods of aging to balance out errors that greatly affect non-continuous tissues (Schug et al., 2012).

In a historical archaeology case, cementochronology was used to determine the ages and season of death at Shiloh Cemetery in Missouri. This rural, historic cemetery was used at the earliest in 1838 and was abandoned in 1876 when the church associated with it was relocated (Wedel and Wescott, 2016). All of the graves were inhabited by African Americans, either free or enslaved, and most of the deceased were adolescents. Macroscopic and microscopic methods were used to determine age-at-death. The ages determined through cementochronology were consistent with ages at eruption and all of the individuals died in the spring or summer of unknown years (Wedel and Wescott, 2016).

Cementum and pathology

Pathology is a major factor to consider when determining the age of an individual. An aging method that is unreliable for remains with evidence of disease limits its use. It has been found that roots affected by periodontal disease do show signs of cementum shape variability and cementum resorption, although there is some debate over this in the literature (Alghonamy, Gaballah, and Labah, 2015). A study by the Oral Surgery and Maxillofacial Department of Tanta University in Egypt looks at the differences of age estimation in healthy individuals and periodontally affected individuals (Alghonamy et al., 2015). The sample included teeth from male and female individuals between the ages of 20 and 70 years old. The age estimates of these teeth varied from having a minor deviation from the known age to a significant deviation from the known age. Statistically, a mean standard deviation for healthy teeth showed no significant difference between known age and estimated age. For periodontally affected teeth, a significant difference between the two ages was observed (Alghonamy et al., 2015). In periodontitis, a bacterial infection of the periodontal ligament, collagen fibers can be destroyed (Broucker, Colard, Penel, Blondiaux, Naji, 2015). This destruction results in the thinning of cementum which causes less incremental lines to be measured, thus an inaccurate age estimate. Three types of microscopes were used in this study: light microscope, polarized microscope, and a phase contrast microscope. Researchers observed teeth with periodontal disease under all three microscopes with the same deviations between ages being seen and confirmed (Alghonamy et al., 2015).

Other studies have found conflicting results. One study that used four women and fourteen men purposefully selected teeth with signs of bone destruction to conduct further testing on how periodontal disease affects age estimates done histologically (Broucker et al., 2015). Forty-one teeth were divided into three groups: group A which had alveolysis on the cervical third of the root, group B with alveolysis on the middle third of the root, and finally group C which had alveolysis on the entire root (Broucker et al., 2015). Six of these teeth were discarded from the study due to factors that obscured cementum measurements. Group A's and group B's results were relatively close to the known age, but group C's estimates were significantly different from the known age (Broucker et al., 2015). The results from group C were not surprising due to the fact that the apical third of the root is not often used in counting cementum annulations. These results state that the cervical third of the root may be used in cementochronology despite periodontal disease, and cementum erosion caused by cervical caries (areas of tooth decay) and extreme tooth brushing (Broucker et al., 2015). Known age and estimated age from counting cementum annulations were also closely related in group B no matter what degree of alveolysis. Group C had various ranges of ages, which is consistent with what has been determined by the literature (Broucker et al., 2015).

Another study done on how cementum annulations are affected by disease found similar results and questioned how the method would work for an individual who has contracted an infectious disease. This study evaluated how well different aging methods worked with a variety of pathologies including achondroplasia, osteogenesis imperfecta and residual rickets, and leprosy (Bertrand, Schug, Polet, Naji, Colard, 2016). Leprosy is a disease that challenges cementochronology, due to alterations the infection makes to the rhino-maxillary portion of the cranium. These alterations include: atrophy of the anterior nasal spine, erosion and remodeling of the nasal aperture, periodontitis, lytic lesions on the palatine bones, and reabsorption of the maxillary alveoli for the incisors (Aufderheide and Rodriguez-Martin, 2011). Leprosy patients have also had circumferential hypoplasia of the cementum; this condition and major destruction to the alveolar bone led to the hypothesis that cementum would either not be present or would not yield accurate results (Bertrand et al., 2016; Ghom, 2005). The pubic symphysis estimated the individual to be 38.2 years old with a margin of error being 10.9 years, counts of cementum annulations estimated the individual to be 42.5 years of age with a margin of error of four years, and dental attrition gave an age estimate of 35 years with a five year margin of error (Bertrand et al., 2016).

The results show that cementum was not affected by periodontitis or by infectious disease of the rhinomaxillary region. The cementum was also not affected by exposure to the individual’s environment, since the roots were exposed with the progression of leprosy. The most affected method seemed to be dental attrition because of antemortem tooth loss (Bertrand et al., 2016). While these studies do show conflicting results, they do provide the possibility that cementochronology can be used for individuals with infectious diseases and oral diseases when there are no other methods available.

The current state of cementochronology in forensics

Forensic anthropology involves forming an osteological profile from the remains of an individual for the criminal justice system or for humanitarian work (such as mass grave and past conflict identification). Victim identification is one of the most popular requests of a forensic anthropologist, and age-at-death is one of the most important parts of this. Although cementochronology would seemingly be an obvious solution to the dilemma of inaccurate techniques for skeletal age estimation, at the time of this thesis and after approximately twenty years of validation studies and research, cementochronology has not been adopted by the forensic community. Practitioners are often concerned about the application of this technique because there are still aspects of the biology that have yet to be learned, they are not trained in histological methods, and they do not have experience observing the annulations. Issues surrounding the protocol, application of the technique, and other concerns will be addressed below. Here I will first briefly describe the potential benefits of the technique and the question of the biological basis of the annulations.

An incorrect age-at-death can cause the parameters in a missing persons search to omit potential victims. Such was the case in 1991 when non-anthropologists claimed that remains belonged to an individual between the ages of 25 and 35 years (Márquez-Grant, 2015). Anthropologists later examined the remains and determined that the age range should have been between 35 and 50 years which resulted in the correct identification of a 42 year old man. In human remains, the pubic symphysis is a reliable way of determining age-at-death, particularly for those under the age of 40 years (Márquez-Grant, 2015). The pubic symphyseal surface begins to erode and deteriorate with age. Aging with this surface involves comparing the surface of the remains with casts or reference photographs (White, Black, and Folkens, 2012). Todd (1920) developed the first formal pubic symphysis aging method based on four parts of the pubic symphysis: the ventral border, the dorsal border, the superior extremity, and the inferior extremity (Todd, 1920). After much refinement of the Todd method, the Suchey-Brooks method was established and is the one used most frequently today (Márquez-Grant, 2015).

The auricular surface of the ilium also erodes and deteriorates with age, although this surface is more likely to be preserved in forensic and archaeological contexts than the pubic symphyseal surface. The auricular surface can be used for age determination with the Lovejoy et al. method (1985). This surface experiences changes past the age of 50 years unlike the pubic symphysis making it useful for older individuals. Lovejoy et al.'s method requires more expertise than the Suchey-Brooks method or the Todd method but margins of error are smaller (White et al., 2012). Lovejoy et al. describes these changes caused by age on the auricular surface: surface granulation, macroporosity, microporosity, transverse organization, striations, and billowing (Lovejoy Meindl, Pryzbeck, Mensforth, 1985).

Age estimation by way of the sternal rib ends uses the right fourth rib, which is collected separately from the rest of the skeleton for easy identification (Márquez-Grant, 2015). This method includes a standard for ancestry and sex, but only for those of European and African American ancestry (White et al., 2012). The fourth sternal rib exhibits age-related changes similar to the auricular surface and pubic symphysis. For the fourth sternal rib, pit depth, pit shape, rim configuration, and wall configuration are the features that are observed to age individuals (Işcan, Loth, Wright, 1984). Problems with this method include that it relies solely on the preservation of this rib and there is debate over whether or not this method works for other ribs (White et al., 2012).

Aging methods for teeth other than cementochronology include looking at the developmental stages of deciduous and permanent teeth. This method only applies to individuals under the age of 21, because by that point all 32 permanent teeth have erupted and are fully developed. For adults, aging is based on wear of the occlusal surface of the tooth using Gustafson's method, or Lamendin's method (Burns and Wallington, 2013). Gustafson's method involves thin sectioning the tooth to examine its microstructure. After thin sectioning has been completed, status of the following microstructures is determined: crown attrition, secondary dentin, periodontosis, root transparency, cementum, and root resorption (Gustafson, 1950, 1962). This information is then used in the Gustafson Formula (Gustafson, 1950, 1962). Lamendin's method cannot be used for anyone under the age of 25 and involves measuring different aspects of the tooth. These measurements are then applied to Lamendin's formula (Lamendin et al., 1992).

The previously mentioned methods regarding the pubic symphysis, sternal rib ends, and auricular surface are currently the most popular methods of aging human remains. If the postcranial skeleton has not been properly preserved, the cranial sutures are preferred to estimate age (Márquez-Grant, 2015). Although it should be noted that cranial suture methods do not seem to be able to reproduce a relationship between known age and suture closure, and that even expanding the age ranges in this method does not appear to be beneficial (Hershkovitz et al., 1997).

The question is raised: Would counting cementum annulations improve upon the accuracy and application of other techniques? One of the negative aspects of cementochronology is that the properties of the material are still not completely understood. The variability of age estimates through this method have not been entirely determined yet, so whether or not cementum annulations vary in one individual or between different individuals is unknown.

Challenges with aging and cementochronology

There are a myriad of factors that can affect aging human remains. Factors that the living individual experiences which can alter results from aging after death are: diet, climate, nutrition, exercise, drug abuse, medication, pregnancy, genetics, illness, child neglect, and working life (Márquez-Grant, 2015). One example is methamphetamine abuse which has been found to cause increased rates of tooth wear, antemortem tooth loss, plaque development, dental decay, and calculus (calcified plaque) development (Ravenel et al., 2012). Geographical location and culture may also be a factor that affects aging. Most aging standards are based on European or American subadults and there are none for Southeast Asian subadults (Halcrow, Tayles, and Buckley, 2006). A study that compares aging standards to aging in Thai sub-adults found that dental formation in the Thai population was delayed when compared with data obtained by Moorrees et al. (1963). Patterns in dental formation seem to suggest a genuine delay as opposed to one caused by differing methodology (Halcrow et al., 2006).

This raises the question of whether or not current information on dental formation, development, and cementum can be applied to cases involving non-Europeans when this information is based on individuals of European descent (Halcrow et al., 2006). Condition of the remains is an obstacle that many face with skeletonized individuals. The remains may only be partially complete and may have been drastically affected by taphonomic processes like animal gnawing or burning. It is difficult to estimate age in individuals belonging to an older age bracket (Márquez-Grant, 2015). Observer bias can greatly affect the estimation of age as well. Scientists attempt to do their work unbiased at all times, but nonetheless discrepancies between estimated ages among observers are common either due to differences in experience or knowledge that is provided before the observations is made (Márquez-Grant, 2015).

Determining formation stages with teeth can be improved through proper training but still it is a visually objective method (Liversidge, Smith, and Maber, 2010). Some solutions to the observer error problem are being researched. A computer program called Auto-TCA for the automated counting of tooth-cementum annulations (TCA) has been developed and is currently being tested (Czermak et al., 2006). A study of teeth from individuals in an early European medieval graveyard has been one of the studies to test this program. Using this software involves five steps: defining a region-of-interest (ROI) by the software user, segmentation and rotation of this region, image analysis, counting grey level peaks row-by-row, and age determination through a read out of the results (Czermak et al., 2006). Five photographs of every sample were counted three times by the program and the mode was added to the age at tooth eruption. These results were compared to manually counted annuli and morphological methods of aging. They found that the three ages matched fairly closely if the individual was between the ages of twenty-five and thirty years or younger (Czermak et al., 2006). For individuals between the ages of 33 and 38 years or older, the manual annuli estimated an age younger than the known age and the Auto-TCA program gave higher estimates. Auto-TCA seemed to save time and produces consistent, independent, and reproducible results. Age estimates made using Auto-TCA were higher than manually counted estimates meaning that the number of annuli counts by Auto-TCA are is not reachable through manual counting (Czermak et al., 2006). One of their 8-bit images with 256 grey scales was counted by the human eye and the program. The program was able to count all of them and observers were able to count between 50 and 60 levels. They concluded that the program would be able to circumvent errors caused observers given that the state of preservation, preparation, and choice of section and image quality were considered acceptable (Czermak et al., 2006). Many issues with cementochronology may be prevented in the future with computer programs such as this, provided that more research is conducted with the software.

Taphonomy is another consideration when aging human remains. Burning is a form of taphonomy that can result from fires caused by: automobile accidents, deliberate attempts to conceal remains, structural fires, and natural circumstances. Teeth are able to withstand high temperatures due to their highly mineralized structure, but enamel explosion may occur and other dental features may also be damaged (Gocha and Schutkowski, 2012). Despite this, teeth can be the best preserved remains from an individual who has been exposed to high heat due to their placement in the jaw (Gocha and Schutkowski, 2012). In order to determine how cementochronology is affected by exposure to high heat, a study was performed using thirty teeth from twenty-four different individuals who had only lived in the United Kingdom, with the exception of two individuals who did not have complete information (Gocha and Schutkowski, 2012). The teeth were separated into three groups and then heated to 600 °C, 800°C, or 1000°C. The crowns of the teeth were removed before heating, which may have altered how the root responded to high temperatures, but this was not related to the purpose of the researcher's study (Gocha and Schutkowski, 2012). The teeth were heated at a rate of 100°C a minute, and once the target temperature was reached the teeth were left in the oven for thirty minutes before the oven was turned off. The samples were left unpolished due to sample fragility (Gocha and Schutkowski, 2012).

The cementum annulations can survive the heating process, however in the above study; the visibility was so poor after heating, that 90 out of 180 thin sections could not be used. Of the remaining sections, 57 came from the 600°C group, 16 were from the 800°C group, and 17 were from the 1000°C group (Gocha and Schutkowski, 2012). The visibility of the sections was graded on a scale from zero to three; teeth heated to 600°C had an average score of 1.3, and teeth heated to either 800°C or 1000°C had an average score of 0.3. Of the heated samples, 90% appeared to be more opaque than unheated samples, and had a “gritty” deposition (Gocha and Schutkowski, 2012). The opaque appearance is thought to have been caused by chemical and physical changes that occur with increased temperature. Hydroxyapatite crystals increase in size as temperature increases and it is believed that this increase in crystal size affects the visibility of the cementum rings (Gocha and Schutkowski, 2012). Radial cracks through the cementum layers were observed in 80% of the thin sections; individually the cracks do not prevent counting but can cause complications when a large number of them are present in one area of the tooth (Gocha and Schutkowski, 2012). The root was measured before and after heating, leading the researchers to find that in a similar fashion to bone, the tooth material does shrink when heated and that greater shrinkage is associated with a high temperature (Gocha and Schutkowski, 2012). This may have caused the cementum rings to appear wider in these teeth than unheated teeth. Although many changes are present in heated cementum, the actual number of cementum rings is not affected by heating. The arrangement of the microstructures does not change; they are just closer together due to shrinkage and changes in crystal size (Gocha and Schutkowski, 2012). It is possible that in a forensic context, cementochronology can still be used. This study did not factor in the conditions of a tooth in a living or recently deceased individual such as alveolar bone and soft tissue surrounding the tooth. These tissues would prevent the tooth from being in direct contact with the heat source, therefore, causing the temperature in the oral cavity to rise at a slower rate. Fires that also are present in a forensic context also are not usually active for thirty minutes; fires are normally extinguished as quickly as possible so the teeth may only be exposed to high temperatures for a few minutes (Gocha and Schutkowski, 2012). This study proves that cementochronology may be less affected by taphonomic processes than other aging methods. Of course, it does not encompass all forms of taphonomy, only exposure to high temperature.

These factors affect all methods of aging, although it has been previously determined that counting cementum annulations may become a more precise and accurate estimate of age performed in tandem with traditional aging methods. There is some debate to whether or not this is true (Kasetty, Rammanohar, and Ragavendra, 2010). In 2010, a study was conducted on 200 teeth that were sectioned longitudinally to about 100 microns of thickness. They were stained and then the cementum lines were measured. They found that the incremental lines were not always distinct, that lines morph together in certain spaces, that any resorption of the cementum reduce its thickness, and that the cemento-dentinal junction is not always distinct resulting lines that are not distinct (Kasetty et al., 2010). This study also contradicts past studies, in that it states: that sectioning maxillary teeth in the mesial third of the root is more accurate; and that the apical third is more accurate for mandibular teeth. Previous studies have reported inaccuracy and increased susceptibility to morphological changes in the apical third of the root (Kasetty et al., 2010). Sexual dimorphism was determined to be statistically insignificant, and for individuals older than 60 years, increased inaccuracy due to aging was observed. They conclude by stating that cementochronology at this time cannot be used in a forensic or medicolegal context (Kasetty et al., 2010). Other concerns lie with the accessibility and appropriateness of cementochronology.

Cementochronology is relatively efficient for anthropologists with training in histology for the analysis of small sample sizes. However, cost can be an issue. It takes about $20 USD to create one slide, but this is after factoring out the cost of the machinery needed to cut the thin sections (Colard et al., 2015). Naji et al. (2016) explained that the saw required for cementochronology is a low speed diamond saw that many researchers do not have unless a histology lab is within access. When cementochronology is needed, it is commonly outsourced to zooarchaeology and wildlife laboratories since they are typically equipped with thin sectioning saws, and use the technique on non-human mammals. Cementochronology is also a destructive process (Naji et al., 2016). Some researchers may find resistance to destructive forms of analysis in certain contexts, although this process does leave the crown and cervical third of the tooth available for DNA and/or isotope testing. The lack of knowledge on how cementum is produced in humans also hinders cementochronology's acceptance in the anthropological world much less the forensic one (Naji et al., 2016).

Whether or not the sections require staining before counting the annulations depends on the researcher and the individual sections. Staining can assist in viewing faint annulations under a microscope. Some anthropologists will decalcify and stain the sections, but others do not. A study can also include both stained and unstained sections (Colard et al., 2015). The thickness of the sections is debated as well. Sections created for cementochronology over different studies can range from five to 200 microns (Colard et al., 2015). The orientation in which the teeth should be sectioned at is also up for debate. Some anthropologists use longitudinal sections while others use cross-sections. Longitudinal sections allow the viewer to observe the entire root surface, and cellular and acellular cementum. However, cross-sections allow for a series of sections to be taken with ease (Aggarwal, Saxena, and Bansal, 2008). The lack of consistency between studies conducted in anthropology makes it difficult to compare studies and difficult for scientists to replicate each other's work. Furthermore, cementochronolgy has not developed a reputation as a common aging method yet, and inter-observer error can cause inconsistencies in studies (Aggarwal et al., 2008). Further research and study will hopefully resolve these technical issues in the future.

ISO-9001 TEST STUDY

Background and goals

One of the biggest obstacles to the widespread adoption of cementochronology for medico-legal casework and bioarchaeological research is uncertainty about whether histological slides can be prepared correctly by individuals who do not have extensive training in hard tissue section preparation. The ISO-9001 protocol was developed in part to allay these fears because a standardized procedure would provide a basis for applying the technique, and theoretically at least, would minimize inter-observer error (Colard et al., 2015). To this end, a validation study was recently conducted to determine the accuracy and precision of the ISO-9001 protocol using teeth from nine individuals of known sex and age (Colard et al., 2015). That study demonstrated the technique was accurate; cementum annulation counts produced estimates close to the known ages of the individuals studied, with the exception of the youngest two individuals. One of the younger individuals was determined to be younger than the known age and the other was considered older than the known age. The precision of cementochronology, which refers to the closeness of all the counts to each other, was noted was also evident (Colard et al., 2015).

However, the protocol has not been tested in regard to the concern of application by inexperienced practitioners. Proponents of the method have claimed that the process of counting cementum annulations can be performed without much experience (Colard et al., 2015).

Proponents of the technique have further claimed that this aging method actually requires less training than traditional, anthroposcopic aging methods. In their view, histological preparation is an easy skill to acquire; experience is preferred for the observation and counting of the annulations more so than the sectioning (Colard et al., 2015). This claim is based on the observation that inter-observer error rates are high; counts made from the same thin sections and the same images have contained disparity between observers (Colard et al., 2015). Observer bias results from lack of familiarity with the microstructure, including disagreements about the location of the cementum dentin junction (Colard et al., 2015).

Materials and methods

This thesis is not testing the accuracy or precision of the protocol and it is not testing potential errors resulting from the process of counting the cementum annulations. In this project, the goal is to test whether the histological techniques are indeed easy for inexperienced researchers to learn and apply using the ISO-9001 protocol (Appendix 1). I chose this protocol because it was published in 2013 and thus is one of the most recent protocols for cementochronology (Bertrand, 2013). It was designed to be a thorough and comprehensive description of the histological technique--how to create thin sections of teeth for cementochronology. To test the hypothesis that not much experience is required to prepare the sections for counting cementum annulations, I collected 29 single-rooted teeth from individuals of varying ages (Table 1): 23 teeth were donated from the Medical Examiner's office in Raleigh, North Carolina and six teeth were from the collection of Professor Gwen Robbins Schug at Appalachian State University in Boone, North Carolina. Six of the samples were from biological females and 23 were from biological males. All samples came from modern individuals who had died within the last 37 years, and the majority of the individuals were visually identified by the medical examiner as either black or white Americans. Eleven teeth came from the right side of the body, eight teeth were from the left side of the body, and 10 teeth came from the center of the dental arcade. There were 14 mandibular teeth and 15 maxillary teeth in this sample. Classes of teeth include: six canines, eight premolars, and 13 incisors. The youngest individual was 16 and the oldest individual was 57. The remaining individuals were between the ages of 27 and 53 years.

Table 1- Side, jaw, class, and age for each sample.

Sample Letter	ID #[1]	Side	Jaw	Class	Age
1.A	B132247	R	Mand.	C	42
2.B	B79277	L	Mand.	P_3	31
3.C	B79277	R	Mand.	I_1	31
4.D	B13-3691	L	Max.	I^1	36
5.E	B08-3853	R	Max.	I^1	33
6.F	B11-3059	R	Max.	I^1	53
7.G	B01-253	L	Max.	C	27
8.H	B10-184	R	Mand.	I_2	47
9.I	B12-2526	R	Mand.	P_3	46
10.J	B78627	R	Mand.	P_3	16
11.K	B78627	R	Mand.	C	16
12.L	B15-4628	R	Max.	I^2	57
13.M	B84427	L	Mand.	C	16
14.N	B961106	R	Max.	I^1	28

[1] Identification numbers were assigned by the Medical Examiner of North Carolina or by Appalachian State University

15.O	B08-3853	L	Max.	I^1	33
16.P	B11-2788	R	Max.	I^2	50
17.Q	B78627	R	Max.	I^2	16
18.R	B06-2194	L	Mand.	I_2	38
19.S	B10-184	L	Mand.	C	47
20.T	B84427	R	Mand.	C	16
21.U	B15-4628	R	Mand.	I_1	57
22.V	B13-3691	R	Mand.	C	36
23.W	95-4	L	Max.	P^3	31
24.X	95-3	R	Max.	C	35
25.Y	94-1	L	Max.	P^4	35-40
26.Z	95-5	L	Mand.	P_3	33
27.Aa	ASU1	L	Max.	P^4	35
28.Bb	ASU10	R	Max.	I^2	27
29.Cc	B13-2247	R	Max.	P^3	42

The ISO-9001 protocol was used to section the teeth. The teeth were sectioned with a Buehler® Isomet® saw fitted with a diamond impregnated blade. The polishing process, in order, was as follows: 600 strokes on 400 grit paper, 900 strokes on 600 grit, six minutes of polishing with six micron diamond paste (DMT Dia-Paste Diamond Compound®), eight minutes with three micron diamond paste, ten minutes with one micron diamond paste, and twelve minutes with 0.5 micron diamond paste. I was trained in thin-sectioning of hard materials with this protocol by Mr. Anthony Love, of the Appalachian State University Geology Department. Mr. Love is the Research Operations and Laboratories Manager for this department. His research focuses on creating thin sections of geological material in order to observe their internal characteristics and mineral composition.

Discussion: What can go wrong?

I discovered that if the protocol was followed by a novice researcher using histological techniques, omissions in the protocol can lead to the following mistakes: uneven section thickness, saw marks on the slides and other artifacts on the sections, and mounting medium complications. Uneven sections (produced by flawed technique in polishing) can result in the adhesive bubbling which can obscure the annulations. Artifacts and saw marks also create problems when counting annulations. This can cause sections to be unusable for age estimates, because obscured annulations cannot be accurately counted. These mistakes occurred despite training with an instructor experienced in thin-section creation, suggesting that cementochronology requires more extensive training than what has been claimed.

The protocol states that an anterior tooth must be chosen, and that the tooth should be marked. The protocol clearly outlines the teeth cleansing process with acetone and distilled water. The protocol recommends that the teeth be outgassed if needed. Outgassing is a procedure where the sample, embedded in epoxy, is placed in a vacuum chamber for a certain amount of time (thirty minutes in this protocol) in order to remove any gases from the epoxy and sample. Epoxy of a lower viscosity is required for outgassing, and in this case the epoxy was too viscous for outgassing to be performed.

The saw required by the protocol is a “Buehler®IsoMet®” with a diamond coated blade that is used to saw the sections at 100 microns (Bertrand, 2013). In making thin sections of teeth for cementochronology, it is important to use the low speed version of the Buehler saw because higher speeds cannot cut thin sections at 100 microns without shredding the sample in the process so a researcher would have to cut the sections thicker and then polish them to the correct thickness, which is very time consuming.

Once sections have been made, the surfaces must be polished to remove excess saw marks and create a clear surface for counting the cementum lines. The polishing section of this protocol states: “to use a polishing machine with Al_2O_3 [aluminum oxide] on both faces to eliminate cutting marks” (Bertrand, 2013). This is not enough detail. The protocol should provide information about grit size of the aluminum oxide. Inexperienced practitioners may not realize that they need to consult published research on polishing methods. If polished by hand, typically the Frost method is used for thin sections of bone, wood, or fruit pits (Frost, 1958). This method involves the use of waterproof carborundum sandpaper of various grit sizes. The sandpaper is wrapped around a 7.62cm by 2.54cm slide, and, using the slide, the sample is polished on one side while being submerged in water. The sample is then mounted, polished side down, and the other side is polished in a similar manner (Frost, 1958). This method also mentions that the section may require staining in order to observe the microstructure of the sample (Frost, 1958). An update and modification of Frost’s method has been created which contains recommendations for fragile specimens such as specimens that have been poorly preserved, inhumed, or cremated (Maat, Bos, and Arrents, 2001).

There are various types of polishing machines that can be used. These are not specifically identified by the protocol but the use of the incorrect technique or machine can be detrimental to the section creation process. A polishing wheel uses rotation and abrasive material. The slide is placed on the wheel, and the polisher rotates the slide counter to the direction of the rotating polishing wheel. The polisher should also rotate the sample in an orbital motion. The polisher available for this study was an automated polisher requires 27mm by 46mm slides due to the specific fixture used to hold the slide. The automated polisher works in the same manner as the polishing wheel except that everything is automated by the machine. The polisher's head moves the material against the rotation of the polishing wheel on the machine and spins the sample orbitally under hydraulically controlled conditions.

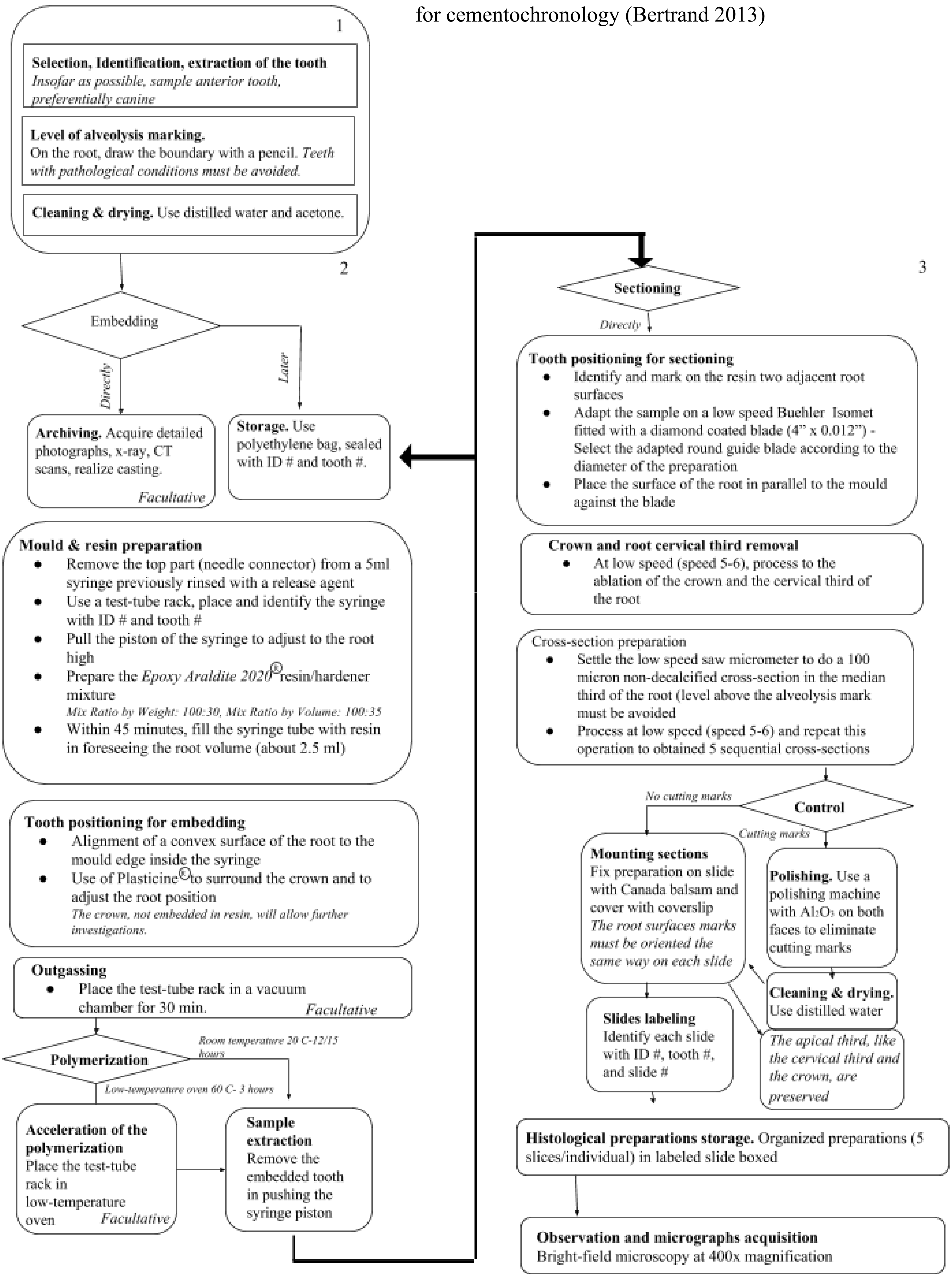
The polishing machine that the ISO-9001 protocol is referring to is the Buehler MiniMet™ 1000 Semi-Automatic Grinder-Polisher. This polishing machine is designed to combine the benefits of a polishing machine and of hand polishing. It has a random polishing action in order to eliminate any artifacts that can occur from polishing. The speed, pressure, and time are adjustable, and the machine can be adapted to polish thin sections (MiniMet™ 1000 Semi-Automatic Grinder-Polisher, 2014). Furthermore, diamond paste is often used in thin sections of bone intended for histological study. Diamond paste is a substance made of industrial grade diamonds of a small diameter, such as 0.5 microns, and it ensures that the sections are completely devoid of saw marks. The polishing section of the protocol mentions that it can be changed based on the circumstance of the experiment.

In terms of mounting medium, the protocol states that Canada balsam should be used, although there has been some debate as to what type of mounting medium is most appropriate for thin sections. Canada balsam has perfect transparency and hardness once it has been set; however it can turn yellow with age and takes longer to set than cyanoacrylate adhesive (Subramanyam, Vangala, Rudraraju, 2016). Cyanoacrylate adhesive is commonly known as "super glue" and is used often when fixing household objects such as glass or china. Cyanoacrylate has been found to result in better visualization of striae of Retzius, enamel lamellae, dentinal tubules, interglobular dentin, Sharpey's fibers, and sclerotic dentin under a polarized light microscope (Subramanyam et al., 2016). Cyanoacrylate is also optically negative, not viscous, clear, fast setting, and easily obtainable making it a superior mounting medium to Canada balsam in this case (Subramanyam et al., 2016). Cyanoacrylate's ability to set quickly can cause issues with being able to remove air bubbles as this must be achieved before the adhesive has dried. UV adhesive may yield similar results to cyanoacrylate, because it is also not viscous and is optically negative, and the complication of the adhesive setting before the air bubbles can be removed is not present since it takes about twenty minutes to harden. UV adhesive is commonly used with geological thin sections, but more research must be conducted in order to determine if it could also be beneficial for thin sections of human bone or teeth. ISO-9001 concludes with information regarding storage and labelling, but does not mention that some sections may require staining in order to observe the microstructure.

CONCLUSION

Cementochronology has potential to serve as a highly accurate and precise method for aging individuals. Although the counting process for the annulations may be subject to inter-observer error, this is the case for any other aging method and it is offset by the high degree of accuracy (two year margin of error). The technique is less frequently used than other methods of aging, which involve the pubic symphyseal surface, auricular surface, fourth sternal rib end or cranial stenosis. There are valid reasons for this. The most widely adopted protocol, the ISO-9001 has flaws and there is a certain amount of specialized equipment required to apply this technique. It has been claimed that cementochronology does not require extensive training to be utilized. Throughout this project, it has been revealed that this claim is not necessarily true. An inexperienced researcher using the ISO-9001 protocol could not produce thin sections that could be used in cementochronology. The protocol contains areas of ambiguity for those who have not had experience in histological techniques. Extensive training in this procedure is recommended and areas of the protocol should be clarified if it is intended to be used by novice researchers. However, it is my opinion that refinements to the protocol will be worthwhile because cementochronology has so much potential for age estimation in a variety of situations.

Appendix 1. ISO-9001 protocol for sectioning teeth for cementochronology (Bertrand 2013)



Literature Cited

Aggarwal P, Saxena S, Bansal P. 2008. Incremental lines in root cementum of human teeth: An approach to their role in age estimation using polarizing microscopy. Indian Journal of Dental Research 19:326.

Alghonamy W, Gaballah O, Labah D. 2015. Age estimation in adult human sound and periodontally affected teeth using tooth cementum annulations. Tanta Dental Journal 12:277–285.

Aufderheide AC, Rodríguez-Martín Conrado, Langsjoen O. 2011. The Cambridge encyclopedia of human paleopathology. Cambridge, UK: Cambridge University Press.

Bertrand B. 2013. Procedure ISO-9001: cementochronologie, référence: MOP-Dap-04. Indice a Communauté d’ Agglomération du Douaisis.

Bertrand B, Schug GR, Polet C, Naji S, Colard T. 2016. Age-at-death estimation of pathological individuals: A complementary approach using teeth cementum annulations. International Journal of Paleopathology 15:120–127.

Broucker ACAD, Colard T, Penel G, Blondiaux J, Naji S. 2016. The impact of periodontal disease on cementochronology age estimation. International Journal of Paleopathology 15:128–133.

Burns KR, Wallington J. 2013. Forensic anthropology training manual. Boston: Pearso.

Charles DK, Condon K, Cheverud JM, Buikstra JE. 1986. Cementum annulation and age determination inHomo sapiens. I. Tooth variability and observer error. American Journal of Physical Anthropology 71:311–320.

Colard T, Bertrand B, Naji S, Delannoy Y, Bécart A. 2015. Toward the adoption of cementochronology in forensic context. International Journal of Legal Medicine.

Colard T, Falgayrac G, Bertrand B, Naji S, Devos O, Balsack C, Delannoy Y, Penel G. 2016. New Insights on the Composition and the Structure of the Acellular Extrinsic Fiber Cementum by Raman Analysis. Plos One 11.

Condon K, Charles DK, Cheverud JM, Buikstra JE. 1986. Cementum annulation and age determination inHomo sapiens. II. Estimates and accuracy. American Journal of Physical Anthropology 71:321–330.

Cool S, Forwood M, Campbell P, Bennett M. 2002. Comparisons between bone and cementum compositions and the possible basis for their layered appearances. Bone 30:386–392.

Czermak A, Czermak Ad, Ernst H, and Grupe G. 2006. A New Method for the Automated Age-At-Death Evaluation by Tooth-Cementum Annulation (TCA). Anthropologischer Anzeiger: 25–40.

Frost HM. 1958. Preparation of Thin Undecalcified Bone Sections by Rapid Manual Method. Stain Technology 33:273–277.

Ghom AG. 2005. Textbook of oral medicine. New Delhi: Jaypee Brothers.

Gocha TP, Schutkowski H. 2012. Tooth Cementum Annulation for Estimation of Age-at-Death in Thermally Altered Remains. Journal of Forensic Sciences 58.

Gustafson GCB, Malmö DO. 1950. Age Determinations on Teeth. The Journal of the American Dental Association 41:45–54.

Gustafson GCB. 1962. Forensic odontology. Australian Dental Journal 7:293–303.

Halcrow SCAE, Tayles N, Buckley HR. 2007. Age estimation of children from prehistoric Southeast Asia: are the dental formation methods used appropriate? Journal of Archaeological Science 34:1158–1168.

Hershkovitz I, Latimer B, Dutour O, Jellema LM, Wish-Baratz S, Rothschild C, Rothschild BM. 1997. Why do we fail in aging the skull from the sagittal suture? American Journal of Physical Anthropology 103:393–399.

Hillson S. 1996. Dental Anthropology.

Işcan MYCF, Loth SR, Wright RK. 1984. Metamorphosis at the sternal rib end: A new method to estimate age at death in white males. American Journal of Physical Anthropology 65:147–156.

IsoMet 1000 Precision Sectioning Saw. 2013.IsoMet 1000 Precision Sectioning Saw. Illinois Tool Works Company.

Kasetty S, Rammanohar M, Ragavendra TR. 2010. Dental Cementum in Age Estimation: A Polarized Light and Stereomicroscopic Study. Journal of Forensic Sciences 55:779–783.

Klevezal G, Shishlina N. 2001. Assessment of the Season of Death of Ancient Human from Cementum Annual Layers. Journal of Archaeological Science 28:481–486.

Lamendin H, Baccino E, Humbert JF, Tavernier JC, Nossintchouk RM, Zerilli A. 1992. A Simple Technique for Age Estimation in Adult Corpses: The Two Criteria Dental Method. Journal of Forensic Sciences 37.

Lieberman DE. 1994. The Biological Basis for Seasonal Increments in Dental Cementum and Their Application to Archaeological Research. Journal of Archaeological Science 21:525–539.

Liversidge HM, Smith BH, Maber M. 2010. Bias and accuracy of age estimation using developing teeth in 946 children. American Journal of Physical Anthropology 143:545–554.

Lovejoy CO, Meindl RS, Pryzbeck TR, Mensforth RP. 1985. Chronological metamorphosis of the auricular surface of the ilium: A new method for the determination of adult skeletal age at death. American Journal of Physical Anthropology 68:15–28.

Maat GJ, Bos RPVD, Aarents MJ. 2001. Manual preparation of ground sections for the microscopy of natural bone tissue: update and modification of Frost's rapid manual method. International Journal of Osteoarchaeology 11:366–374.

Márquez-Grant N. 2015. An overview of age estimation in forensic anthropology: perspectives and practical considerations. Annals of Human Biology 42:308–322.

MiniMet™ 1000 Semi-Automatic Grinder-Polisher. 2014. MiniMet™ 1000 Semi-Automatic Grinder-Polisher. Illinois Tool Works Company.

Moorrees CF, Fanning EA, Hunt EE. 1963. Age Variation of Formation Stages for Ten Permanent Teeth. Journal of Dental Research 42:1490–1502.

Naji S, Colard T, Blondiaux J, Bertrand B, D'Incau E, Bocquet-Appel J-P. 2016. Cementochronology, to cut or not to cut? International Journal of Paleopathology 15:113–119.

Raman FAQs - What is polarised Raman spectroscopy?, HORIBA. http://www.horiba.com/scientific/products/raman-spectroscopy/raman-academy/raman-faqs/what-is-polarised-raman-spectroscopy/, accessed April 9, 2017.

Ravenel MC, Salinas CF, Marlow NM, Slate EH, Evans ZP, Miller PM. 2012. Methamphetamine abuse and oral health: A pilot study of "meth mouth". Quintessence International 43(3): 229–237.

Robbins G, Misra VD, Pal JN, and Gupta MC. 2003. Mesolithic Damdama, dental histology and age estimation. Allahabad: Dept. of Ancient History, Culture & Archaeology, University of Allahabad.

Schug GR, Brandt E, Lukacs J. 2012. Cementum annulations, age estimation, and demographic dynamics in Mid-Holocene foragers of North India. HOMO - Journal of Comparative Human Biology 63:94–109.

Subramanyam R, Vangala M, Rudraraju A. 2016. Mounting ground sections of teeth: Cyanoacrylate adhesive versus Canada balsam. Journal of Oral and Maxillofacial Pathology 20:20.

Ten Cate, AR. 1998. Oral histology: development, structure, function. St. Louis, MO: Mosby.

Todd TW. 1920. Age changes in the pubic bone. I. The male white pubis. American Journal of Physical Anthropology 3:285–334.

Wedel VL. 2007. Determination of Season at Death Using Dental Cementum Increment Analysis. Journal of Forensic Sciences.

Wedel VL, Wescott DJ. 2016. Using dental cementum increment analysis to estimate age and season of death in African Americans from an historical cemetery in Missouri. International Journal of Paleopathology 15:134–139.

White TD, Black MT, Folkens PA. 2012. Human osteology. 3rd ed. San Diego, CA: Academic Press.

Wittwer-Backofen U, Gampe J, Vaupel JW. 2004. Tooth cementum annulation for age estimation: Results from a large known-age validation study. American Journal of Physical Anthropology 123:119–129.