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Institut für Rechtsmedizin

Direktor: Prof. Dr. med. K. Püschel

„Age Estimation of Living Individuals and Identification of Unknown Deceased by Means of Forensic Odontological Investigations„

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Hussam Mansour

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1 Synopse

1.1 Introduction

1.1.1 The critical necessity of human identification

The discovery of scattered human remains or an unknown corpse exposed to various post-mortem conditions such as severe decomposition, incineration or skeletization is a common scenario in routine forensic practice. Moreover, in recent decades, there have been mass disasters across the world much more frequently, which have caused a great number of fatalities with many unidentified victims [1].These disasters can be categorized into: (i) natural events such as tsunamis, earthquakes, hurricanes or flooding, (ii) accidental events (airplane or train crashes), (iii) various terrorist attacks (explosions or bombings), especially in populated areas, and (iv) other fatal incidents as a consequence of enlarged armed conflict [1, 2]. An increasing number of unidentified corpses has therefore become apparent in forensic practice [3].

Identification is one of the fundamental human rights adopted by the United Nations. According to Article 6 of the Universal Declaration of Human Rights, "*everyone has the right to recognition everywhere as a person before the law*" [4]. Without establishing positive human identification, several critical issues concerning legal, criminal, financial, social, religious and humanitarian necessities cannot be established [5]. In the context of criminal investigation, identifying an unknown victim is the first essential step in commencing an investigation. In many societies, the inability to issue a death certificate can impede the procedures of re-marriage or receiving pensions and other benefits [5]. Moreover, the relatives of a missing person will continue to suffer from mournful condition until recovering the body and confirming the identity [5, 6]. Authorities and societies thus have a moral and ethical duty to make the necessary efforts towards identifying unknown decedents in order that they can be buried with their own names and mourned by their relatives and loved ones after the certainty of the death [7].

1.1.2 Primary and secondary means of human identification

Internationally, three methods - DNA profiling, dental identification and fingerprint analysis (dactyloscopy) - are reliably used as primary means of human identification and recognized by the International Criminal Police Organization (Interpol) [8, 9]. This thesis focuses on dental and DNA identification methods, since they are very important for various fields of forensic sciences and widely used in forensic practice.

1.1.2.1 DNA-based identification:

Since the early 1990s, thanks to unprecedented evolution in DNA technologies, DNA-based approaches have been playing a crucial role in several forensic aspects including human identification, crime scene investigations, legal issues such as paternity testing, and other important forensic applications [10-14].

As a rule, forensic genetic identification is based on two fundamental requirements. These are (i) post-mortem DNA samples extracted from high quality tissues, if possible, from the corpse or the available human remains, and (ii) reference samples for matching [15]. These reference samples can be from one of the following categories: (i) samples obtained from the relatives of unknown corpses [2], (ii) ante-mortem self-samples (for instance: preserved biopsy, stored blood cards, stored serum samples, bone marrow, cytological smears or primary teeth preserved as mementoes) [2, 16, 17], or (iii) direct reference samples obtained from personal items and belongings of unknown corpses such as personal tooth brushes, combs, razors or lipstick [2, 16].

Genomic DNA profiles can be generated from any post-mortem, non-degraded, nuclear biological sample [14]. According to recommendations from the DNA Commission of the International Society for Forensic Genetics (ISFG) [16], in fresh (not decomposed) cases, it is recommended to sample blood, buccal swabs or deep red muscular tissues [16]. However, soft tissues suffer from accelerated post-mortem decay [18]. When all soft tissue becomes decomposed or fails to yield sufficient DNA, bones and teeth are the main feasible source of DNA for identification purposes [19]. Thanks to their particular structure, being highly mineralized, dental tissues can survive post-mortem decay and endure the adverse effects of extreme post-mortem conditions [20, 21]. Some reported publications [10, 21, 22] referred to teeth as a valuable source, which succeed in generating DNA profiles and overcoming challenging post-mortem conditions, such as severely incinerated cadaver [21], adipocere cadaver immersed in water for approximately 2 years [10], human remains exhumed 2.8 years after death [10] and skeletonized, exhumed human remains from the Second World War [22].

1.1.2.2 Dental DNA-based identification:

In 1990, Hänni et al. [23] reported the first study concerning DNA extraction from dental tissue in ancient human teeth [24]. Afterwards, many publications addressed teeth as a source of DNA. Despite the utmost importance of dental DNA-based identification, some difficulties and potential drawbacks should be considered when isolating DNA from teeth, such as the need for time-consuming preparation, long preliminary procedures, a comparatively more laborious process for isolating DNA from dental tissue [18, 25, 26], unevenly and sparsely distributed cellularity in dental tissues when

compared to other body tissues, and the presence of a considerable amount of non-DNA components in teeth [25, 27, 28, 94].

To enhance dental DNA-based identification and optimize DNA sampling from dental tissues, many studies, for instance [28-34], have discussed the potential factors affecting dental DNA yield in specific experimental conditions. However, the aforementioned studies based on extracted teeth directly worked upon in the experimental conditions without considering the role of anatomical protection provided by the surrounding periodontium components, the surrounding facial and oral muscles and anticipated changes in the surrounding environmental conditions which occur through the passing of time [25]. Mansour et al. [25] have investigated various ante and post-mortem factors which affect dental DNA in various real post-mortem conditions. Similarly to Higgins et al. [28], our study [25], described in detail in Chapter 4, divides potential factors which affect dental DNA into two groups (ante and post-mortem factors), as seen in Fig. 1.

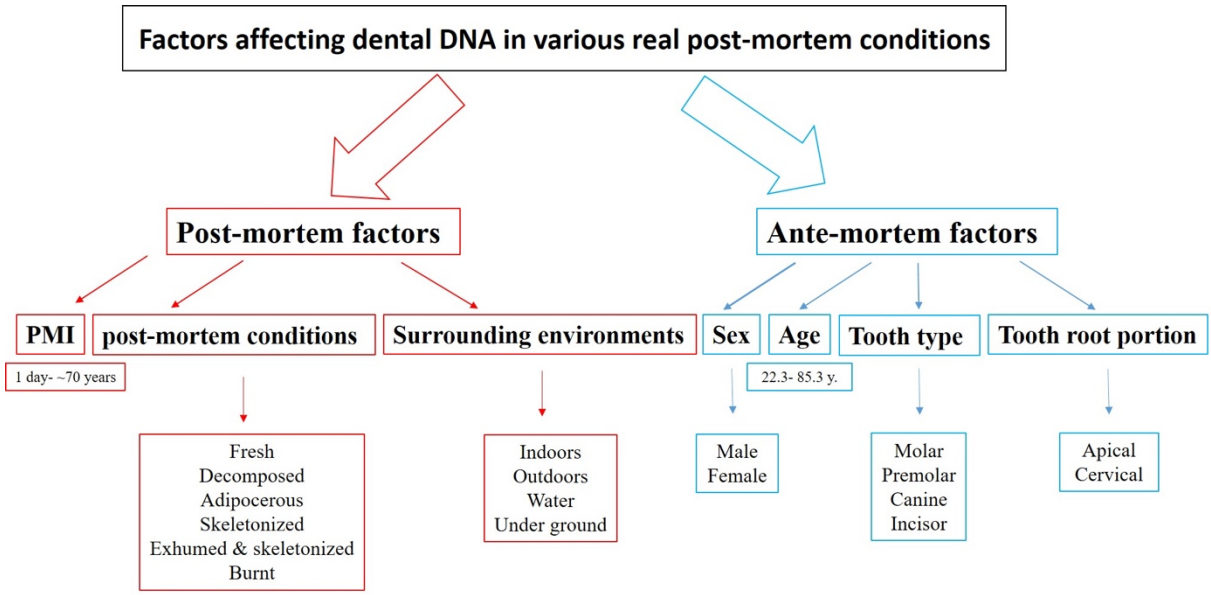

Figure 1: The categorization of factors affecting dental DNA, assigned by Mansour et al. [25]

Comparative dental identification:

This is based essentially on comparing post-mortem dental findings with ante-mortem dental data derived from the utilizing of various available materials, such as [5, 35]: (i) different dental radiographs (intra-oral, OPG (Orthopantomogram) or CBCT (Cone beam computed tomography)), (ii) dental charts and available written descriptions of dental status, (iii) dental casts, (iv) palatal rugae, (v) photographs (including clinical pictures or snapshots taken of smiling individuals), and (vi) important clues derived from dental implants.

This comparison depends on differentiating the similarities or discrepancies between both ante and post-mortem data. It focuses on the individuality of many combinations of dental characteristics in the same person with unique hand crafted restorations and the peculiarity of surrounding anatomical structures [5, 36, 111]. As a result of several advantages, dental identification is used in forensic institutions across the world. However, some limitations should be taken into account (Table 1).

Table 1 shows an overview of the advantages and limitations of comparative dental identification [8, 20, 32, 36-44].

Advantages	Limitations
Reliability and high accuracy [8, 36, 43]	Ineligible or low quality antemortem data [38, 39, 40]
The high value of dental evidence, thanks to the durability and high resistance of dental tissue, as well as dental treatments against various adverse post-mortem effects [20, 44]	Difficulty of identifying children or adolescents as a consequence of developmental changes and a lack of potential treatments [41]
Simplicity of implementation [37] No need for a variety of complicated devices	Difficulty in identifying edentulous individuals [42]
Time and cost-effectiveness [43]	Non-uniform dental charting [38]
	Mass fatality incidents, including fragmented or commingled corpses [32]
	The need for combining dental and DNA-based identification to identify scattered skeletons [36]

Dental implants in human identification:

Implant dentistry can make a significant contribution to contemporary forensic odontology and human identification [45]. Thanks to advanced surgical techniques and a significant improvement in materials industry, a remarkable increase in the prevalence of implanted medical and odontological devices has become apparent [46]. Today, dental implants are considered highly specific findings and presenting a new avenue for facilitating the identification process, being frequently encountered in both antemortem and postmortem dental data [45, 47]. In addition, dental implants can resist various adverse post-mortem conditions, such as thermal insults, since they are characterized by peculiar physical and chemical properties [48]. To this end, many recent publications have addressed the importance of dental implants in identifying unknown corpses. Comprehensive investigations concerning the physical and radiological features of dental implants have been reported in [47, 49, 50],

while a computer software program expediting the identification of dental implants has been developed by Michelinakis et al. [51]. The recognition of the characteristic morphological pattern of osseointegration with the respective dental implant was addressed by Angelis et al. [52], and Berketa et al. [48] investigated the effect of heat exposure on the ability to decipher the batch numbers engraved in the implants manufactured by particular companies. Mansour et al. [45] identified not-engraved manufacturers' item numbers of both dental implant components (implant fixtures and prosthetic abutments) using microscopic analysis together with clinical and radiological examinations after post-mortem extraction of dental implant, the respective details can be seen in Chapter 7.

Combined dental and DNA-based identification:

It is well known that each identification casework has its own uniqueness and poses a particular approach to solving it. In some scenarios, dental evidence alone is not sufficient for reaching the confidence level of personal identification. Additionally, dependence on genetic identification alone (without considering dental evidence) could complicate the identification process and consume more time, money and effort [36]. Therefore, a combination of DNA and dental identification was used to re-associate and identify scattered human skeletal remains confidently [36]. This is described in details in Chapter 6.

1.1.3. Secondary methods of human identification:

When the aforementioned primary identification methods fail to confirm an identity, other avenues representing secondary identifiers can provide meaningful information to reconstruct the identity, narrow down the search to a specific population pool and, accordingly, facilitate the identification process and maximize identification ability [8]. Following the Interpol Disaster Victim Identification (DVI) Guide, secondary means can be exemplified by (i) an individual description of the biological profile, including sex, age, height and ethnicity, (ii) any available personal effects, including ID cards, clothing, hand watches or jewellery, (iii) medically distinctive findings, such as prosthetic devices, (iv) pathologically distinctive findings, such as skeletal deformities, tumors or surgical scars, and (iv) other characteristic findings, such as tattoos or moles [8].

1.3. Forensic age estimation:

Thank to its key role in establishing our biological profile, age is considered a fundamental part of our identity. Forensic age estimation for both living individuals and unidentified cadavers is one of the main responsibilities of forensic scientists and practitioners. In recent years, as a consequence of several sociopolitical changes, the number of young immigrants and unaccompanied minors arriving in Europe without official documents validating their ages has increased exponentially

[3, 54]. Most recently, statistics from UNHCR [55] have revealed high levels of displacement conditions in the world. UNHCR have estimated that there are, worldwide, approximately 26 million refugees out of about 70 million people, who were forced out of their home [55]. More than 50% of these refugees are minors (younger than 18 years) [55].

An increase in armed conflicts has played a crucial role in aggravating this phenomenon. Refugees from three countries, Syria (in the first place), Afghanistan and South Sudan, comprise 57% of UNHCR refugees [55]. Moreover, according to Unicef, only 50% of births are registered in developing countries [56]. Forensic age assessment is, therefore, very important, since serious consequences of false age estimation can be anticipated, especially, for unaccompanied minors treated as adults when applying for asylum.

Ages from 14 to 22 represent critical age thresholds for judicial and non-judicial proceedings in many countries [57]. In Germany, 14, 16, 18, and 21 are the most important age thresholds [58]. Thus, attaining the age thresholds relevant for criminal responsibility demonstrates the importance of forensic age assessment [59]. For other civilian, social, legal, and administrative purposes concerning the individual's life, forensic age estimation of living individuals is required in the following example cases [60, 61]:

- Issues concerning attendance at school, child labor, employment, military service, marriage and sexual relationships/ exploitation.
- Rationality of consent to/refusal of healthcare.

1.3.2. German Study Group on Forensic Age Diagnostics (AGFAD):

On March 10, 2000, the interdisciplinary "German Study Group on Forensic Age Diagnostics" (Arbeitsgemeinschaft für Forensische Altersdiagnostik, AGFAD) was established in Berlin [62]. This event was a landmark in the historical development of forensic age estimation practice aimed to standardize the process, enhance professional expert reports, and conduct required quality assurances [62]. AGFAD suggested important recommendations for enhancing the criteria of age estimation. Within the frame of judicial proceedings, the first AGFAD recommendations for age assessment were published in 2001, followed by an updated set of recommendations in 2008 [62, 63].

The main AGFAD recommendations [62] can be summarized as follows:

- The justification of forensic age estimation examinations should in advance be provided through a court order.

- The clarification of the procedures and purposes of the examination for individuals should be carried out. When necessary, an interpreter is required.
- When using X-ray examinations, the respective legitimization is required and the national regulations must be considered.
- These methods include:

a. Clinical-physical examinations with the medical history acquired, in order to diagnose any disorder which could affect physical development.

b. An anthropometric evaluation of different parameters (height, weight and body constitution).

c. Evaluating dental development using Orthopantomograms (OPGs).

d. Evaluating skeletal development using an X-ray of the left hand. If the development of the hand is completed, the skeletal development of the medial clavicular epiphyses should be evaluated using either thin-slice computed tomography-scan of medial clavicular epiphysis (CT-clavicle) or clavicular projection radiography (clavicle-PR).

- The expert report should provide the most probable age with respect to specific reference data for the relevant population, socioeconomic factors, the presence of developmental disorders, and other related concerns.
- The expert report should assess the probability that the given age is the actual age.
- The expert report should mention the cited publications.

1.4. General Objectives

The published research included in this thesis aims to address various objectives related to human identification and forensic age estimation in different aspects.

1.4.1. The main objectives of addressing "Factors affecting dental DNA in various real post-mortem conditions" (Chapter 4):

1. Studying the correlation between dental DNA quantity and variables, including sex and age, in real forensic casework.
2. Evaluating dental DNA yield from different tooth types and tooth root portions in real-life conditions.
3. Evaluating the correlation between dental DNA quantity and post-mortem variables, including PMI, post-mortem conditions and various surrounding environments.

4. Comparing the results of research based on real post-mortem conditions with previous relevant reported literature based on experimental conditions.

1.4.2. The main objectives of addressing "Cementum as a source of DNA in solving challenging forensic cases" (Chapter 5):

1. Underlining the utmost importance of using cementum as a profitable source of DNA in challenging post-mortem conditions.
2. Approaching the effect of intra and inter-individual variation in terms of anatomical and histological dental structure on dental DNA using microcomputed tomographic imaging (μCT) and histological dental examination.

1.4.3. The main objectives of addressing "Identification of scattered skeletal remains: Combined dental and DNA-based identification" (Chapter 6):

1. Highlighting the effectiveness of combining two primary identifiers, dental and DNA identification, in identifying scattered skeletal remains.
2. Emphasizing of the dual role of teeth in matching different scattered human remains and comparative dental identification.

1.4.4. The main objectives of addressing "New aspects of dental implants and DNA technology in human identification" (Chapter 7):

1. Suggesting a new strategy, utilizing the presence of dental implants as important findings in ante and post-mortem data.
2. Identifying un-engraved batch numbers of dental implants to facilitate the identification of the dentist who ordered the implant article, and accordingly, facilitating the identification process.
3. Providing the biological profile with supporting information, using novel DNA approaches including DNA methylation, mtDNA analysis and YSTR analysis.

1.4.5. The main objectives of addressing "The role of Forensic Medicine and Forensic Dentistry in estimating the chronological age of living individuals in Hamburg, Germany" (Chapter 8):

1. Presenting an overview of the methods used in the forensic age assessment of living individuals in Hamburg over 25 years.
2. Approaching the important respective changes in different concerned authorities, legal regulations and sociopolitical conditions.

3. Approaching the scientific background of the methods used in Hamburg and other published studies concerning forensic age estimation practice.

1.2 Material and Methods

1.2.1 DNA extracted from dental tissues

Various methods and techniques for isolating dental DNA have been reported in literature, some examples of which are illustrated in Table 2.

Table 2 An overview of the different methods and techniques used to extract DNA from dental tissues, as well as the respective advantages and disadvantages of each technique

The method	Advantages	Disadvantages	Reference
Occlusal trepanation (occlusal access) for dental pulp extraction	✓ relatively minimal-invasive and more conservative ✓ the ability to utilize the teeth for further radiological, morphological or biochemical investigations ✓ better results in terms of DNA quantity and quality in comparison to the crushing method ✓ less risk of contamination than the crushing method	•the study did not discuss samples retrieved from corpses where pulp is subjected to post-mortem decay in long post-mortem intervals. •it can not be used for teeth undertaken to endodontic treatment or severely pathologic carious teeth	Tilotta et al. [20]
Crushing the entire tooth	✓ a widely used method ✓ maximizes yielded dental powder ✓ increases the surface area which reacts with biochemical DNA extraction kit materials [5]. ✓ simplifies exposing the trapped cells inside the mineral matrix to conduct the genetic material [5].	•includes non-DNA components, such as enamel •a destructive method	Smith et al. [64]
Cryogenic grinding of the entire tooth using a freezer mill (in Nitrogen liquid)	✓ relatively simple method and less time consuming. ✓ less risk of contamination ✓ liquid nitrogen minimizes the damage resulted from the heat of drilling [5]	•the method is destructive, meaning that further dental investigation of the respective tooth is no longer possible •special devices and materials are required	Sweet et al. [65]

Scraping cementum tissues from tooth through using a scalpel blade	✓ sampling is quick ✓ no need for special equipment ✓ reduces the laborious procedures required in other methods ✓ can be used for teeth undertaken by endodontic treatment ✓ optimizes dental samples through increasing cellularity in the specimen by targeting cementum	•less DNA samples can be obtained (only cementum samples)	Higgins et al. [66]
Non-powdering technique, based on the demineralizing of root apices	✓ no need for special expensive equipment ✓ as "a low-tech method", it can be used in laboratories in developing countries	•time consuming (the demineralization process using EDTA solution takes approximately one week, with the need to change the solutions every day)	Corrêa et al. [24]
Retrograde access of pulp and dentin tissues via the apex of the tooth through the root canals and pulp cavity using endodontic dental files	✓ is considered minimally invasive, since it avoids damage to the crown morphology used for other investigations. ✓ avoids damage from the heat of drilling ✓ yields better amount of amplifiable DNA than that yielded from grinding method ✓ yields better quality DNA than the grinding method ✓ less risk of contamination	•laborious method •requires more time than the grinding method •less dental powder can be expected to be obtained •it is not feasible in mass fatality incidents	Hughes-Stamm et al. [19]
Horizontal sectioning the teeth and then cryogenic grinding each section into dental powder	✓ can exclude poor DNA areas (tooth crown) ✓ both cellular components, such as odontoblasts or cementocytse, can be utilized	•destructive method •relatively time consuming (sectioning and grinding)	Gaytmenn et al. [32]

In Chapter 4 of this thesis, similarly to the technique suggested by Gaytmenn et al. [32] and with few modifications, each tooth was divided into 3 portions (the crown, the cervical portion of the root and the apical portion of the root) through two horizontal cuts, which are rather perpendicular to the tooth axis. Since DNA yield in tooth roots is better than in the crown [27, 32, 34], the crown was excluded from DNA extraction while both root portions were included (Fig. 2).

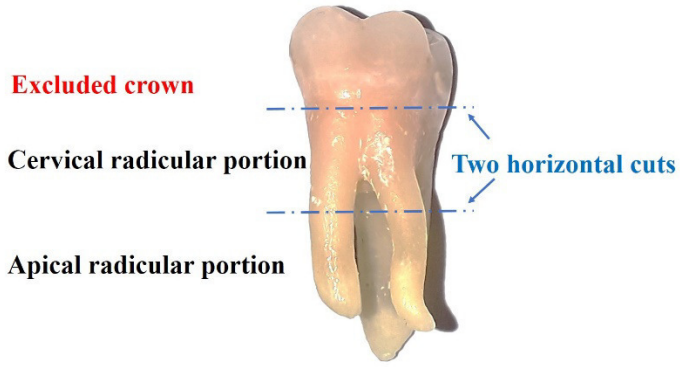

Figure 2: Similar to Gaytmenn et al.'s guidelines [32], tooth sectioning prior to grinding for DNA extraction.

To evaluate DNA amounts in real-life forensic casework, dental samples were collected from unknown corpses which were later identified using dental or DNA identification and prepared for DNA extraction over the last 3 years. In total, 95 teeth were extracted from 39 corpses found in various post-mortem conditions and a wide PMI, from 1 day to approximately 70 years. All the descriptive statistics are illustrated in Chapter 4. After the preparation procedures and tooth grinding, DNA extraction from 179 dental samples was carried out using the Crime Prep Adem-Kit (ADEMTECH SA, France) [67]. Afterwards, DNA quantification was determined by real-time polymerase chain reaction (PowerQuant™ System/Promega) using the ABI 7500 RT PCR System. A statistical analysis was then completed, in order to interpret the results. Details of the preparation procedures, DNA extraction and the statistical analysis used in this investigation are described in Chapter 4.

1.2.2 Dental implant investigation in human identification:

The investigation, described in Chapter (7) [45], depends on utilizing the reported literature about the morphological and radiological features of dental implants. Besides these methods, new approaches such as microscopic examination, metric analyses and data analyzing were carried out. Fig. 3 illustrates all the procedures performed in these investigation [45, 47, 49, 50, 68, 69].

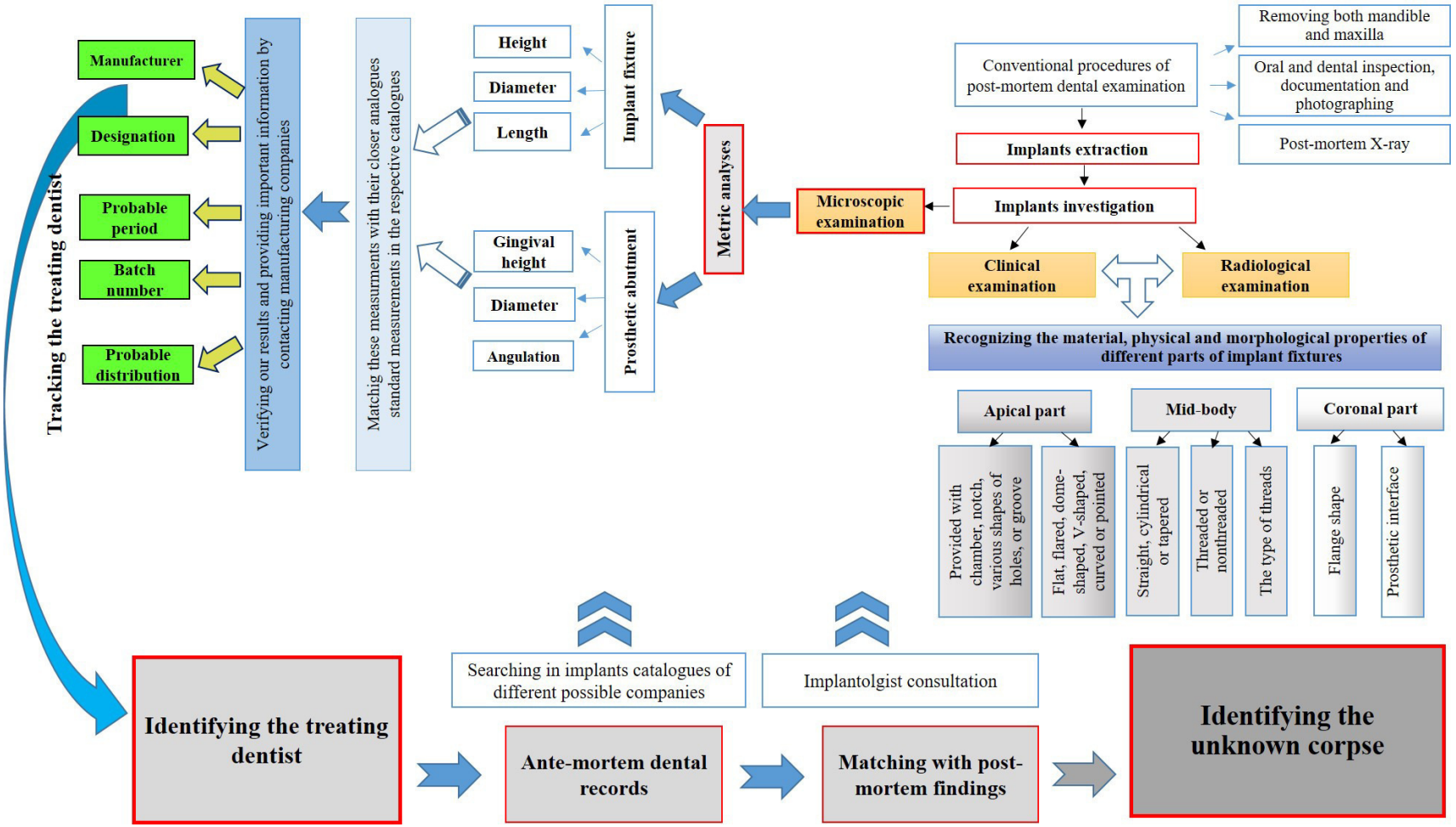

Figure 3: Dental implant investigation for human identification as described in [45, 47, 49, 50, 68, 69].

1.2.3 Forensic age estimation methodology for living individuals

Several approaches and methods are used in age estimation for living adolescents and young adults. According to the results of a European Asylum Support Office (EASO) survey [60], a combination of non-medical approaches, medical methods or other kind of approaches are used in different countries.

Although non-medical methods (such as considering the available document, conducting an interview and the visual evaluation of behavior and body appearance) are not physically invasive, they are subjective and result in a significant error range [60]. The medical methods can be shown through dental examination based on clinical inspection without carrying out radiological examinations, clinical evaluations of the physical growth by pediatricians, estimation of the sexual maturity, mental evaluation by conducting psychological interview, ionizing imaging methods such as hand X-ray, orthopantomogram (OPG), clavicular projection radiography (Clavicle-PR) and thin-slice computed tomography-scan of medial clavicular epiphysis (Clavicle-CT), or recently developed non-ionizing imaging methods, which are based either on ultrasoundsonography or on magnetic resonance imaging [60, 70-75].

At the Institute of Legal Medicine in Hamburg-Eppendorf, a comprehensive investigation of the course of development of forensic age assessment of living individuals has been discussed by Mansour et al. [76]. This investigation approached the age estimation over a period of 25 years, from 1990 to 2015. It was divided into three main phases, 1990- 2000 [77], 2001- 2008 [78] and 2009 -2015 [76, 79], and gave an overview of the methods and various authorities concerned. In addition to physical examination, ionizing imaging methods including hand X-ray, OPG, Clavicle-PR and Clavicle-CT were variedly used with respect to the period of time and the category of the examination either in the context of judicial or non- judicial proceedings [76] (as illustrated in Chapter 8). In general, X-rays of left hand, collar bone and teeth are scientific methods with a clear error range [60].

1.2.3.1 Hand X-ray

The Greulich and Pyle Atlas method [80] is one the most commonly used methods for skeletal age assessment purposes, and has been for a long time [81]. Between 1931 and 1942, radiographs of 1000 American children with good socioeconomic status, whose age ranged from 0 to 18, established the aforementioned atlas [82, 83]. The method essentially depends on a comparison with the standard reference radiographs with respect to age and sex [59]. Similar atlas methods have been reported in literature, such as [84-86]. The main criteria in estimating the skeletal age using a left wrist/hand X-

ray is to determine the size, shape, mineralization and ossification degree of the epiphyseal plates [59, 60, 84].

1.2.3.2 Orthopantomogram (OPG)

OPG is the fundamental approach used for forensic age diagnostics for living individuals in Hamburg [76] and is a basic method adopted by AGFAD [62]. Furthermore, the effective radiation dose of OPG is 26 microSv (μSv), which is a relatively low dose of radiation when compared to Clavicle-PR (220 μSv) or Clavicle-CT (400 μSv) [53, 59, 87, 88].

For age assessment purposes, several criteria can be evaluated in digital OPG to provide a comprehensive view of dental status [76]. These criteria are the mineralization stage of third molars [89-91], the chronological course of third molars eruption [92, 93, 95, 103] and other additional age-related features such as periodontal recession [96], attrition [97], DMFT-Index (Decayed Missing Filled Tooth-Index) or special status of third molars, like inclination, migration, elongation or coronal morphological changes of third molars [76].

Third molar mineralization:

Estimating dental development through evaluating the degree of mineralization is the most critical criterion and the basic principle for dental age assessment [76, 98]. Many different staging systems for teeth mineralization have been reported in literature, for example in the 14 developmental stages described by Moorees et al. [99], the 10 development stages described by Nolla [100] and the 8 developmental stages described by Demirjian et al. [101] (Table 3). Demirjian et al.'s method, the most common classification system of dental development, is considered the most accurate [98]. To estimate dental age, Olze et al. [98] recommended adopting Demirjian et al.'s classification system, as it provides sufficient intervals between stages and avoids estimating the respective length speculatively.

Table 3 shows the dental development stages described by Demirjian et al. [101], with illustrations from [102].

Stage	**Stage A**	**Stage B**	**Stage C**	**Stage D**
Description	Initial calcification at the tip of the crypt, without fusion in the calcified points.	Fusion of the calcified cusps, forming a definable contour on the occlusal surface.	Completely formed enamel, beginning of dentin deposition and curve shape of the occlusal outline of the pulp camber.	Completely formed crown up to the cementoenamel junction (CEJ) and initial formation of the root.
Stage	**Stage E**	**Stage F**	**Stage G**	**Stage H**
Description	Definable formed bifurcation of the molars and the crown height is still greater than the root length.	The formed root has a more definite outline. It is at least as long as the crown. The apical end of the root has a funnel shape.	The walls of the root canal become parallel. The apex is still partially open (especially in distal root of molars).	Completely formed root, with completely closed apices. Uniform width of the periodontal membrane around the entire root.

Eruption status of the third molars:

The progression of the wisdom tooth's journey, from its initial formation in the relevant alveolar crypts toward the occlusal plane, can provide additional helpful information about dental development [103]. Unlike third molar mineralization, the eruption status of the third molars can be clinically and/or radiologically evaluated [103, 104]. Based on alveolar, gingival, and complete emergence of wisdom tooth, the third molar's eruption status can be evaluated using Olze et al.'s staging system [93, 95] (Table 4).

Table 4 illustrates the different stages of third molar's eruption status, as defined by Olze et al. [93, 95].

Stage A	The occlusal surface of the third molar is still covered with alveolar bone.
Stage B	Alveolar emergence: the alveolar bone over the occlusal surface of the third molar is completely resorped without penetrating the gingiva.
Stage C	Gingival emergence: at least one cusp of the third molar has penetrated the gingiva.
Stage D	The third molar reaches the occlusal plane without elongation.
Elongation	The occlusal surface of the third molar is over the occlusal plane.

Periodontal recession:

As a physiological age-related change [105], periodontal recession can be used as a supportive indicator in forensic age diagnostics [96]. The general horizontal periodontal recession can be clearly estimated using digital OPGs [76]. Olze et al. [96] defined a staging system to estimate periodontal recession, as shown in Table 5.

Table 5 illustrates the stages of periodontal recession according to Olze et al. [96].

Stage 0	No periodontal recession
Stage 1	Initial periodontal recession represented in less than half of the coronal third of the tooth root
Stage 2	Advanced periodontal recession represented in up to the coronal third of the tooth root
Stage 3	Considerable periodontal recession represented in exceeding the coronal third of the tooth root

Richel [97] described a staging system of the horizontal periodontal recession measured in mm. below the cement-enamel junction (CEJ), as shown in Table 6

Table 6 presents the different stages of periodontal recession described by Richel [97].

Healthy periodontium represents alveolar bone located 1-2 mm below CEJ.	
Stage 1	Periodontal recession is up to 2 mm below CEJ.
Stage 2	Periodontal recession is up to 4 mm below CEJ.
Stage 3	Periodontal recession is up to 6 mm below CEJ.

Attrition:

Attrition, the physiological loss of dental hard tissue as a consequence of tooth-to-tooth contact, begins on the tips of the occlusal surfaces of opposing teeth, usually as soon as reached by the occlusal plane [106, 107]. In some cases, this physiological age-related change can be used to support the decision of forensic age diagnostics. Richel [97] classified the attrition into 4 stages, as illustrated in Table 7

Table 7 shows the classification of dental attrition, as defined by Richel [97].

Stage 0	No noticeable attrition.
Stage 1	Attrition in enamel with flattening of the cusps of the teeth, except third molars.
Stage 2	Attrition in the enamel of the third molars.
Stage 3	Attrition in enamel and dentin.

1.2.3.3 Thin-slice computed tomography scan of medial clavicular epiphysis (CT-clavicle)

In the context of judicial proceeding, the ability to estimate an age threshold of 21 using ionizing imaging methods such as left hand X-ray or OPG is unavailing, since the development of the hand and teeth is fully completed [108]. Here, the radiological investigation evaluating the ossification pattern of the medial clavicular epiphyseal cartilage is the only appropriate method [108]. Either projection radiography (PR) or computed tomography (CT) of medial clavicular epiphysis were thus recommended by the AGFAD [62, 109].

In 2004, Schmeling et al. [108] established a classification system of five stages to evaluate the degree of ossification of the medial clavicular epiphyseal cartilage (Table 8). However, the interval of the estimated age for both stages 2 and 3 described by Schmeling et al. [108] was overly broad for diagnosing the age threshold of 18 [110]. Therefore, Kellinghaus et al. [110] divided each stage

(stages 2 and 3) into a sub-classification (Table 8). This can in turn enhance the age majority decision for the individuals whose CT-clavicle reveals stage 3C [110], with a minimum age of 19 [110, 126].

Table 8 shows the two most common classification systems of the developmental stages of the medial clavicular epiphyseal cartilage: five staging system, defined by Schmeling et al. [108], and the six staging system described by Kellinghaus et al. [110] (sub-classifying stage 2 and 3). Additionally, the table features the relevant illustrations and preconditions described by Wittschieber et al. [109, 112].

Stage I	Non-ossified epiphyseal ossification center, irregular metaphyseal surface with edgy boundary lines	
Stage II	The epiphyseal ossification center has ossified, but it is still isolated without any osseous connection (fusion) to the adjacent metaphysis. This stage is sub-classified by Kellinghaus et al. [110] into 3 sub-classification stages:	
	Stage 2a	The lengthwise epiphyseal measurement does not exceed 1/3 of the metaphysis width.
	Stage 2b	The lengthwise epiphyseal measurement is between 1/3 and 2/3 of the metaphysis width.
	Stage 2c	The lengthwise epiphyseal measurement exceeds 2/3 of the metaphysis width.
Stage III	Partial ossification of the epiphyseal cartilage (partial fusion between epiphysis and metaphysis). This stage is further sub-classified by Kellinghaus et al. [110] into 3 sub-classification stages:	
	Stage 3a	The epiphyseal–metaphyseal fusion does not exceed 1/3 of the metaphysis width.
	Stage 3b	The epiphyseal–metaphyseal fusion is between 1/3 and 2/3 of the metaphysis width.
	Stage 3c	The epiphyseal–metaphyseal fusion exceeds 2/3 of the metaphysis width.
Stage IV	Complete epiphyseal–metaphyseal fusion with presence of discernible epiphyseal scar. The medial ending of the clavicle is provided by rounded boundary lines.	
Stage V	Completely fused epiphyseal cartilage with convex surface on the medial ending of the clavicle is provided by rounded boundary lines. The epiphyseal scar is no longer discernible.	

1.3 General results and discussion

1.3.1 Factors affecting dental DNA

1.3.1.1 Ante-mortem factor effects on dental DNA

The statistical analyses in Chapter 4 [25] show that all the ante-mortem factors (sex, age, tooth type and tooth root portions) investigated in our study did not show significant relationships to DNA concentration. The comparison of our results, based on investigating dental DNA in real conditions [25] with other reported studies [28, 30, 34] and based on experimental conditions, is shown in Table 9.

Table 9 A comparison of ante-mortem factors (sex, age, tooth type, and tooth root portions) investigated in our study [25] (Chapter 4, in real conditions) and other studies (in experimental conditions) [28, 30, 34].

Factor	Higgins et al. [28]	Rubio et al. [30]	Corte-Real et al. [34]	Mansour et al. [25]
	Experimental conditions			Real conditions
Age	positive significant effect with ageing	no significant effect	not investigated	no significant effect
Sex	no significant sex-related differences	no significant sex-related differences	no significant sex-related differences	no significant sex-related differences
Tooth type	not investigated	DNA yield in posterior teeth higher than that in anterior ones	no significant effect	no significant effect
Tooth portion	cementum yielded better genomic DNA than dentine	not investigated	the apical part of the root yielded better than remaining root body	no significant effect

The explanation of insignificant effects of the aforementioned ante-mortem factors is the remarkable effect of intra- and inter-individual variations concerning the dental structure [10, 28, 32] rather than any external variables. This variation can be seen in the different thicknesses of cementum in different tooth root regions in different tooth types, which can even be observed in the same person through the micro-computed tomography (μCT) imaging presented in Chapter 5 [10]. The histological findings in various levels of post-mortem extracted teeth revealed diverse cellularity, especially in

cementocytes density, (Chapter 5 [10]). Therefore, the anatomical and histological individual variations should be considered when approaching the dental DNA yield [10].

1.3.2 The effect of post-mortem factors on dental DNA

Unlike the ante-mortem factors, post-mortem factors (PMI, post-mortem conditions and various surrounding environments) investigated in our study (Chapter 4) revealed noticeably and statistically significant impacts on DNA yield [25]. The most important factor affecting DNA degradation was PMI. This effect is compatible with the results of previously reported studies in Table 10.

Table 10 A comparison of the post-mortem factors (PMI and the surrounding environment) investigated in our study (Chapter 4 [25], real conditions) and other studies [30, 31, 33] (experimental conditions).

Factor	Pfeiffer et al. [33]	Rubio et al. [31]	Rubio et al. [30]	Mansour et al. [25]
	Experimental conditions			Real conditions
PMI	Teeth storage in soil for more than 6 weeks resulted in a considerable reduction of dental DNA concentration (more than 90%)	The first 2 years of teeth storage at room temperature after extraction demonstrated the most significant DNA degradation, while the following period of 2-10 years displayed no significant decrease	Significant degradation of dental DNA concentration was observed during the first month following extraction, followed by a stabile concentration for 1-12 months. DNA concentration revealed another reduction again, at 18 months after the extraction.	The highest DNA concentration was yielded from dental samples in the first 10 days after death. After 10 days, dental DNA yield dropped considerably.
Surrounding environment	**Garcia et al. [113]**		**Mansour et al. [25]**	
	Experimental conditions		Real conditions	
	The best dental DNA yields were observed in teeth subjected to outdoor conditions, followed by teeth buried in the soil or sand, then teeth submerged in water.		Samples subjected to indoor conditions showed the best results, followed by water, outdoors and under the ground.	

Interestingly, we found (in Chapter 4 [25]) that among different post-mortem conditions, the highest amount of DNA was obtained from teeth extracted from fresh and burnt corpses. In burning cases, we suggested that the root teeth remained anatomically protected from severe incineration, though other soft tissues were severely damaged. Furthermore, the relevant PMIs in most cases of burning did not exceed 1-2 days, most likely thanks to the particular circumstances pertaining to the burning [25].

1.3.2.1 DNA extracted from cementum

Dental tissues show different histological structures. According to the cellularity of each tissue, enamel does not contain cells [19, 27]. Dentine usually consists of dental tubules provided with odontoblasts' processes without nucleated cell bodies [27]. While, odontoblasts' nucleated bodies are located in the odontoblastic zone in the pulp [114]. Cementum has two types – (i) cellular cementum, usually located in the apical region and furcation, and (ii) acellular cementum, usually located the cervical area of the tooth [114]. Pulp, the soft tissue, is rich in cells but degrades rapidly after death and can be exposed to different diseases during life [27, 66, 113]. The dental histological investigation carried out by Vavpotič et al. [115] displayed a significant drop of odontoblasts density, with a proximal average of about 11000 per mm^2 in healthy vital teeth [115, 116], with up to 5 days after death being the point at which odontoblasts is no longer discernible [115]. To this end, in adverse post-mortem conditions, cementum is the principal source of nucleated cells for DNA profiling.

In Chapter 5 [10], two forensic cases of challenging conditions used cementum as a source of DNA for DNA typing, for (i) identification of an unidentified adipocere corpse submersed in water inside a car for approximately 2 years, and (ii) proof of the paternity of an exhumed man around 2.8 years after his death. The dental histological findings showed organized structure in cementum and recognizable cementocytes, while the pulp was mostly destroyed, displaying indefinite structure with an absence of odontoblasts. Similar histological findings were described by Higgins et al. [28]. Additionally, remarkable variations between teeth and different tooth root areas [66, 32] in cementocytes density and cementum thickness was observed in dental histological examination and micro-computed tomography (μCT). This illustrates respective histological and anatomical variations.

1.3.3 Alternative methods facilitating human identification using dental implants and new DNA technology

The recent emergence of methods such as determining epigenetic age using DNA methylation (DNAm) [117] and inferring genetic ancestry using Y-Haplotype and mitochondrial DNA (mtDNA) [118] could support the identification process with meaningful information to reconstruct the biological profile, when traditional comparative identification fails as a consequence of DNA reference samples being unavailable. Based on the method described by Bekaert et al. [117] and the

formula of the regression model built by Smeers et al. [119], we calculated the epigenetic age after determining the percentage values of DNA methylation of CpG6 ELOVL2, CpG1 EDARADD, CpG1 PDE4C and CpG1 ASPA. Our results from Chapter 7 [45] demonstrated that the calculated epigenetic age provided proper lower and upper age limits. Moreover, the difference between the calculated epigenetic age based on the formula of weighted least squares (WLS) regression recommended by Smeers et al. [119] and the actual chronological age was 5.43 years. This deviation is much better than the potential error range expected when using other anthropological dental and skeletal methods based on physiologically degenerative age-related changes. Although the result of Y-STR Haplotype did not show any matches, even with Minimal Haplotype (9 Y-STR systems), mtDNA provided proper biogeographical origin.

Our results of dental investigation, as described in Chapter 7 [45], highlight new dimensions for utilizing dental implants through identifying the manufacturers' item number, considered the key step to identifying the treating dentist and ultimately recovering antemortem dental records. In this case, based on the criteria identified in our method, 9 batch numbers out of 11 concerning both prosthetic abutments and implant-fixtures could be identified. Any mistakes during the microscopic measurement of any parameter of the implant fixture (length, diameter or height) or any parameter of the prosthetic abutments (diameter, angulation or gingival height) can lead to an improper match with the analogue in the relevant catalogue of manufacture, and consequently lead to improperly inferred batch numbers. Our mistake in measuring the diameter of two implant-fixtures thus resulted in discrepancies in the relevant batch numbers (2 batch numbers out of 11).

Matoso et al. [120] utilized the traceability of an implanted orthopedic device's batch number to establish positive human identification. However, the difference with our reported case (in Chapter 7 [45]) is that no batch was engraved in our dental implants. Nevertheless, we could successfully infer the batch numbers (9 batch numbers out of 11) after performing the microscopic metric measurements described in Chapter 7. Such a conclusion can effectively facilitate identifying the dental clinic or dentist who ordered the respective implant article if the data saved in manufacturing companies' databank is used to track the dentist or person who ordered them. Therefore, when the conventional dental identification can not be established as a consequence of missing antemortem dental data, extracting the inserted dental implant and enhancing the clinical and radiological examination with microscopic measurements and microscopic metric analyses is recommended [45]. In this way, the probability of identifying the manufacturers' item number and the treating dentist can be increased. Data provided from dental implants manufacturers' databank was very supportive. Such data (the implant distribution, the mode of production, and the costumers/individuals ordering these implants) can be used as "reference data". Prospectively, this data can effectively orient the police in the context of identification investigation.

To this end, when the conventional primary identifiers (comparative dental and DNA identification) fail to establish identity, other avenues like implant investigation in dental identification or/ and new DNA-based approaches in DNA identification should be utilized as alternative methods facilitating human identification.

1.3.4 Forensic age estimation in Hamburg, Germany

The results of the investigation presented in Chapter 8 [76] demonstrate that 4223 age estimations were carried out in the Institute of Legal Medicine in Hamburg over a period of 25 years, from 1990 to 2015. According to the period of time, the number of age estimations differed. The frequency of age estimations in 1990-2000, 2001-2008, and 2009-2015 was 699, 946, and 2578, respectively [76-79]. Furthermore, the method used for age assessment changed noticeably over time. Subjective and unscientific estimation was used by the staff of the Federal Office for Migration and Refugees (Landesamt für Migration und Flüchtlinge) before 1990 [77]. Hand X-ray and OPG were used in most cases by the Institute of Legal Medicine in Hamburg from 1990 to 2000 [77]. After 2000, OPG played a crucial role in age assessment in Hamburg. 84% of all cases recorded between 2001 and 2008 were examined through OPGs, while hand X-ray was used in 30% and clavicular projection radiograph (clavicle-PR) in 10% of cases in this period [78]. After 2009, as with clinical-physical examinations, OPG was used in every forensic estimation case, while hand X-rays and clavicle PRs were rarely used [76, 79]. After 2015, CT-clavicle investigations were used as an alternative method of clavicle-PR. CT-clavicle has become the exclusive method for evaluating the developmental stages of the medial clavicular epiphyseal cartilage [121, 76] as a result of the potential superimposition effects of clavicle-PR, leading to improper assessment of the developmental stages of clavicular medial ending [121].

In general, due to the diverse national legislations of each country, several approaches and methods are used in age estimation for living adolescents and young adults [60]. As a non-medical indication, using X-rays in the context of age diagnostics requires a legal justification, according to respective legal regulations [59]. In Hamburg, three different authorities - courts, the foreigners' registration office (Zentrale Ausländerbehörde), and the state office of education and consultation (Landesbetrieb Erziehung und Beratung) - requested expert opinion of forensic age diagnostic over 25 years [76]. The overall comparison between the number of asylum applications and age estimations shows remarkable correlation, as illustrated in figures 4 and 5.

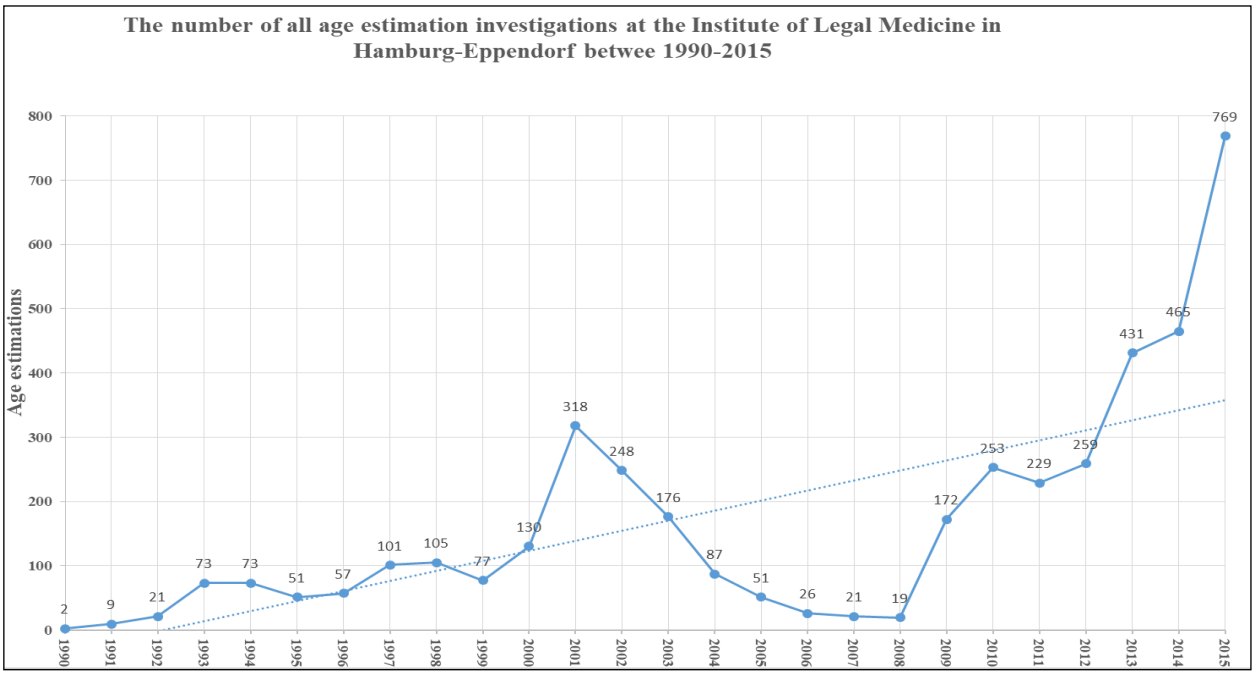

Figure 4 Statistical distribution of the number of age estimation investigations at the Institute of Legal from 1990 to 2015 [76-79].

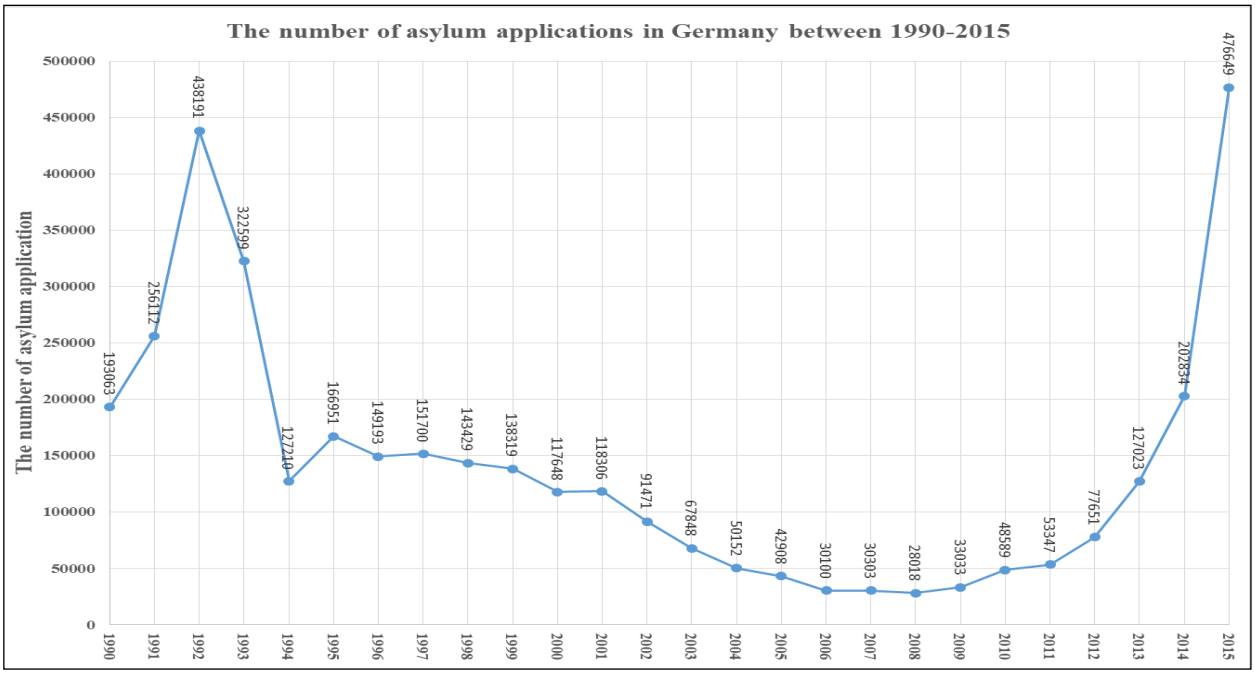

Figure 5 Statistics for asylum applications in Germany between 1990 and 2015, according to the Federal Agency of Migration and Refugees (Bundesamt für Migration und Flüchtlinge) [122].

From 1990 to 2015, the greatest total number of asylum applications in Germany was 476,649, recorded in 2015 by the Federal Agency of Migration and Refugees “Bundesamt für Migration und Flüchtlinge” [122]. Similarly, the highest number of age estimations was 769 cases, recorded in the

Institute of Legal Medicine in Hamburg for the same period [76]. The lower number of asylum applications in Germany in 2008 (28018 applications [122]) was also consistent with the lowest total number of age estimations (19) for the same year [76, 78].

1.3.4.1 The combined individual findings and minimum-age concept:

Since the chronological age can not be determined exactly using any of the current scientific methods [123], the combination of multiple age markers can narrow the error margins and improve decisions about the legally-relevant age threshold [54, 81, 124]. In 2016, Schmeling et al. [59] suggested the minimum-age concept, based on approaching the minimum limits determined in the respective studies of the reference population to provide the highest standard of proof. This approach is based on combining the values of the minimum limits of multiple age markers and adopting the largest minimum value as minimum age [54, 59]. Likewise, the maximum age should be the lowest maximum value of the maximum limits of multiple age markers in the respective study of the reference population [54, 59]. Fig. 6 clarifies the minimum-age concept using a combination of more than one age marker. In addition, Table 11 shows an overview of different statistical parameters stated in various important studies about the respective stages of the different age markers to estimate age thresholds of 18 and 21.

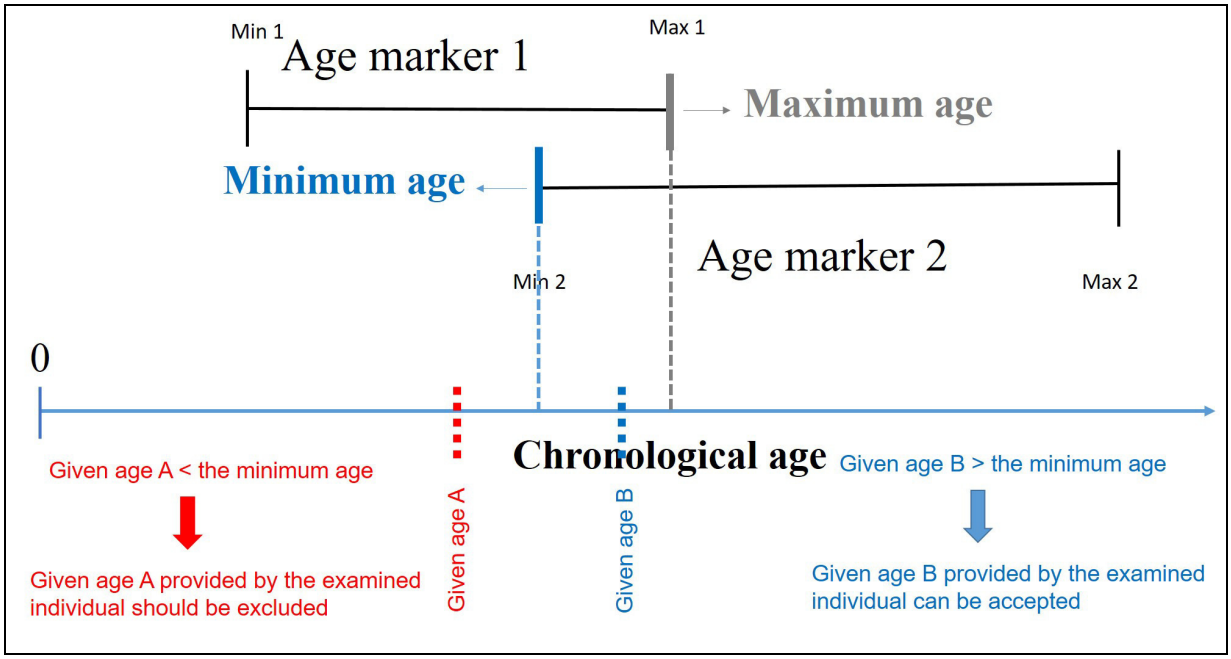

Figure 6 illustrates the concept of minimum age, using a combination of multiple age markers as described in [54, 59].

Table 11 Overview of different statistical parameters stated in various studies regarding the respective stages of the different age markers concerning age thresholds 18 and 21.

Age marker	Stage	Gender	Min	Median	Max	Ref.
Wrist and hand bones	full ossification of the hand skeleton	Male	**16.1**	18.8	19.9	Tisè et al. [81]
		Female	**16.2**	18.7	20	
Third Molar mineralization	H 38	Male	**17.38**	22.88	26.95	Olze et al. [125]
	H 48	Male	**17.38**	23.12	26.91	
	H	Male	**17.65**	_	25.94	Guo et al. [89]
	H	Female	**17.23**	_	25.82	
	H	Male	**18.1**	_	26.9	Zeng et al. [91]
		Female	**17.6**	_	26.9	
The medial clavicular epiphysis	**3a**	Male	**17.5**	**18.6**	20.7	Kellinghaus et al. [110]
		Female	**16.8**	**19.6**	22.1	
	3a	Male	**16.4**	**19.5**	22.3	Wittschieber et al. [126]
		Female	**15.5**	**18.8**	23.3	
	3b	Male	**18.3**	**21.1**	25.4	Kellinghaus et al. [110]
		Female	**17.8**	**21.1**	24.4	
	3b	Male	**17.6**	**20.6**	36.5	Wittschieber et al. [126]
		Female	**16.4**	**20.2**	23.3	
	3c	Male	**19.7**	**23.3**	26.2	Kellinghaus et al. [110]
		Female	**19.5**	**22.1**	26.2	
	3c	Male	**19**	**23.2**	30	Wittschieber et al. [126]
		Female	**19.4**	**21.5**	26.5	
	4	Male	**21.3**	**26.7**	30.9	Schmeling et al. [108]
		Female	**20**	**26.7**	30.9	
	4	Male	**21.6**	**29.8**	35.8	Kellinghaus et al. [127]
		Female	**21.3**	**27.9**	35.2	
	4	Male	**21.6**	**28.7**	40.5	Wittschieber et al. [126]
		Female	**21.1**	**26.3**	37.3	
	5	Male	**26.4**	**31.8**	35.8	Kellinghaus et al. [127]
		Female	**26.1**	**31.2**	35.7	
	5	Male	**26**	**28.3**	30.4	Schmeling et al. [108]
		Female	**26.7**	**29.1**	30.9	

2 List of Abbreviations

OPG	Orthopantomogram
CBCT	Cone beam computed tomography
AGFAD	German Study Group on Forensic Age Diagnostics (Arbeitsgemeinschaft für Forensische Altersdiagnostik)
Clavicle-PR	Clavicular projection radiography
CT-Clavicle	Thin-slice computed tomography-scan of medial clavicular epiphysis
µSv	MicroSv
DMFT-Index	Decayed Missing Filled Tooth-Index
CEJ	Cement-enamel junction
PR	Projection Radiography
CT	Computed Tomography
PMI	Post-mortem interval
DNAm	DNA methylation
mtDNA	Mitochondrial DNA
CpG	Cytosine-phosphate-guanine dinucleotide

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4 Publication 1: Factors affecting dental DNA in various real post-mortem conditions

4.1 Publication

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ORIGINAL ARTICLE

Factors affecting dental DNA in various real post-mortem conditions

Hussam Mansour[1] · Oliver Krebs[1] · Hans O. Pinnschmidt[2] · Nadine Griem[1] · Ilona Hammann-Ehrt[1] · Klaus Püschel[1]

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Abstract

Post-mortem DNA degradation is still the real challenge of DNA-based identification in forensic practise. It is a complicated multifactorial process occurring as a result of the combination of several different environmental effects along with the crucial effect of the elapsed post-mortem interval (PMI). The main purpose of the present study is to evaluate the effect of ante- and post-mortem factors on dental DNA in real forensic cases. Ninety-five teeth extracted from 39 corpses, whose bodies were subject to 6 different post-mortem conditions, were used to evaluate dental DNA amount. In total, 179 DNA extracts isolated from the root of the teeth were examined after removing the crown and sectioning each root into apical and cervical portions. DNA concentration was measured using real-time polymerase chain reaction DNA quantitation kit (PowerQuant™ System/Promega). Our results indicate that the post-mortem interval (PMI) is the most important influential factor on dental DNA quantification ($p < 0.001$). However, in the actual data set, it was confounded with several ante- and post-mortem factors, rendering its actual net effect difficult. The time period of the first 10 days after death yielded the best DNA results from all analysed dental samples. Afterwards, a dramatic decrease in dental DNA was observed in the following time period. Teeth extracted from burnt and fresh corpses yielded the highest amount of DNA, while skeletonized exhumed corpses resulted in the lowest DNA amount. Indeed, dry and indoor conditions demonstrated better results than those in water, outdoors, or buried in the ground. On the other hand, ante-mortem factors including sex, age, tooth type, and tooth root portions did not reveal significant effect on dental DNA yield. We suggest that ante-mortem factors are considerably more subjected to individual variations. Post-mortem factors including PMI, post-mortem conditions, and the relevant surrounding environments have substantial influence on the dental DNA amount yielded.

Keywords Teeth · DNA quantification · Genetic identification · Post-mortem interval (PMI)

Introduction

The specificity of anatomical, histological, physical, and chemical structure of dental tissues explains the reasons of the critical need for dental evidence in identification investigations. Even if teeth fail to be utilized in successful comparative dental identification as a consequence of missing or ineligible ante-mortem dental records, teeth

Hussam Mansour and Oliver Krebs contributed equally to this work.

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✉ Hussam Mansour
Hussam.Mansour@uke.de

Oliver Krebs
oliver.krebs@uke.de

Hans O. Pinnschmidt
h.pinnschmidt@uke.de

Nadine Griem
n.griem@uke.de

Ilona Hammann-Ehrt
Ilona.Hammann-Ehrt@uke.de

Klaus Püschel
pueschel@uke.de

[1] Institute of Legal Medicine, University Medical Center Hamburg-Eppendorf, Butenfeld 34, 22529 Hamburg, Germany

[2] Center of Experimental Medicine, Institute of Medical Biometry and Epidemiology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

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can offer an exceptional source of the genetic material for DNA genotyping [1–3].

Compared with soft tissues, teeth used as a source of DNA in genetic identification have both advantages and disadvantages. The genetic material in soft tissues suffers from a rapidly accelerated post-mortem degradation while teeth still have more resistance and stability comparatively [4]. On the other hand, potential drawbacks for using teeth in DNA-based identification should be taken into account. Such drawbacks include the large volume of non-DNA components [3, 5, 6], sparse and irregular distribution of cellularity, the adverse effect of dental diseases or dental therapy [3], the availability of relatively small amounts of dental DNA samples [7], and the laborious procedures of dental DNA extraction [4].

In general, regardless of the source of DNA, post-mortem DNA degradation is still the real challenge of DNA-based identification in extremely difficult post-mortem conditions [5]. DNA degradation is a complicated multifactorial process that occurs as a result of the combination of several different environmental effects including humidity, temperature, pH, ultraviolet radiation, natural and artificial inhibitors, and the nature of existed microorganisms along with the substantial effect of the elapsed post-mortem interval [6, 8–11]. This damage downgrades both the quality and quantity of DNA extracted from forensic samples and, consequently, minimizes the high discrimination power of routine forensic STR analyses, which are used worldwide for genotyping in forensic laboratories by resulting in partial or no STR profiles [9, 12, 13]. It is well known that DNA quantity, the level of DNA degradation, and the coexistence of PCR inhibitors impact crucially on the success rate of PCR amplification when forensic samples are subjected to such harsh environmental conditions [13, 14]. A comprehensive investigation of different factors affecting the amount and quality of extracted DNA can provide a proper explanation for possible disappointing results, save both time and the consumption of expensive materials, avoid potential misinterpretation of results, and optimize DNA sampling [3, 15]. However, simultaneous control of experimental conditions for all potential factors seems to be impossible [15–17]. In reality, no controlled environmental conditions affecting post-mortem DNA damage can be expected since considerable changes in these conditions can occur by the passage of time. In addition, the teeth had remained protected by the supporting periodontium (alveolar bones, gingival tissues, periodontal ligament, and cementum [18]) and the surrounding oral muscular structures will be affected differently by the post-mortem environments in comparison with the extracted teeth directly worked upon in the experimental conditions [6, 19].

We sought in this research to investigate dental DNA content from corpses subjected to various real post-mortem conditions during the last 3 years. Similar to Higgins et al. [6], the potential factors attributed to DNA yield were categorized in two types: (i) ante-mortem factors including sex, age, tooth type, and tooth root portion and (ii) post-mortem factors including PMI, post-mortem conditions, and various surrounding environments.

Materials and methods

Sampling and data distribution During the last 3 years, 95 teeth were extracted from 39 corpses, which were subjected to different post-mortem conditions in real forensic caseworks to isolate DNA and evaluate DNA quantification. Most of these cases were unidentified corpses, which later had been identified using either DNA genotyping or comparative dental identification. This study was approved by the Ethics Committee of the State Chamber of Medicine in Hamburg "Ethik-Kommission der Ärztekammer Hamburg", approval no. WF- 40/19. Furthermore, consent from the responsible authorities for unidentified dead bodies was provided. The collective included 29 males and 10 females whose age ranged from 22.3 to 85.3 years (M = 46.87 years). PMI ranged widely, from 1 day to approximately 70 years. Table S1 shows descriptive data about the distribution of corpses' relevant data points including sex, post-mortem conditions, and the various surrounding environments.

The extracted teeth were distributed as shown in Table 1. In total, 179 DNA extracts were obtained from these teeth to evaluate DNA amount in varying real conditions. Table 2 shows the distribution of DNA analyses. Detailed descriptions about the number of teeth extracted from each corpse and the distribution of different tooth types and tooth portions used from each corpse are illustrated in Table S2.

Preparation procedures, DNA extraction, and DNA quantification

In accordance with the manufacturer's directions of the Crime Prep Adem-Kit (ADEMTECH SA, France) [20], genomic DNA was extracted from dental roots after doing the preparation procedures shown in Fig. 1.

Afterwards, the concentration of the pure extracted DNA was measured using real-time polymerase chain reaction DNA quantitation kit (PowerQuant™ System/Promega) with ABI 7500 RT PCR System. Table S3 presents the amplicon size of the targets amplified in the PowerQuant™ System [23–25].

Statistical analysis

For statistical analyses, SPSS (Statistical Package Social Science) 25.0 software was used. To process the data with SPSS, all categorical data saved in an Excel file were coded

Table 1 The distribution of the extracted teeth according to different factors

Variables	Distribution of different variables concerning the DNA samples analysed	Number of teeth
Sex	Males	63
	Females	32
	Total	95
Post-mortem conditions	Fresh (non-decomposed) corpse	7
	Advanced decomposition	40
	Adipocerous	8
	Completely skeletonized	15
	Exhumed and completely skeletonized	13
	Burnt	12
	Total	95
Surrounding environment	Indoors	24
	Outdoors	23
	Water	33
	Underground	15
	Total	95
Tooth type	Molar	43
	Premolar	20
	Canine	14
	Incisor	17
	Missing	1
	Total	95

into numerical values (Table S4). Data distributions of continuous variables were assessed by boxplots and histograms. Bivariate relationships among variables were examined by means of Spearman correlations, Fisher's exact tests, and Mann-Whitney *U* tests, depending on the measurement scales of the respective variables.

Table 2 The distribution of DNA extracts according to different variables

Variables	Distribution of different variables concerning the DNA samples analysed	Number of DNA extracts
Sex	Males	118
	Females	61
	Total	179
Post-mortem conditions	Fresh (non-decomposed) corpse	12
	Advanced decomposition	75
	Adipocerous	14
	Completely skeletonized	30
	Exhumed and completely skeletonized	25
	Burnt	23
	Total	179
Surrounding environment	Indoors	40
	Outdoors	46
	Water	60
	Underground	33
	Total	179
Tooth type	Molar	79
	Premolar	35
	Canine	28
	Incisor	35
	Missing	2
	Total	179
Tooth part	Apex	89
	Rest root	90
	Total	179

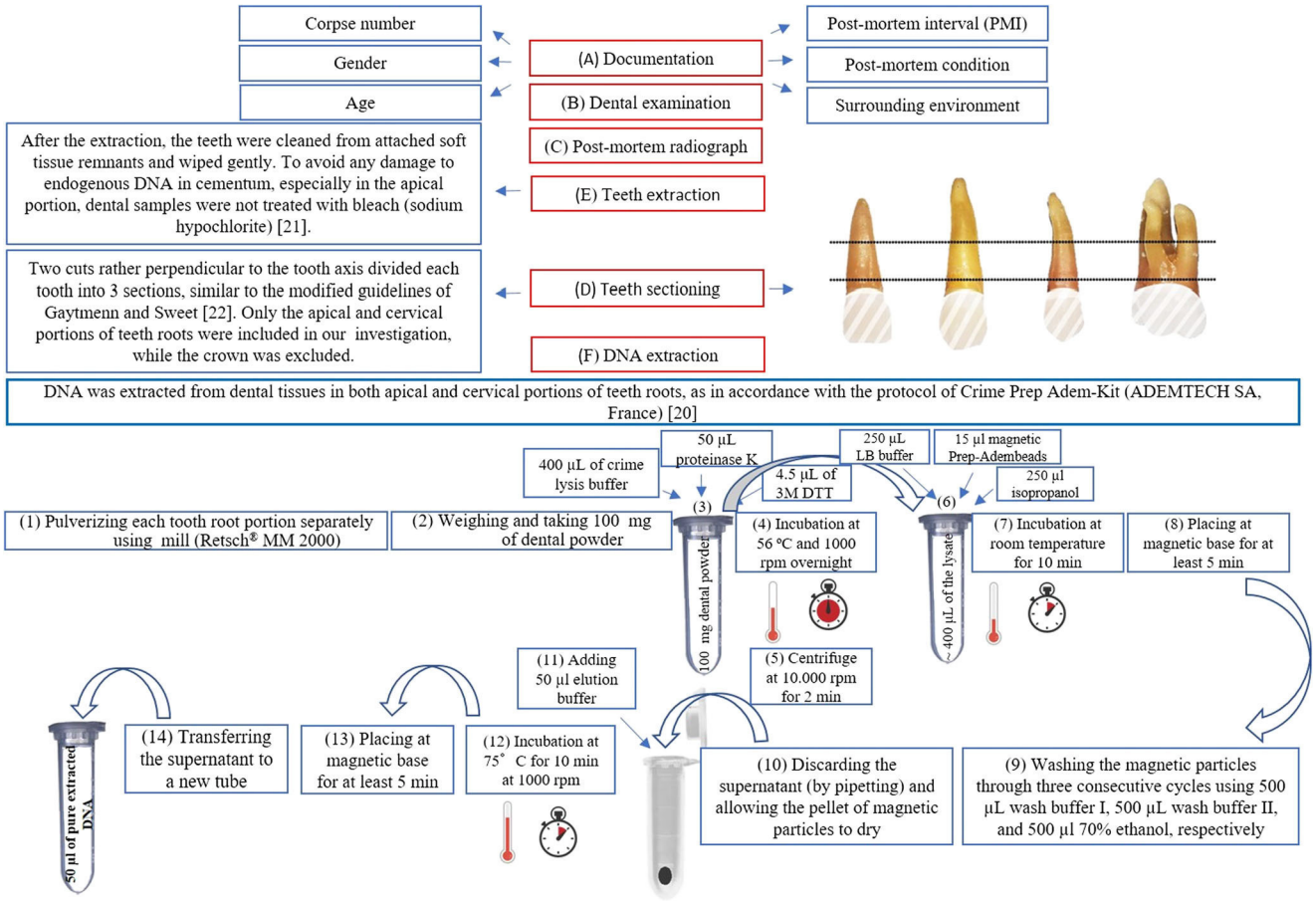

Fig. 1 Preparation procedures of dental samples and the steps of DNA extraction [20–22]

Using independent variables individually in univariate analyses, general linear mixed-effects models were fitted to the double-ln-transformed dependent variable "autosomal DNA concentration" (pg/100 mg/µL), considering the corpses' identities as subjects and sample identity as repeated measures within subjects. To estimate the effect of PMI (independent variable) on DNA concentration, PMID (post-mortem interval in days) was recoded into a categorical variable consisting of four categories (Table 4) prior to using it in the mixed model. Multivariate mixed model analyses were also done. However, high interrelations among some of the independent variables rendered interpretations of the results of multivariate analyses difficult.

Results

The different statistical analyses revealed marked relations between DNA quantification and post-mortem interval, various post-mortem conditions, and surrounding environment (Fig. 2). Spearman's correlation indicated a considerable negative correlation between the post-mortem interval and DNA quantification ($r = -0.68$, $p < 0.001$).

Results of tests of independent variables with F-statistic of the univariate analyses are presented in Table 3.

Table 4 shows that group 1 yielded the highest DNA concentration of all PMI groups. In comparison with all groups, the difference in DNA concentration between group 1 and the next DNA yielding group (group 2) was the highest. Thus, the substantial DNA degradation occurs within 10 days after the death. As anticipated, the lowest DNA yield was in group 4.

DNA concentration was not significantly related to corpses' age and sex as seen in Table 5 and Fig. 2e, f. Furthermore, no significant differences were found for the amount of DNA extracted from the corpses' different tooth types or root regions (Table 5, Fig. 2c, d). As estimated by a mixed model, random effect variance was 7.383 while residual effect variance was 2.504. We thus estimate that about 0.25 (= 2.504 / (2.504 + 7.383)) of the total variance was due to intra-corpse variation.

In different post-mortem conditions, univariate analyses showed that the highest DNA quantification was observed in burnt and fresh corpses, while skeletonized exhumed corpses exhibited the lowest DNA amount (Table 5 and Fig. 2a).

Dental DNA extracts from corpses exposed to indoor conditions demonstrated the best results followed by water and outdoors, while the poorest dental DNA was retrieved from corpses buried in the ground (Table 5, Fig. 2b).

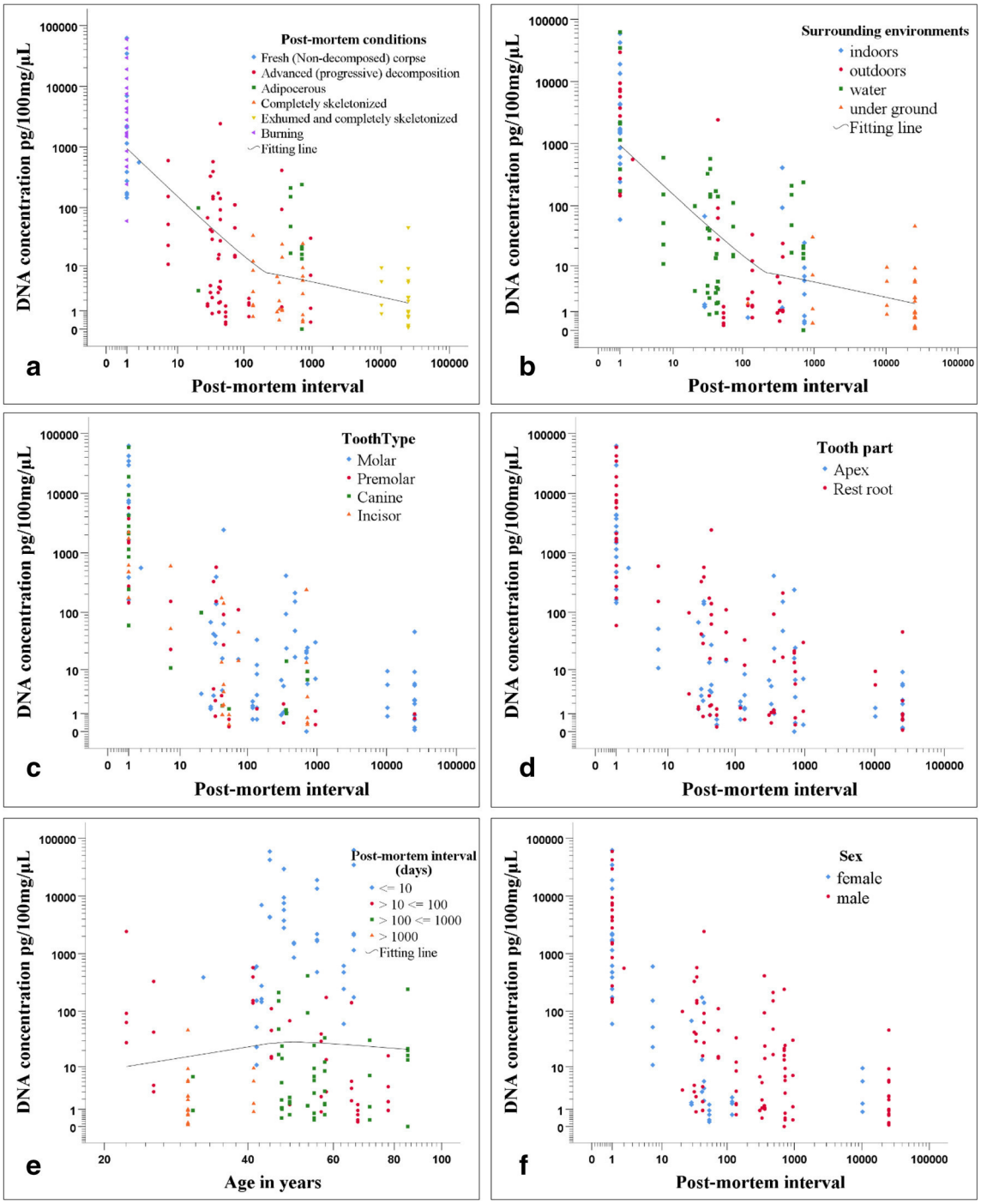

Fig. 2 Dental DNA concentration versus various ante- and post-mortem factors. DNA concentration and post-mortem interval are presented on a logarithm scale (base 10)

Discussion

In comparison with previous relevant studies based on controlled environmental conditions, this investigation, based on real forensic cases, showed some consistencies as well as some inconsistencies in its results. Ante-mortem factors (sex, age, tooth type, and tooth portion) displayed no significant relations to dental DNA yield, in contrast to post- mortem factors (PMI, post-mortem condition, and the surrounding environment) that did show such relations.

Tooth type Rubio et al. [17] found that DNA content in molars and premolars was higher than that in canines and incisors. Similarly, other studies [3, 26] referred to molars as a preferable choice among different tooth types for several reasons such as their higher potential cellularity resulting from

Table 3 Results of tests of independent variables with F-statistic of the univariate analyses

Independent variables	*F*	df1	df2	*p*
PMI in 4 categories	68.613	3	149	0.000
Post-mortem conditions	46.010	5	147	0.000
Surrounding environment	10.008	3	149	0.000
Sex	2.074	1	151	0.152
Age/years	0.003	1	149	0.956
Tooth type	1.835	3	147	0.143
Tooth root portion	0.500	1	151	0.481

comparatively larger pulp volume and larger size of cementum, more stability in their alveoli, and more anatomical protection and, consequently, molars are less prone to post-mortem loss or contamination [3, 16, 27]. Furthermore, Raimann et al. [28] reported that independent of the elapsed PMI, molars, and premolars yielded satisfying DNA profiles. However, Corte-Real et al. [29] did not find any influence of tooth type on dental DNA. Similarly, our results indicate that there is no significant tendency in DNA yield with respect to different tooth types. This can be explained as a result of a rapidly accelerated decay in pulp tissues. Accordingly, no impact of the pulp volume on dental DNA retrieved from non-fresh corpses can be expected. Odontoblasts, the main cells in the pulp-dentin complex, degraded rapidly and are not existent after 5 days after the death [30]. Moreover, histological findings showed remarkable pulp destruction after different PMIs [2, 6, 30].

Tooth part Despite the trend of revealing higher DNA content in the cervical root part (rest root) in comparison with the apical one (apex), the results of the present investigation did not demonstrate significant quantitative differences in DNA yielded in both root portions. The study of Gaytmenn et al. [22] showed that the concentration of DNA yielded from the cervical portion of the root was significantly higher than that yielded from the apical one. Nevertheless, they stated that both root portions revealed sufficient yield of DNA. Corte-Real et al. [29] found the apical radicular portions showed higher DNA quantification than cervical radicular portions.

The non-significant pairwise difference in both radicular portions can be explained as a result of a wide intra- and inter-individual variation with respect to the histological and anatomical structures [2, 6, 22]. Both pulp and cementum are the main dental sources of genomic DNA [3]. The histological findings for cementum [2, 21] demonstrated that the distribution of cellularity in cementum is uneven and its thickness varies widely even between different tooth regions in the same individual. Stamfelj et al. [27] stated broad maximal cementum thickness ranges (25–1140 μm in upper molars and 20–700 μm in lower molars) in the measurements on transverse sections of teeth roots in their histometric study. Moreover, Bosshardt [18] suggested the influence of molecular factors on synthesizing different types of cementum.

Sex As anticipated, no significant sex-related differences were observed in DNA amount. This is consistent with findings reported in previous studies [6, 15, 21]. Martins et al. [31] found few differences with respect to the number of roots and root canal morphology between males and females. Moreover, the reference population may play an important role in the amount of different dental tissues in both genders [32].

Age In general, two main contradictory physiological changes attributed to dental DNA occur during ageing process: (i) decrease in both pulp volume and pulp cellularity and (ii) increase in cellular cementum thickness [3, 33, 34]. Similar to the corpse's gender, our results did not show a significant relationship between age and DNA yield. A controversial interpretation of the interaction between age and DNA yield from teeth has been reported in different studies. For instance, Rubio et al. [15] reported that age is not significantly correlated with DNA concentration, while Higgins et al. [6] found a positive effect with ageing on DNA isolated. Higgins et al. [6] suggested that the cementum deposition with age can increase the thickness of cellular cementum and enhance the protection from microorganisms.

Table 4 Model-estimated marginal means with 95% confidence intervals (CI) of DNA concentration in 4 categorized groups of post-mortem intervals ($p < 0.001$, F test) after back-transformation*. All means are significantly different from each other at $\alpha = 0.05$, according to pairwise contrast

Categorized groups	PMI	DNA extracts	Percentage of DNA extracts	Mean of DNA concentration pg/100 mg/μL	95% CI	
					Upper limit	Lower limit
Group 1	PMI < = 10 days	40	22.3%	1227.9	2870.1	546.8
Group 2	10 < PMI ≤ 100 days	56	31.3%	9.5	17.0	5.4
Group 3	100 < PMI ≤ 1000 days	58	32.4%	4.2	7.1	2.5
Group 4	PMI > 1000 days	25	14%	1.4	3.0	0.7

*Dependent variable was double-ln-transformed prior to subjecting it to general linear mixed model analyses

Table 5 Univariate model estimated marginal means with 95% confidence intervals (CI) after back-transformations*. Category means of a variable followed by the same letter are not significantly different from each other at $\alpha = 0.05$, according to pairwise contrast

Categorical variables		Mean	95% CI	
			Upper limit	Lower limit
Post-mortem conditions $p < 0.001$ (F test)	Burning	2656.1 c	8330.2	907.0
	Fresh (non-decomposed) corpse	1145.7 c	5271.8	282.2
	Adipocerous	18.7 b	59.7	6.4
	Advanced (progressive) decomposition	8.8 b	14.3	5.5
	Completely skeletonized	2.6 a	5.1	1.4
	Exhumed and completely skeletonized	1.4 a	2.9	0.7
Sex $p = 0.152$ (F test)	Female	28.5 a	73.8	11.7
	Male	13.0 a	23.4	7.5
Tooth type $p = 0.143$ (F test)	Canine	78.7 b	359.7	19.8
	Premolar	14.0 ab	45.6	4.7
	Incisor	13.8 ab	44.9	4.7
	Molar	13.7 a	27.3	7.1
Tooth part $p = 0.481$ (F test)	Rest root	19.5 a	39.4	9.9
	Apex	13.7 a	27.9	7.0
Surrounding environment $p < 0.001$ (F test)	Indoors	59.3 c	174.9	21.7
	Water	29.5 bc	66.8	13.6
	Outdoors	14.1 b	34.7	6.1
	Underground	1.6 a	4.1	0.6

*Dependent variable was double-ln-transformed prior to subjecting it to general linear mixed model analyses

In brief, the effect of the aforementioned ante-mortem factors (sex, age, tooth type, and tooth root portion), referred to as individual factors by Leo et al. [16], varies obviously in different studies due to the wide variation from person to person [22]. On the other hand, the effects of post-mortem factors are remarkable and statistically significant.

Surrounding environment Our results revealed that dental samples retrieved from corpses which had been buried in the ground yielded the lowest amounts of DNA. Similarly, Imaizumi et al. suggested in their study [35] that the soil was a potential contributor to strong PCR inhibition in their buried samples. In this study, dental samples retrieved from corpses that remained in water yielded better DNA than those buried in the ground (Table 5). Nevertheless, DNA extracts from teeth in a water surrounding environment showed also poor DNA yield. This is consistent with findings presented by Garcia et al. [19]. They proposed that the rate of DNA hydrolysis in water, which is higher than that of other environments, resulted from a dilution effect of water, which can explain the poor DNA yield from teeth preserved in water [19]. Interestingly, the best results were observed in corpses that had remained indoors, where the surrounding environment is dry and relatively away from environmental chemicals and UV light exposure and where no dramatic change in pH, humidity, or temperature [8] in comparison with outdoor conditions can be expected. Ambers et al. [8] called attention on these adverse environmental implications on DNA fragmentation damage such as double-strand breakage through elevated temperature and high humidity conditions, deamination alteration through environmental chemicals, and intra- and inter-strand crosslinking in low-level pH conditions.

PMI and post-mortem conditions The most remarkable factor associated with DNA degradation is the elapsed post-mortem interval. The highest DNA amount in our data was observed in dental samples representing the shortest PMI (group 1). DNA concentration dropped substantially in the next period (group 2) after 10 days after death (Table 4). The early period after death is the most critical period with respect to yielding dental DNA. DNA isolated from dental samples older than 10 days was vulnerable to considerable damage. Similarly, Pfeiffer et al. [36] found that dental DNA obtained from teeth stored in soil garden for more than 6 weeks suffered from a considerable decrease in DNA concentration by more than 90%. Higgins et al. [6] indicated a rapid decrease in DNA extracted from dental pulp in the early 8 months of PMI.

However, in our study, PMI was confounded with other ante- and post-mortem factors and moreover, its role may considerably interact with other factors such as post-mortem conditions. Its true "net effect" on DNA degradation can therefore not easily be determined but would

require additional work, perhaps on specimen derived from samples representing more evenly distributed combinations of conditions of factors affecting DNA degradation. Our study nonetheless demonstrated that high DNA concentrations were obtained from burnt and fresh corpses, whose PMIs did not exceed 1–2 days (Fig. 2a). Despite the severe degree of fire damage to the bodies, our clinical and radiological post-mortem examinations revealed a relatively good status of the roots of the extracted teeth. These roots had remained in a good status due to the anatomical protection provided by the surrounding alveolar bones and other supporting periodontium components as well as the surrounding oral muscular structures. Normally, in forensic practise, it is more likely to discover burnt bodies only a short time after death, thanks to circumstantial conditions pertaining to the burning. In contrast, the lowest DNA concentrations were found in completely skeletonized and skeletonized exhumed corpses, which were left in their respective environment for a long time after death (PMI exceeds several years in most cases) as seen in Fig. 2a.

Conclusion

Dental DNA-based identification is a valuable tool for forensic identification. However, several factors attributed to post-mortem DNA degradation should be taken into account. The aforementioned post-mortem factors are more influential than ante-mortem ones. Promising dental DNA can be obtained from corpses with a short PMI. Dental DNA yield in corpses found in dry indoor conditions is superior to those found in other surrounding environments. In contrast, sparse dental DNA amount can be expected from cases buried in the ground and forensically skeletonized after a long PMI.

However, in our data set, the different factors associated with DNA degradation were not independent of each other, rendering it difficult to assign independent quantitative effects to individual factors. Ante-mortem factors may, on average, not play an important role for dental DNA content. The findings of the present investigation provide additional contributions to estimating the outcome gained by DNA extracted from teeth in various real forensic conditions.

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Compliance with ethical standards

This study was approved by the Ethics Committee of the State Chamber of Medicine in Hamburg "Ethik-Kommission der Ärztekammer Hamburg", approval no. WF- 40/19. Furthermore, consent from the responsible authorities for unidentified dead bodies was provided.

Conflict of interest The authors declare that they have no conflict of interest.

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4.2 Supplementary material

Table S 1 The distribution of the corpses in terms of sex, post-mortem conditions and the various surrounding environments.

Variables	The different variables of the dental DNA Samples	Number of corpses
Sex	Males	29
	Females	10
	Total	**39**
Post-mortem conditions	Fresh (non-decomposed) corpse	4
	Advanced decomposition	13
	Adipocerous	3
	Completely skeletonized	5
	Exhumed and completely skeletonized	9
	Burnt	5
	Total	**39**
Surrounding environment	Indoors	7
	Outdoors	9
	Water	13
	Under ground	10
	Total	**39**

Table S 2 The number of teeth extracted from each corpse and illustration of the various tooth types and tooth portions used from each corpse. A: apical root part (apex); B: cervical root part (rest root); *: missing value (indeterminated).

Corpse	Teeth	Tooth type	Tooth part	DNA extracts
1	2	27, 35	27A, 27B, 35A, 35B	4
2	4	11, 22, 17, 46	17A, 17B, 46A, 46B, 11A, 22B	6
3	2	24, 38	24A, 24B, 38A, 38B	4
4	2	16, 26	16A, 16B, 26A, 26B	4
5	5	27, 42, 43, 11, 32	27A, 27B, 42A, 42B, 43A, 43B, 11A, 11B, 32A, 32B	10
6	1	18	18B	1
7	1	47	47A	1
8	1	37	37A, 37B	2
9	1	37	37A, 37B	2
10	1	35	35A, 35B	2
11	1	47	47A, 47B	2
12	1	17	17A, 17B	2
13	2	17, 26	17A, 26A, 26B	3
14	1	37	37A, 37B	2
15	1	*	A, B	2
16	2	14, 27	14A, 14B, 27A, 27B	4
17	2	34, 47	34A, 34B, 47A, 47B	4
18	5	36, 45, 43, 46, 36	36A, 36B, 45A, 45B, 43A, 43B, 46A, 46B, 36A, 36B	10
19	9	17, 18, 31, 44, 16, 35, 37, 47, 24	17A, 17B, 18A, 18B, 31B, 44A, 44B, 16 A, 16B, 35A, 35B, 37A, 37B, 47A, 47B, 24A, 24B	17
20	2	18, 44	18A, 18B, 44A, 44B	4
21	2	27, 43	27A, 27B, 43A, 43B	4
22	2	23, 47	23A, 23B, 47A, 47B	4
23	2	17, 46	17A, 17B, 46A, 46B	4
24	2	23, 44	23A, 44A, 44B	3
25	3	11, 27, 35	11A, 11B, 27B, 35B	4
26	2	21, 43	21A, 21B, 43A, 43B	4
27	4	27, 28, 35, 43	27A, 27B, 28A, 28B, 35A, 35B, 43A, 43B	8
28	3	23, 35, 47	23A, 23B, 35A, 35B, 47A, 47B	6
29	3	33, 32, 16	33A, 33B, 32A, 32B, 16A, 16B	6
30	2	37, 43	37A, 37B, 43A, 43B	4
31	2	28, 34	28A, 28B, 34A, 34B	4
32	2	47, 13	47A, 47B, 13A, 13B	4
33	4	11, 17, 23, 21	11A, 11B, 17A, 17B, 23A, 23B, 21A, 21B	8
34	3	23, 34, 32	23A, 34A, 34B, 32A, 32B	5
35	3	16, 17, 15	16A, 16B, 17B, 15A, 15B	5
36	3	13, 42, 48	13A, 13B, 42A, 42B, 48A, 48B	6
37	2	42, 11	42A, 42B, 11A, 11B	4
38	2	42, 34	42A, 42B, 34A, 34B	4
39	3	11, 33, 35	11A, 11B, 33A, 33B, 35A, 35B	6
Total	95			179

Table S 3 The amplicon size of four targets amplified in the PowerQuant™ System used in this study [23- 25].

Small autosomal DNA target*	84 bp
Y-chromosomal target	81 bp and 136 bp
Large degradation target	294 bp
Internal PCR Control (IPC)	435 bp

*Only the measurement of autosomal DNA amount was considered in the statistical analyses of this study.

Table S 4 Coding all categorical variables into numerical values.

Abbreviation code	Sex	Tooth type	Tooth part	Post-mortem conditions	Surrounding environment
0	Female				
1	Male	Molar	Apex	Fresh (non-decomposed) corpse	Indoors
2		Premolar	Rest root	Advanced (progressive) decomposition	Outdoors
3		Canine		Adipocerous	Water
4		Incisor		Completely skeletonized	Under ground
5				Exhumed and completely skeletonized	
6				Burning	
999	missing value				

5 Publication 2: Cementum as a source of DNA in solving challenging forensic cases

5.1 Publication

Mansour H, Krebs O, Sperhake J, Augustin C, Koehne T, Amling M, Püschel K (2018) Cementum as a source of DNA in challenging forensic cases. J Forensic Leg Med 54:76–81. doi.:10.1016/j.jflm.2017.12.015

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Cementum as a source of DNA in challenging forensic cases

Hussam Mansour[a,*], Oliver Krebs[a], Jan Peter Sperhake[a], Christa Augustin[a], Till Koehne[b], Michael Amling[c], Klaus Püschel[a]

[a] University Medical Center Hamburg-Eppendorf, Institute of Legal Medicine, Butenfeld 34, 22529 Hamburg, Germany
[b] University Medical Center Hamburg-Eppendorf, Department of Orthodontics, Martinistr. 52, 20246 Hamburg, Germany
[c] University Medical Center Hamburg-Eppendorf, Institute of Osteology and Biomechanics, Lottestraße 59, 22529 Hamburg, Germany

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ABSTRACT

Each forensic case is characterized by its own uniqueness. Deficient forensic cases require additional sources of human identifiers to assure the identity. We report on two different cases illustrating the role of teeth in answering challenging forensic questions. The first case involves identification of an adipocere male found in a car submersed in water for approximately 2 years. The second scenario, which involves paternity DNA testing of an exhumed body, was performed approximately 2.8 years post-mortem. The difficulty in anticipating the degradation of the DNA is one of the main obstacles. DNA profiling of dental tissues, DNA quantification by using real-time PCR (PowerQuant™ System/Promega) and a histological dental examination have been performed to address the encountered impediments of adverse post-mortem changes. Our results demonstrate that despite the adverse environmental conditions, a successful STR profile of DNA isolated from the root of teeth can be generated with respect to tooth type and apportion. We conclude that cementocytes are a fruitful source of DNA. Cementum resists DNA degradation in comparison to other tissues with respect to the intra- and inter-individual variation of histological and anatomical structures.

1. Introduction

Since the early 1990s, DNA technology has been utilized as a powerful tool for identification, paternity testing, criminal investigation, and other forensic investigations.[1–3] As the genetic material in soft tissues rapidly degrades post-mortem, teeth and bones are of interest to yield sufficient DNA for identification.[4,5] Due to their longevity, durability, and endurance against postmortem degenerative changes, teeth were recognized as a valuable source of forensic data many years ago.[6–8] Adverse environmental conditions and associated post-mortem changes play a critical role in determining the proper forensic method for investigation.[9] Prior to analyzing DNA, it is sometimes difficult to estimate the effect of environmental factors on tissues and whether these structures are still sufficient for genotyping.[9]

Interestingly, the yield of DNA isolated from various dental tissues is significantly variable in quantity and quality.[10] Being an acellular tissue, enamel is devoid of DNA.[4,11] As a rule, dentin does not contain nucleated cell bodies. Rather, dentin consists of parallel dental tubules containing the processes of the odontoblasts, which are the cells responsible for dentin formation.[12] Furthermore, according to the presence or absence of cells, two types of cementum can be recognized. First, acellular cementum predominates in the cervical portion of the root and plays a key role in connecting the tooth to the adjacent periodontal ligament.

Second, cellular cementum mainly covers the apical root and can be encountered in the furcation area.[12] It has been hypothesized, that the latter plays a role in the adaptation to occlusion and post-eruptive movement of the tooth.[13]

In the light of this information, pulp and cellular cementum are the main sources of nuclear DNA in dental samples.[11,14] Although pulp is considered the best source of DNA in intact fresh teeth, the yield of DNA is significantly affected by several factors, such as dental diseases, advanced age, and post-mortem cellular degeneration especially in moist environments.[10,14,15] This publication seeks to highlight the importance of cementum as a source of nuclear DNA and to clarify the variability of DNA yield from dental tissues. Cementocytes, our target cells, are comparably well preserved due to the protection afforded by the cementum matrix itself.

* Corresponding author.
E-mail addresses: Hussam.Mansour@uke.de (H. Mansour), oliver.krebs@uke.de (O. Krebs), sperhake@uke.de (J.P. Sperhake), augustin@uke.de (C. Augustin), t.koehne@uke.de (T. Koehne), amling@uke.de (M. Amling), pueschel@uke.de (K. Püschel).

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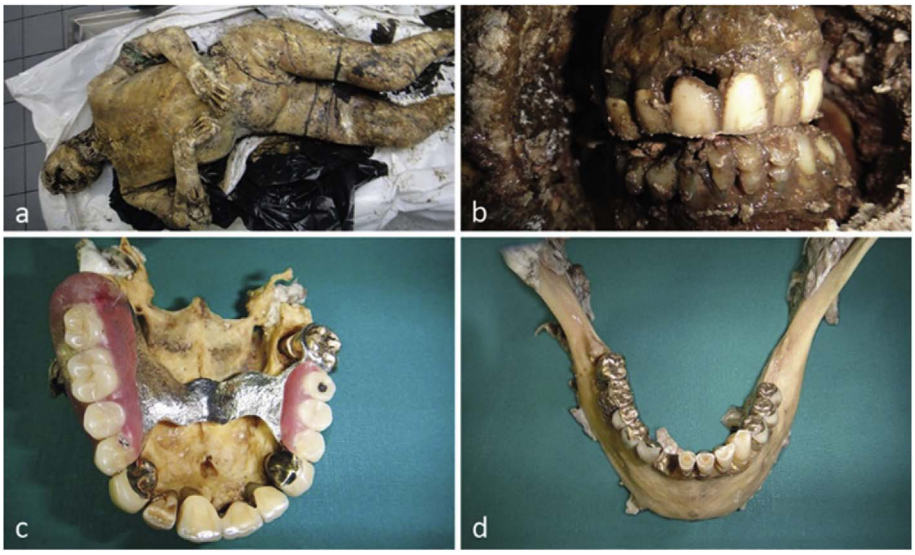

Fig. 1. (a) The adipocere male body. (b) The teeth in situ. (c) Resected upper jaw. (d) Resected lower jaw.

2. Case reports

2.1. Case 1

In October 2015, during sonar training of a diving group, six cars were located under water at the east bank of the Elbe River, which is a large river in the northeastern part of Germany. Five of these sites were well known by the Water and Shipping Authority (Schifffahrtsamt) for about 20 years. However, one vehicle was unknown. The diving group sought the unknown car and found a male human body inside of the car. After recovering the car from the water, the plate number of the car was deciphered; however, the adipocere male body was unrecognizable (Fig. 1). The corpse was transferred to the department of Legal Medicine for routine autopsy, identification, and estimation of the time of death. According to the police report, the owner of the car had been missing since November 2013. He was 87 years old.

To validate the identity by odontological means and DNA testing, several procedures were undertaken: post-mortem removal of both the mandible and maxilla for dental examination (Fig. 1), post-mortem panoramic radiographs (Fig. 2), and DNA analysis of dental samples as well as the bone of the femur.

2.2. Case 2

Following a court order, the body of a deceased male individual (73 years old), who died in November 2013, was exhumed in July 2016 (Fig. 3). The purpose of this exhumation was to determine whether the exhumed man was the biological father of a young woman. For posthumous paternity testing, the genetic profile of osseus or dental tissues has to be established.[16]

Multiple sample types (Fig. 3) including the following were collected: four teeth (14, 15, 25, and 27), muscle tissue, and a piece of bone from the distal left femur (5 cm).

3. Materials and methods

After extracting the selected teeth, horizontal sections were cut. Similar to the Gaytmenn and Sweet guidelines,[17] with little modification, two horizontal sections were made to separate the teeth into three main parts: the crown, the coronal part of the root, and the apical part of the root (Fig. 4a). Several studies have found that DNA extracted from the root is better than DNA extracted from the crown.[11,17,18] Therefore, DNA was isolated only from the root portion.

Following the manufacturer's instructions, the Crime Prep Adem-Kit (ADEMTECH SA, France) was used to isolate DNA from the teeth. After grinding each part of the tooth (Fig. 4b), 400 µL of Lysis Buffer, 50 µL of Proteinase K and 4.5 µL of DTT (3M) were added to 100 mg of tooth powder. The samples were incubated overnight at 56 °C. After incubation (Fig. 4c), about 400 µL of the lysate was transferred to a new microtube. Afterwards, 250 µL LB Buffer, 250 µL Isopropanol and 15 µL magnetic Prep-Adembeads were added and incubated at room temperature for 10 min.

Microtubes were placed in the sample holder and then inserted into the magnetic base for at least 5 min (Fig. 4d). To eliminate inhibitors and impurities, three consecutive wash cycles of the magnetic particles were performed by using 500 µL Wash Buffer I, 500 µL Wash Buffer II, and 500 µL 70% Ethanol, respectively. After washing, the particle suspension was magnetized for 5 min, the supernatant was carefully removed and the pellet of magnetic particles was dried. DNA was eluted by adding 50 µL Elution Buffer.

DNA quantification was performed with real-time PCR (PowerQuant™ System/Promega) by using TaqMan®. Multiplex PCR was performed with a Universal PCR Master Mix, using a 7500 Real-time PCR System (Applied Biosystems). Samples were subjected to short-tandem repeat (STR) DNA analysis with multiplex kits (Powerplex® ESI 17, Powerplex® ESX 17/Promega) and were applied by using genetic analyser sequencer ABI 3130 (Applied Biosystems, Foster

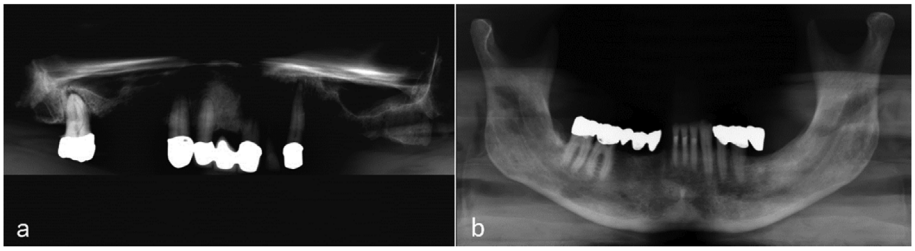

Fig. 2. Post-mortem panoramic radiographs.

Fig. 3. Photos of the exhumation and the samples retrieved from the corpse.

City, USA). Data were processed with ABI GeneMapper™ ID v3.2. software (Applied Biosystems, Foster City, USA).

For microcomputed tomographic imaging (μCT), specimens were scanned with a micro-CT system (micro-ct 40, Scanco Medical, Brüttisellen, Switzerland) at a 15-μm voxel resolution and analyzed by using the software provided by the manufacturer.

4. Results

4.1. Case 1

The geographical location, the plate number of the car, and the wallet of the dead driver effectively accelerated the search for ante-mortem records.[19] However, these means of identification are supportive but not conclusive. Thus, DNA is required for identification.

The genetic profile of the missing elderly man had been generated from samples retrieved from his hair comb in the criminal laboratory. Compared with the genetic profile of the missing person, the DNA profile retrieved from the teeth extracted from the adipocerous corpse matched in 16 loci (including the Amelogenin gender-determining marker) out of 17, while only one allele in locus SE33 could not be detected as shown in Table 1. Statistical calculations were performed by applying Statistefix software version 2.3. The value of the random match probability in the European population was 2.12×10^{-24}.

Additionally, the comparative identification of dental data and the DNA results confirmed conclusively the positive identification.

Table 1
Short tandem repeat analysis of case 1.

Loci	The genetic profile generated from cells retrieved from the missing person's hair comb	The genetic profile generated from teeth of the unknown adipocere male
Amelogenin	XY	XY
D19S433	14/15	14/15
D2S1338	25/26	25/26
D16S539	9/13	9/13
D22S1045	15/17	15/17
D12S391	19/20	19/20
D10S1248	13/14	13/14
D2S441	11/14	11/14
D1S1656	13/19.3	13/19.3
D18S51	12	12
D8S1179	13	13
D3S1358	15/16	15/16
FGA	21/22	21/22
TH01	8/9.3	8/9.3
VWA	17/19	17/19
D21S11	28/33.2	28/33.2
SE33	16/31.2	16

4.2. Case 2

Depending on the interpretation of the STR analysis, after extracting DNA from the selected teeth of the exhumed corpse, the derived DNA profile was compared with the profile of the alleged daughter (Table 2).

The probability of paternity value was calculated to be

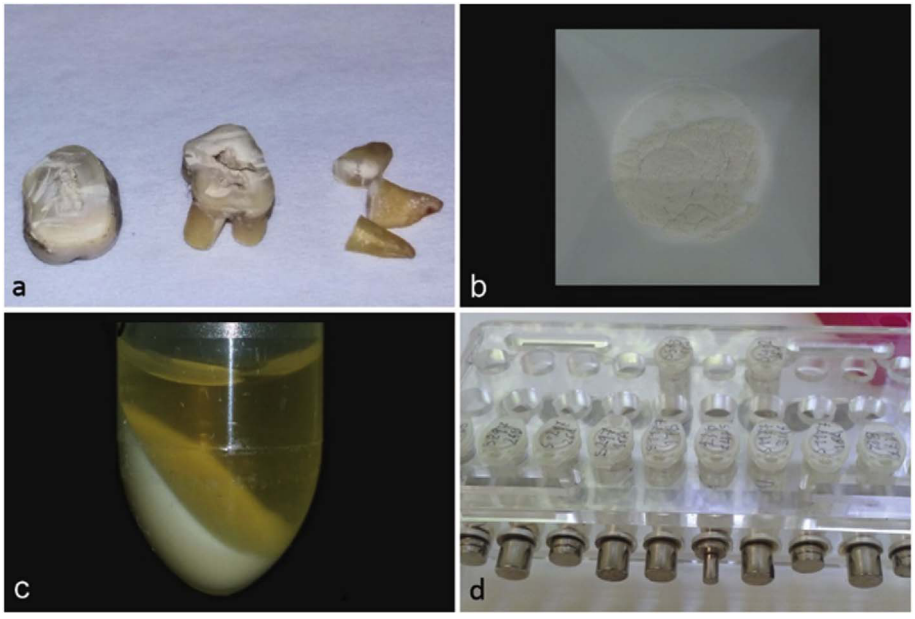

Fig. 4. (a) An illustration of horizontal sections through the teeth. (b) Teeth grinding. (c) After incubation. (d) DNA extraction by using the Crime Prep Adem-Kit.

Table 2
Short tandem repeat analysis of case 2.

Loci	The genetic profile generated from teeth of the exhumed male	The genetic profile generated from the alleged daughter
Amelogenin	XY	XX
D19S433	12/14.2[a]	12/15.2
D2S1338	23[a]	23/24
D16S539	12	12
D22S1045	14/15	15/16
D12S391	21	17/21
D10S1248	13/14	14/16
D2S441	11/14	14
D1S1656	12/15.3	15/15.3
D18S51	15.2/17	15.2/17
D8S1179	13/14	12/14
D3S1358	15/18	15/18
FGA	21/24	22/24
TH01	6/9	6
VWA	14	14/17
D21S11	29/33.2	28/29
SE33	–	17/22.2

[a] Not included in the probability of paternity calculation because the results were obtained only once for the tooth sample and verified with other sample material.

99.999897%, with Genoproof 3. Thus, paternity was proved in this relationship.

4.3. Histological findings

The general histological features of the pulp dentinal complex for both cases were characterized by an utmost destruction of the predentin layer, an irregular structure in the odontoblastic zone, a significant disfigurement observed in the odontoblast layer and severe degradation in the odontoblast nuclei (see Fig. 5g , 5h, Fig. 6d, and Fig. 7d). The residual pulp components showed an indefinite appearance (Figs. 6c and 7d). In contrast, in both cases, the cementum revealed a more organized structure than the pulp dentinal complex, recognizable

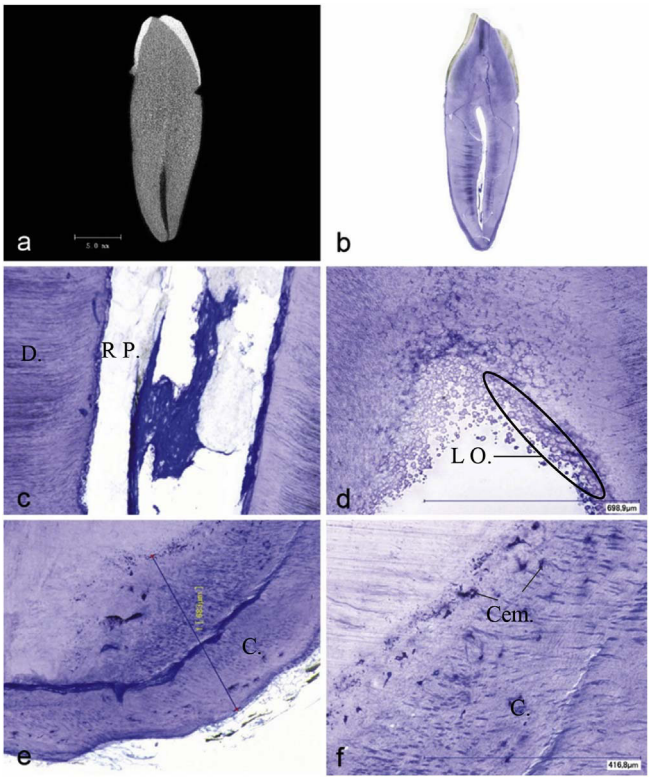

Fig. 6. (a) An image from a right mandible canine (tooth 43), from case 1, obtained by microcomputed tomographic imaging (μCT). (b) Photomicrograph shows an overview of longitudinal sections stained with toluidine blue (tooth 43). (c) Root canal. (d) Pulp horn and the front of canal. (e, f) The apex with different magnifications. D.: Dentin, R P.: Residual pulp tissue, C.: Cementum, Cem.: Cementocytes, L O.: Layer of odontoblasts.

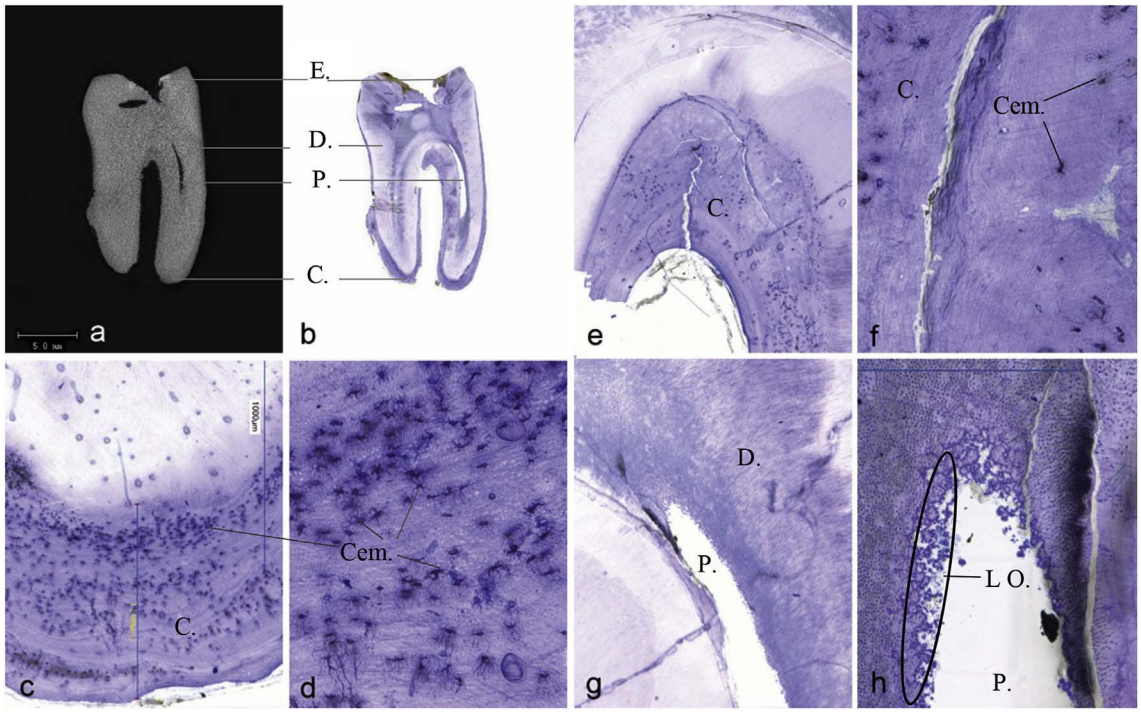

Fig. 5. (a) An image from a second right mandible molar (tooth 47), from case 1, obtained by microcomputed tomographic imaging (μCT). (b) Photomicrograph shows an overview of longitudinal sections stained with toluidine blue (tooth 47). (c, d) The apex with different magnifications. (e, f) Furcation area with different magnifications. (g, h) Pulp horn and the front of canal with different magnifications. E.: Enamel, D.: Dentin, P.: Pulp, C.: Cementum, Cem.: Cementocytes, L O.: Layer of odontoblasts.

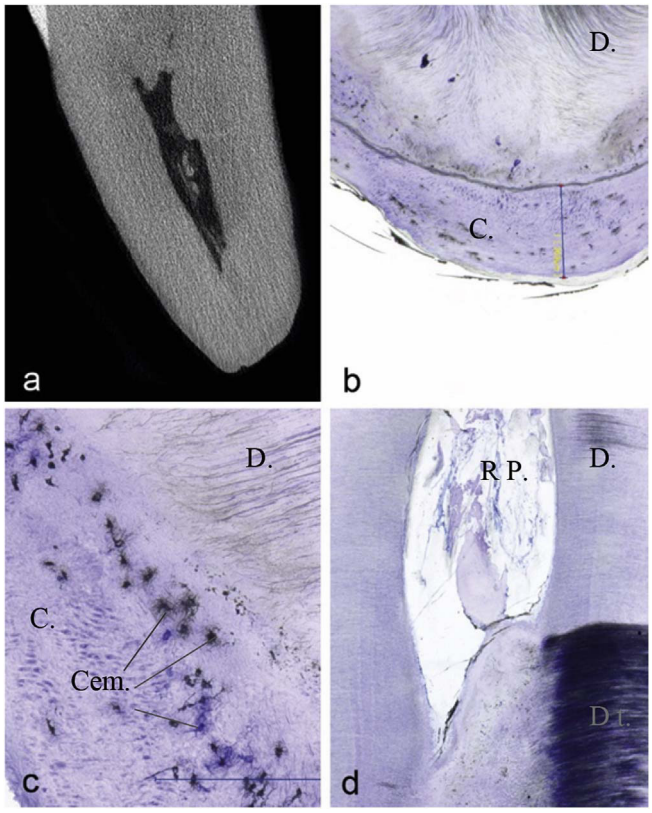

Fig. 7. (a) An image from a right second maxillary premolar (tooth 15), from case 2, obtained by microcomputed tomographic imaging (μCT). (b, c) Photomicrograph of the apex with different magnifications. (d) Root canal. D.: Dentin, R P.: Residual pulp tissue, C.: Cementum, Cem.: Cementocytes, D t.: Dead tracts.

cementocytes, distinct lacunae and canaliculi in cellular cementum (Fig. 5d) and incremental lines (see Fig. 5c, 5d, 5e, 5f, Fig. 6e, 6f, and Fig. 7b, 7c). Thus, unlike pulp and dentine, cementum was more structurally stable and less affected. These results are in agreement with Higgins et al.[20]

In addition, micro-computed tomography (μCT) imaging (Figs. 5a, 6a and 7a) and histological findings (Figs. 5c, 6e and 7b) revealed diverse thicknesses in cementum even in the same individual. Remarkably, cellular density of cementum varies significantly between different types of teeth (Figs. 5c, 6e and 7b) and between different tooth regions (Fig. 5c, 5e).

5. Discussion

Owing to their morphologically, anatomically, and histologically unique structure and their protected location,[14] teeth are more likely than bones to withstand environmental adversities over long periods. Therefore, teeth demonstrate less DNA degradation and contamination than bones.[10,21–23] However, the quantity of DNA retrieved from teeth varies significantly; not only between teeth or between comparable teeth from the same person, but also in different parts of the same tooth.[10]

Several ante- and post-mortem factors crucially affect the quality and quantity of dental DNA. Post mortem interval (PMI) and surrounding environmental conditions, such as humidity, temperature, light, and pH[11,15,20] represent the most critical post-mortem factors. While dental diseases, cellularity, and chronological age represent ante-mortem factors, which can also affect the DNA.[10,11,24]

In our cases, mainly two complicating factors have to be addressed, namely the adverse surrounding conditions (submersed within water in case 1 and buried in the ground in case 2) and the relatively long post mortem intervals (about 2 years in case 1 and about 2.8 years in case 2).

Garcia et al.[15] investigated the influence of different environments (sand buried, soil buried, fresh water, sea water, and outdoors) and different PMIs on DNA typing of teeth. They stated that water resulted in the poorest yield of DNA, whilst teeth placed outdoors offered the best results. Furthermore, Pfeiffer et al.[25] found that longer PMIs resulted in significantly less DNA yield from teeth stored in garden soil. On the other hand, Rubio et al.[26] found that the most degradation of DNA extracted from dental tissues occurs during the first two years after death. Thereafter, no significant decrease in DNA concentration was observed between 2 and 10 years.

Histologically, it is well acknowledged that the dental root is covered by a calcified tissue called cementum, which is structurally and chemically similar to bone. However, cementum does not contain innervation, blood vessels or marrow spaces. Additionally, it has a relatively smaller amount of mineral salts than bone.[12,27] As a rule, sources of genomic DNA in dental and periodontal cells include cementocytes in cementum, odontoblasts either located in pulp tissue or incorporated within reparative dentine, white blood cells located within blood vessels in accessory canals, and periodontal cells trapped in the cementum.[10] Compared to other body tissues, pulp has a relatively sparse cellular composition.[28] Furthermore, its cellularity is subjected to dramatic degradation in a relatively short post mortem interval.[10,29] Vavpotič et al.[28] found that the density of odontoblasts in pulp varies inversely with the elapsed time since death. Furthermore, the study found that at room temperature, the decrease in the relative density of odontoblasts was estimated on average to be 1.025% points per hour and after 5 days no odontoblasts remained in the pulp. Similarly, we found significant degradation in the pulp's cellular components in both cases as well as in different tooth types (Fig. 5g, 5h in case 1, tooth 47, Fig. 6c, 6d in case 1, tooth 33 and Fig. 7d in case 2, tooth15).

Compared with the cellular components of soft tissue samples (including pulp), cementum is more resistant to the aforementioned conditions. Pulp represents a poor sample quantity and quality for DNA analysis. Additionally, cementum was not negatively affected by the advanced age of the individuals in both cases (the first case was 87 years old and the second was about 73 years old). These results are consistent with other studies.[10,14,20]

On the other hand, histological findings (Figs. 5c, 6e and 7b) and micro-computed tomography (μCT) imaging (Figs. 5a, 6a and 7a), which is a non-destructive technique to morphologically visualize the external and internal root structure,[30] revealed that the thicknesses of cementum vary even in the same individual. Interestingly, the cementocyte density differs remarkably not only between different teeth types from the same person, but also in different tooth regions. This can in turn justify the noticeable intra individual variation of DNA yield from different teeth. The greatest density of cementocytes was observed in the molar's apices (Fig. 5c, 5d), while the interradicular area had a lower cementocytes density (Fig. 5e, 5f). Both apices in the canine tooth (case 1) (Fig. 6e, 6f) and the premolar tooth (case 2) (Fig. 7b, 7c) contained fewer cementocytes than in the molar's apex (Fig. 5c, 5d). Gaytmenn et al.[17] and Tilotta et al.[31] found significant inter-individual variation in the number of cells containing DNA. Similarly, we found that the density of cementocytes was variable not only between different individuals but also in the same individual in different teeth.

6. Conclusion

Cementum represents an essentially lucrative source of DNA to maximize DNA yield from dental samples, especially in challenging conditions. Moreover, the anatomical and histological intra- and inter-individual variations of the cementum structure in terms of thickness and density of cellular inclusion have to be taken into consideration when interpreting the results.

Ethical approval

All procedures performed in this study were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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6 Publication 3: Identification of scattered skeletal remains: Combined dental and DNA-based identification

6.1 Publication

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H. Mansour · O. Krebs · J. P. Sperhake · A. Fuhrmann · K. Püschel
Institute of Legal Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Identification of scattered skeletal remains

Combined dental and DNA-based identification

Introduction

In the course of history, dental identification has been commonly used since Roman times [1]. Compared to other identification means, dental identification is relatively less time and money consuming [2] and characterized by its simplicity [3], high efficiency and procedural rapidity [4]; however, several challenges and obstacles can be encountered during dental identification, such as the possibility of charting mistakes in antemortem records [5, 6], the low quality of antemortem records, for instance, the lack of dental X-rays, unintelligible abbreviations on dental charts and confined documentation on the areas of treatment disregarding the material used, treated surfaces and other existing conditions [2], identification of children, adolescents or edentulous individuals [4, 7], difficulty to conduct quantitative interpretations presented to the court [8], adverse postmortem conditions, such as fragmented or commingled corpses in situations after explosions or airplane crashes [9], considerable number of missing teeth postmortem, severe antemortem bimaxillary trauma [4] or post-mortem animal scavenging negatively influencing dental evidence and scattered skeletal fragments resulting in laborious matching between different components. Therefore, a combination of DNA analysis and conventional dental identification is required to confirm the identity under special conditions, such as identifying disarticulated scattered human skeletal elements and to exclude false conclusions.

Due to the fact that DNA fingerprinting can be generated from any nucleated biological sample [10, 11], DNA methods can effectively be used to re-associate scattered or fragmented elements while other techniques cannot; however, DNA degradation still represents the main limitation. DNA degradation causing a downgrading in quality and quantity of DNA under adverse post-mortem conditions makes bones and teeth samples almost the best biological material available for DNA profiling [12] owing to the protection afforded by their mineral matrix [13, 14].

Case report

In December 2016 a corpse of an unidentified man was found in a small forest

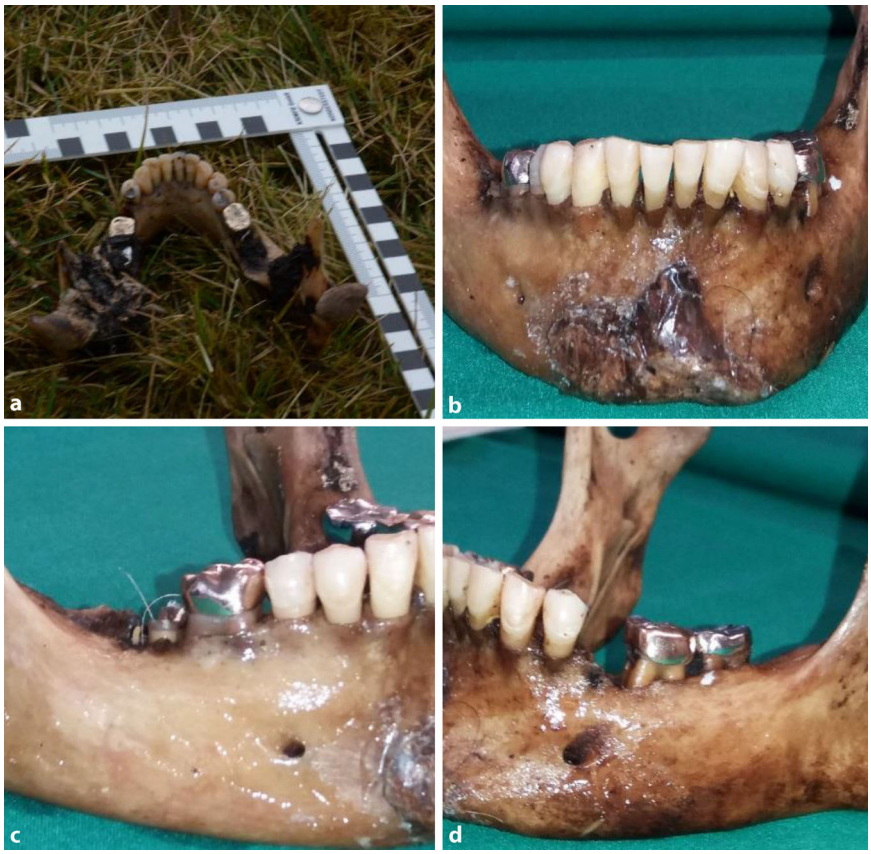

Fig. 1 ▲ Retrieved mandible from different views. **a** The lower jaw at the location of discovery. **b** Frontal view. **c** Lateral right view. **d** Lateral left view

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Fig. 2 ◀ Scattered human remains at the scene of discovery

Fig. 3 ▲ The reconstruction of the skeletal remains for anthropological analysis

in the north of Germany. A mandible (**Fig. 1**) from human remains was incidentally discovered by a dog accompanying a passer-by. Near to location of discovery incomplete scattered human bones (without a skull), remains of shoes and clothing but no ID card or other personal belongings were found by the local German Criminal Police in Cuxhaven during the crime scene search (**Fig. 2**). The preliminary police investigations were oriented to the assumption that the missing person was a male who had been missing since August 2016 from his dormitory. The presumptive missing man was 59 years old. All human remains were sent to the department of Legal Medicine for purpose of identification and routine autopsy procedures (**Fig. 3**).

Material and methods

The anthropological parameters of the pelvis, the long bones and the lower jaw were compatible with a male person. To establish the identity, dental identification and DNA profiling were undertaken.

Dental identification. Forensic odontological examination by visually detecting and describing each tooth of the mandible, taking photographs, and applying radiological methods of panoramic radiography has been utilized to match the mandible to the data of the presumed missing person. Antemortem dental records comprising 10 intraoral x-rays (5 for lower teeth and 5 for upper teeth) were retrieved during the criminal police investigations to be compared with the post-mortem panoramic radiograph (**Fig. 4**).

DNA testing. Prior to DNA extraction, to eliminate environmental contaminants and exogenous DNA [15], the external surface of bone samples (from femur) were mechanically removed. The teeth (36, 45 and 46) were extracted and air-dried. Being preserved in their alveolar sockets, dental samples did not require treatment with bleach or root surface removal to avoid any negative impact of cementum on DNA [16]. Thereafter, the teeth were horizontally sectioned and the crowns were removed. Only the roots were used as a source of DNA, since the roots of teeth yield better DNA than the crowns [9, 17, 18].

Bone and teeth samples were ground into powder and 100 mg of each sample was used for extraction according to the manufacturer's instructions of the Crime Prep Adem-Kit (Ademtech SA, Pessac, France) and 50 µl of the final eluate obtained was used for analysis. For DNA quantification for both teeth and bone samples, real-time PCR (PowerQuant™ System, Promega, Madison WI, USA) using a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) was applied. After multiplex PCR amplification, capillary electrophoresis was performed using an ABI 3130 Genetic Analyser (Applied Biosystems). For short-tandem repeat (STR) DNA analysis multiplex commercial kits (Powerplex® ESI 17, Powerplex® ESX 17, Promega) were used. The ABI GeneMapper™ ID v3.2. software was utilized for data analysis and visualization.

A set of 17 markers (16 autosomal markers as well as the amelogenin marker) were used for matching DNA profiles generated from a toothbrush and beard hairs of the potential missing person. The latter profiles were generated by the police laboratory and were sent to be compared with teeth and bones DNA profiles. Statistefix software version 2.3 was applied for statistical calculations.

Results

Comparative dental identification

As shown in **Table 1**, positive identification could be confirmed as a result of sufficient matching similarities and no unexplainable discrepancies discerned between antemortem and postmortem dental records [19]. Further features could also be included and the high individual discrimination potential of dental details is represented in the morphological characteristics of the dental treatment of tooth 37. This revealed uniqueness in position, design, shape, extension and dimensions.

According to Keiser-Nielsen [20] regardless of the frequency of occurrence in several age groups, race, and sex, the combinations representing the simultaneous occurrence of 7 intact teeth, 3 crowns, 2 missing teeth, 1 filling, 1 root filling and 1 post within one and the same person in the mandible during life can be obtained by multiplying the possible combinations of each dental feature C (n, k), where n represents the maximum and k represents the variable:

$$\begin{aligned} &C\ (16,7) \times C\ (9,3) \times C\ (6,2) \times \\ &C\ (4,1) \times C\ (3,1) \times C\ (2,1) \\ &= 11{,}440 \times 84 \times 15 \times 4 \times 3 \times 2 \\ &= 345{,}945{,}600 \end{aligned}$$

This value of the random match probability in an unidentified population, without consideration of dental status related to certain populations, generations and age can be enormously increased if tooth surfaces, materials of the filling, materials of the crown and a root of a tooth presenting with a post are included.

This leads to the conclusion that the lower jaw matched to the missing person; however, the assignment of both the jaw and the bones to the missing person could only be verified by DNA analyses which is considered the best tool to match different body parts.

DNA findings

Reference samples of the missing person including a toothbrush and beard hairs were obtained from his bathroom. The genetic profiles of the aforementioned samples generated in the police laboratory could be directly compared with the DNA profiles of the bones and teeth. The genotype comparison revealed a complete match of 17 markers (including the amelogenin sex-determining marker) out of 17 between the beard hairs and the bone samples as shown in **Table 2**. The toothbrush had a mixed sample of at least two persons, one of which was matched

Abstract · Zusammenfassung

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Identification of scattered skeletal remains. Combined dental and DNA-based identification

Abstract

The resistant nature of bones and teeth to environmental insults highlights their importance in identification investigations. The DNA preserved in bones and teeth can play a crucial complementary role to comparative dental identification in some forensic scenarios. We report on a case where an isolated mandible and scattered skeletal remains without a skull, were found in a small forest after a post-mortem interval of approximately 4 months. This case illustrates a situation in which two reliable identification modalities, dental identification and DNA profiling, were necessary to reach the confidence level of personal identification and to exclude any false conclusions. Dental identification was established by sufficient concordant dental features in the lower jaw. A comparison of DNA profiles generated from teeth and bone samples of the human remains with DNA profiles generated from a toothbrush and beard hairs as reference samples showed matching profiles. This emphasizes the effectiveness of combining DNA and dental identification for assigning scattered skeletal fragments and identifying human remains. To economize efforts dental comparison, if available, should be performed as a first step prior to DNA genotyping.

Keywords

Short tandem repeats · DNA profiling · Odontological identification · Forensic dentistry · Forensic anthropology

Identifizierung eines verstreuten Skeletts. Kombinierte dentale und DNA-basierte Identifikation

Zusammenfassung

Die Widerstandsfähigkeit von Knochen und Zähnen gegenüber zersetzenden Umwelteinflüssen unterstreicht ihre Bedeutung in Identifizierungsuntersuchungen. Die in Knochen und Zähnen erhaltene DNA kann in manchen forensischen Situationen eine wesentliche Ergänzung zur vergleichenden dentalen Identifizierung darstellen. Im hier vorgestellten Fall wurden ungefähr 4 Monate post mortem ein einzelner Kiefer und verstreute Skelettreste ohne Schädel in einem kleinen Waldstück gefunden. Mit der dentalen Identifizierung und dem DNA-Profiling waren zwei verlässliche Identifizierungsverfahren erforderlich, um die Person mit hinreichender Sicherheit zu identifizieren und falsche Rückschlüsse auszuschließen. Die dentale Identifizierung wurde durch die ausreichende Übereinstimmung dentaler Merkmale des Unterkiefers erzielt. DNA-Profile aus Zahn- und Knochenproben der menschlichen Überreste stimmten mit den DNA-Profilen einer Zahnbürste und von Barthaaren überein. Der Fall verdeutlicht, wie effektiv die Kombination der DNA-basierten und dentalen Identifizierung bei der Zuordnung verstreuter Skelettfragmente und bei der Identifizierung menschlicher Überreste ist. Um den Aufwand zu reduzieren, sollte der dentale Vergleich, soweit durchführbar, in einem ersten Schritt vor der DNA-Genotypisierung erfolgen.

Schlüsselwörter

Mikrosatelliten · DNA-Profiling · Odontologische Identifizierung · Forensische Zahnmedizin · Forensische Anthropologie

Table 1 Comparative dental examination of the antemortem and post-mortem dental charts

Antemortem dental chart in July 2012		Code DVI system	Post-mortem dental chart in December 2016		Code DVI system	Comparison	Concordant dental features
31	Intact	nad	31	Intact	nad	Similarity	1
32	Intact	nad	32	Intact	nad	Similarity	1
33	Intact	nad	33	Intact	nad	Similarity	1
34	Amalgam filling	mcf	34	Amalgam filling	mcf	Similarity	1
35	Intact	nad	35	Missing post-mortem	mpm	Explainable discrepancy	–
36	Crown	mtc	36	Crown	mtc	Similarity	1
37	Root filling, metallic post, crown	rfx, pox, mtc	37	Root filling, metallic post, crown	rfx, pox, mtc	Similarity	3
38	Extracted[a]	mam	38	Missing ante-mortem	mam	Similarity	1
41	Intact	nad	41	Intact	nad	Similarity	1
42	Intact	nad	42	Intact	nad	Similarity	1
43	Intact	nad	43	Intact	nad	Similarity	1
44	Intact	nad	44	Intact	nad	Similarity	1
45	No information	non	45	Amalgam filling	mcf	–	–
46	Crown	mtc	46	Crown	mtc	Similarity	1
47	No information (according to x-ray no root filling)	non	47	Root filling, retained root	rfx, rov	Explainable discrepancy	–
48	Extracted[a]	mam	48	Missing ante-mortem	mam	Similarity	1
Concordant dental features in the lower jaw							15

DVI Disaster victim identification, ***nad*** no abnormality detected, ***mcf*** metal coloured filling, ***mtc*** metal crown, ***rfx*** root filling, ***pox*** post; ***mam*** missing antemortem, ***non*** no information, ***mpm*** missing post-mortem, ***rov*** retained root

[a]According to the dentist's handwritten notes in antemortem records

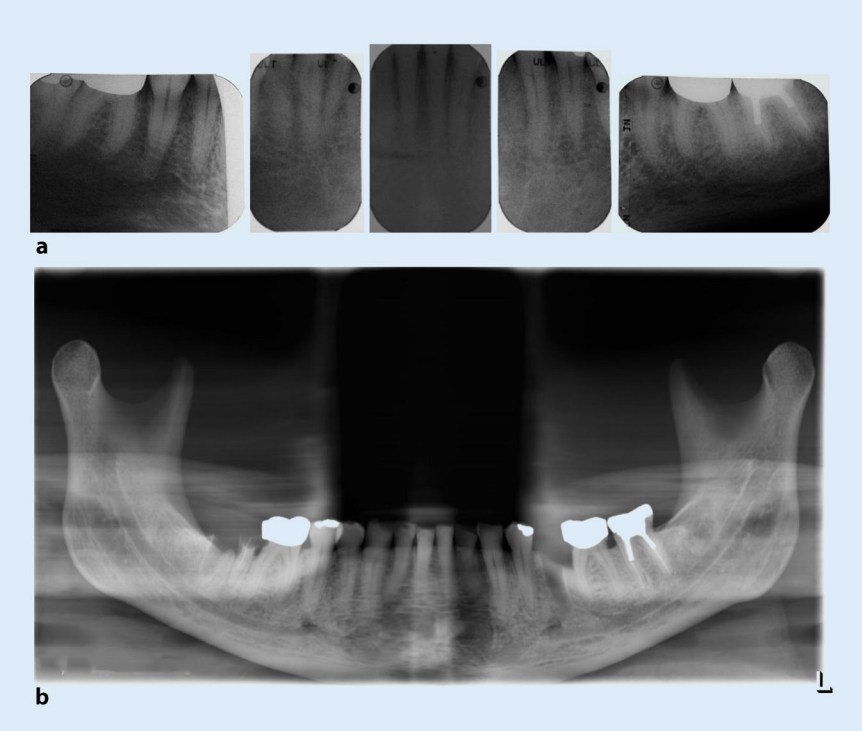

Fig. 4 ▲ **a** Antemortem intraoral x-rays from July 2012. **b** Post-mortem panoramic radiograph performed in December 2016

to the bone sample as well. Compared with the DNA profile obtained from dental samples 14 markers out of 17 were matched, while one allele in locus SE33 could not be detected and for FGA and D21S11 one additional allele could be detected.

According to the guidelines of German Committee for Genetic Diagnostics [21] the DNA results conclusively confirmed that the recovered mandible and the scattered human skeleton bones originated from the same person (◘ **Table 2**).

Discussion

To ensure these bones were from a single person, it was verified if every bone of the human skeleton was represented only once. Furthermore, several correspondences in the biological profiles could be observed in the recovered skeletal remains. The harmony of left and right sets of remains (◘ **Fig. 3**), the consistency of the observed degenerative age-related morphological changes, the consensus of morphological sex parameters

Table 2 Short tandem repeat genotyping and statistical analysis using Statistefix software

Loci	Missing person's toothbrush	Missing person's beard hairs	Unknown corpse bone samples	Unknown corpse teeth samples
Amelogenin	XY	XY	XY	XY
D19S433	13/14/*15*	13/14	13/14	13/14
D2S1338	18/*23*/*24*/25	18/25	18/25	18/25
D16S539	*9*/11/12	11/12	11/12	11/12
D22S1045	15/16	15/16	15/16	15/16
D12S391	*18*/19/21	19/21	19/21	19/21
D10S1248	13/14	13/14	13/14	13/14
D2S441	14	10/14	10/14	10/14
D1S1656	12/*13*/14/*16*	12/14	12/14	12/14
D18S51	11/*13*/*15*/*16*	11/15	11/15	11/15
D8S1179	*10*/13/14	13/14	13/14	13/14
D3S1358	15/16	15/16	15/16	15/16
FGA	*20*/*21*/21.2/22.2	21.2/22.2	21.2/22.2	(21/21.2)/22.2
TH01	6/7/9/9.3	6/9.3	6/9.3	6/9.3
VWA	15/16/*17*/*19*	15/16	15/16	15/16
D21S11	*27*/28/*29*/30	28/30	28/30	(28/29)/30
SE33	*19*/29.2	17/29.2	17/29.2	17/–
Random match probability			6.893×10^{-24}	
Likelihood ratios (1)	The probability that beard hairs (reference sample) and bone samples are from the same person (hypothesis H1)/the probability that someone other than the provider of the beard hairs (reference sample) is the unknown dead person (H2)			1.43204×10^{23}
Likelihood ratios (2)	The probability that beard hairs and teeth samples (H1) are from the same person/the probability that someone other than the provider of the beard hairs is the unknown dead person (H2)			3.84377×10^{16}
Likelihood ratios (3)	The probability that one of the persons contributing to the mixed sample of the toothbrush (reference sample) and the one providing the bone sample is the same person (H1)/the probability that someone other than the persons contributing to the mixed sample of the toothbrush (reference sample) are the unknown dead person (H2)			7.28983×10^{11}

in the bones and the consistent length of long bones indicated that the bones likely belonged to the same individual [22, 23].

Despite the missing maxilla, it was certainly prudent to begin with dental identification since antemortem records were available. Determining a certain number of concordant features for dental identification is a controversial issue. Pretty and Sweet [24] stated that positive dental identification can be established without fulfilling a minimum number of concordant dental features. Whereas Keiser-Nielsen [20] mentioned that 12 concordant features, even being uncharacteristic, are the minimum requirement to conclude a proof of identity. Moreover, Adams [25] emphasized the high individuality of the combinations of dental features. The distinct prevalence of dental caries or injuries in different teeth should be considered. Modesti et al. [26] utilized the statistical information about the dental status of the Brazilian population in calculating the frequency of forensic dental features. In our case 15 consistent dental features were identified and no unexplainable discrepancies could be discerned. This confirmed the identity of the lower jaw.

Despite the positive comparative dental identification, other skeletal elements, which might belong to another person, led to keeping the case still open to question. Therefore, DNA genotyping was required. Personal effects such as a toothbrush [27–29] or hair comb [29] are commonly considered a potential source for recovering reference samples. Birngruber et al. [30] found in their retrospective study that nearly 66% of antemortem DNA samples included personal belongings (e. g. hairbrush, toothbrush and razor) for comparative DNA identification; however, using personal belongings as sources of antemortem DNA samples is currently under discussion [31]. Schwark et al. [32] concluded that personal hygiene items should be restricted in use for DNA identification to cases of no known blood relatives as they can complicate DNA investigations [32] and are a potential risk for a cross-contamination or false profiles [33–35]. In the presented case no known biological relatives were available. Beard hairs found in the bath of the dormitory where the missing person lived, yielded an uncontaminated full DNA profile. A complete match between the missing person's beard hairs and the bone samples from the corpse could be confirmed.

Regarding the DNA profile generated from dental samples, additional alleles were found in the FGA and D21S11 loci. Those alleles are most likely artifacts due to degradation and do not represent a triallelic patter [36–38] as the markers are not present in the non-degraded samples. On the other hand, the DNA profile generated from the missing person's toothbrush showed a mixture of DNA of most probably two individuals (the potential missing person as well as another individual). This leads to the possibility of a contaminated toothbrush (i. e. it has also been used by an individual other than the missing person). Although the comparison with toothbrush and teeth samples revealed a lower probability because of the contamination and the additional alleles, the identity could be confirmed. Thus, re-association of the scattered skeleton elements and the mandible with the potential missing person could be legally validated.

Conclusion

This case report emphasises the value of teeth not only as a powerful tool in the conventional comparative dental identification but also in matching different scattered human remains. To reach a high confidence of correct identification of scattered human skeletal remains, a combination of DNA and dental identification is required.

Corresponding address

H. Mansour, DDS
Institute of Legal Medicine, University Medical Center Hamburg-Eppendorf
Butenfeld 34, 22529 Hamburg, Germany
Hussam.Mansour@uke.de

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7 Publication 4: New aspects of dental implants and DNA technology in human identification

7.1 Publication

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Case Report

New aspects of dental implants and DNA technology in human identification

Hussam Mansour [a,*], Jan Peter Sperhake [a], Bram Bekaert [b,c], Oliver Krebs [a], Peter Friedrich [d], Andreas Fuhrmann [a], Klaus Püschel [a]

[a] University Medical Center Hamburg-Eppendorf, Institute of Legal Medicine, Butenfeld 34, 22529 Hamburg, Germany
[b] KU Leuven - University of Leuven, Department of Imaging & Pathology, Campus St-Rafaël, Kapucijnenvoer 33, Leuven, Belgium
[c] KU-Leuven - University of Leuven, University Hospitals Leuven, Department of Forensic Medicine, Laboratory of Forensic Genetics and Molecular Archeology, Campus St-Rafaël, Kapucijnenvoer 33, Leuven, Belgium
[d] State Criminal Investigation Department of the City of Hamburg (LKA 41), Bruno-Georges-Platz 1, 22297 Hamburg, Germany

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ABSTRACT

Missing, ineligible or delayed reference data to establish conventional dental or DNA identification are common scenarios in forensic practice. Therefore, it is worthwhile to explore new avenues that facilitate human identification. Due to the recent remarkable evolution in the prosthetic dental restorations based on dental implants and the emergence of novel DNA technologies utilized to infer the biological profile, the identification process has become easier than ever before. We report on a characteristic case, which highlights the particular importance of dental implants and DNA approaches in the prospective investigations for human identification. The aim of this publication is to focus on the possibility of identifying the batch numbers, even if they were not engraved in dental implants, making antemortem dental records of dental implants more easily accessible to establish a comparative dental identification. In addition, the reported case presents the supplementary data yielded through estimating the epigenetic age using DNA methylation as well as the biogeographical origin using Y-Haplotype and mitochondrial DNA analyses. Our results demonstrate that expanded oral implant investigations that also include implants extraction and comprehensive microscopic measurements can lead to identifying their batch numbers despite the numerous number of implants systems manufactured and distributed worldwide. Data saved by dental implant manufacturers can be very supportive and represent additional reference data for dental identification, when antemortem dental records are still missing. Furthermore, DNA methylation and mitochondrial DNA analyses can support the progress of investigation.

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1. Introduction

The remarkable evolution in implant dentistry and DNA technology during the last three decades has had a significant impact on forensic odontology and human identification. The increased prevalence of implant prostheses has become apparent in many clinics worldwide. For instance, in Switzerland, the rate of restorations based on dental implants rose about three times from 2002 to 2012 [1]. Similarly, the German Oral Health Study (DMS) revealed that in Germany, from 2005 to 2014, the number of patients rehabilitated with dental implants in age cohorts of adults aged 35–44 increased more than double, while in senior citizens aged 65–74 it nearly tripled [2,3]. Furthermore, due to the tremendous demand for dental implants, the implant industry has evolved into a global business [4]. This in turn will lead to an increase in the frequency of encountered dental implants as high characteristic findings in both antemortem and postmortem dental data [5].

The fundamental requirement of establishing a comparative dental identification is the availability of antemortem dental records. This underlines the critical need for identifying the treating dentist during the police investigation. In some conditions, due to the increasing mobility of patients [6], the task of identifying the treating dentist and recovering the required antemortem dental data is challenging [7]. Therefore, an implant's identifying aspects, such as material, design, type, implant dimensions and the manufacturing company's name can all help ultimately in identifying the batch number, which is the key step in the right direction of the identification process, even if the batch numbers are not engraved in the dental implants. Several attempts using radiographical and morphologic approaches to identify dental implants for human identification have been reported in the literature [5,6,8–12]. However, identifying the batch number of dental implants through elaborately microscopic measurements and the comparison with catalogues of implants' manufacturing companies has not been reported in the literature yet. Data saved in manufactur-

* Corresponding author.
Email addresses: Hussam.Mansour@uke.de (H. Mansour); sperhake@uke.de (J.P. Sperhake); bram.bekaert@uzleuven.be (B. Bekaert); oliver.krebs@uke.de (O. Krebs); peter.friedrich@polizei.hamburg.de (P. Friedrich); a.fuhrmann@uke.de (A. Fuhrmann); pueschel@uke.de (K. Püschel)

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ing companies' databank as "reference data" can offer invaluable information for tracking the treating dentist.

Parallel to dental investigations, DNA represents the ideal reliable forensic evidence for identification. Today, if a DNA donor is still without any match in the national genetic databank of missing persons, other novel DNA-based approaches would be a powerful assist tool for supporting the progress of the investigation [13]. Besides conventional autosomal short tandem repeat (STR) genotyping, age estimation based on DNA methylation and biogeographical ancestry assessment based on the sequence of the mitochondrial DNA (mtDNA) along with Y-Haplotype can create a pivotal dimension in the police investigation. To date, DNA methylation (DNAm), one of the main age-associated changes on the molecular level, has become the focus of interest as a promising biomarker for forensic age estimation [14,15]. DNAm demonstrated a relatively acceptable accuracy with a mean absolute deviation from chronological age in the range of 3–5 years [15–18]. Since mtDNA and human Y chromosome have special features, which are not found in autosomes, the origin of maternal lineage using mtDNA and the paternal lineage using Y-Haplotype can be reconstructed [19,20]. Supplementary information about possible sex-biased genetic structure can be inferred, since both genders are descended from different demographic histories and both Y-Haplotype and mtDNA are characterised by a high polymorphism in different populations [13,20].

The case reported in this paper aims to provide a new strategy utilizing each possible forensic approach in identification, when the reference data are unavailable at least in the early stages of investigation process. Therefore, we focused on: (i) identifying the batch numbers of dental implants making antemortem dental records of dental implants more easily accessible to establish a comparative dental identification, (ii) reconstructing the biological profile using novel DNA technologies.

2. Case history

In March 2016, a corpse of an unknown man was found about 3 meters from the east bank of the river Elbe, one of the main rivers in Germany. The man was presumably moved by the current towards the beach. The corpse was sent to the department of Legal Medicine for all routine forensic procedures including identification. Due to the advanced stage of putrefaction, facial recognition and visual identification could not be established. Clothing, personal effects, and belongings were documented. No ID card or other personal information were found.

After being discovered, the man was still unidentified for longer than one year despite thorough parallel dental and DNA investigations being done. An autosomal STR profile of the missing person had been generated by a police laboratory (in a different federal state) through analysing samples retrieved from his tooth brush and other personal belongings. Finally, he was conclusively identified by DNA genotyping (in December 2017) and the dental identification confirmed a positive identification after recovering the dental records.

3. Materials and methods

3.1. Dental implants investigation

For dental identification, routine procedures, including removing both jaws, visual inspection, photographing, postmortem radiography and documentation, were carried out. At first, the antemortem dental records were missing. However, the dental finding indicated the presence of dental implants (Fig. 1). Therefore, thorough investigations of

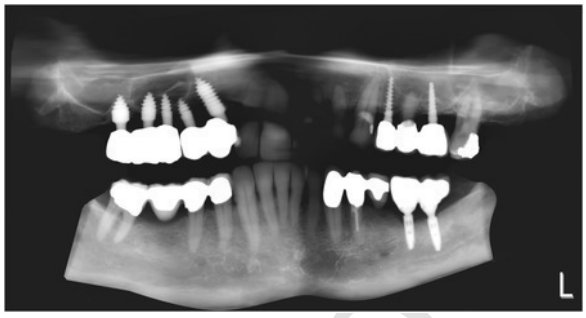

Fig. 1. Post-mortem panoramic radiograph performed in April 2016.

dental implants including implants extraction, clinical and radiological along with microscopic examination, microscopic metric analyses — one of which concerns length, diameter and height for implant fixture the other, diameter, angulation and gingival height for prosthetic abutments (Fig. 2, Table 1) — implantolgist consultation and searching in implants catalogues of different possible companies, were performed. Our measurements were rounded up to the closer standard measurements (Table 1). To identify the batch number of each implant, different inferred designations and measurements were matched with their analogues in the relevant catalogues of different possible manufactures. Lastly, our results were verified and we were provided with probable periods of producing and marketing (probable periods of ordering the relevant implants) by contacting the potential manufactures (Table 1).

3.2. DNA techniques

3.2.1. STR analysis

According to standard procedures, genomic DNA was extracted from blood samples of the decomposed corpse and quantified by real-time PCR (PowerQuant™ System/Promega) with RT PCR (7500 RT PCR System/ABI). DNA concentration was 2.74 ng/µl. After PCR amplifications, STR analysis using multiplex commercial kits (Powerplex® ESI 17, Powerplex® ESX 17/Promega) was performed. For capillary electrophoresis, a genetic analyser (ABI 3130) was used to separate PCR products.

3.2.2. DNA methylation

The epigenetic age has been estimated using DNA methylation levels at 4 specific CpG sites located in 4 age-associated markers APSA, PDE4C, EDARADD and ELOVL2 according to Bekaert et al. [15] (Table 2). After DNA extraction and quantification, maximum input (24 µl) was used for bisulfite conversion using the Methylamp DNA Modification Kit (#P-1001-2, Epigentek). Due to DNA degradation and low DNA concentration (less than 500 ng in 24 µl), the final elution of converted DNA was 10 µl. PCR conditions and the amplification programs are previously outlined in Ref. [15].

3.2.3. mtDNA analysis

To amplify the hypervariable regions HV1 and HV2 of mtDNA, the following sequencing primers were used: F15971/R16410 (HV1) and F15 / R389 (HV2). Samples were sequenced on an ABI3130XL genetic analyser. Sequences were interpreted using Sequencing Analysis Software 5.2.

3.2.4. YSTR analysis

For Y-STR typing, 22 Y-STR loci (DYS 576, DYS389I/II, DYS448, DYS 19, DYS391, DYS481, DYS391, DYS549, DYS533, DYS438, DYS437, DYS570, DYS635, DYS390, DYS439, DYS392,

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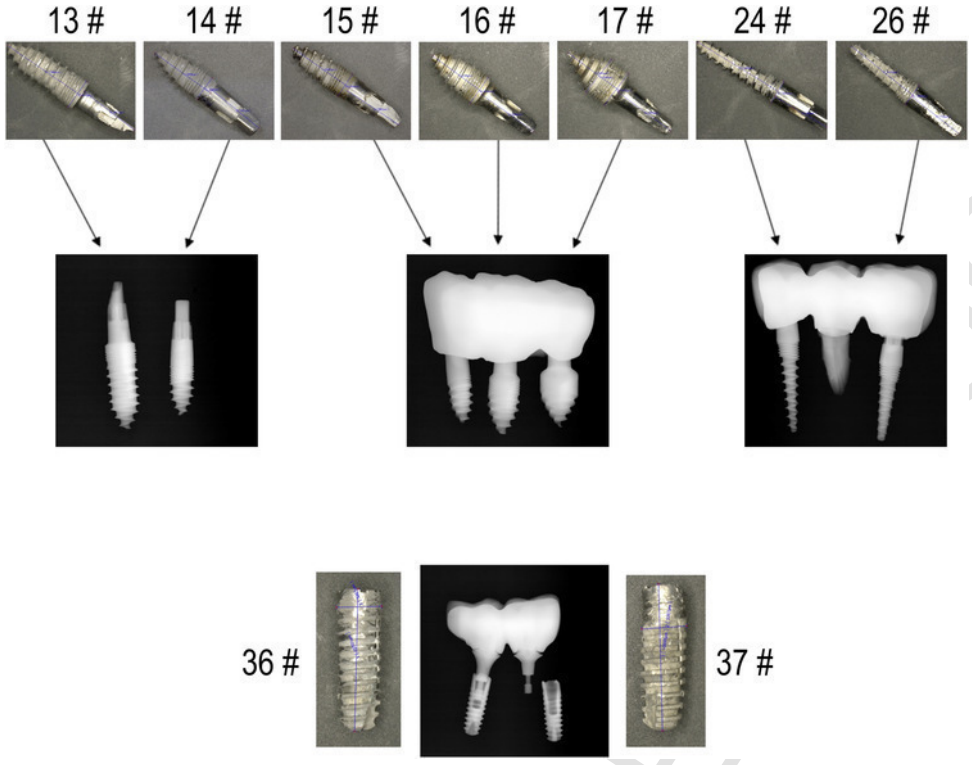

Fig. 2. Post-mortem radiological examination and microscopic measurements of the extracted dental implants.

Table 1
Microscopic measurements referred to as "Measured" and the match with the probable closer analogue in the potential manufactures referred to as "Standard" using manufacturers' catalogues [21,22].

Tooth	Length		Diameter		Height		Manufacturer	Designation	Art.-Nr.	Probable period *[a]
	Measured	Standard	Measured	Standard	Measured	Standard				
13#	11.7	12	4.1	4.5	8.4	8.6	*[b] Champions	Vierkant New Art	1060	Since 2010 **(2010–2016)**
14#	9	8	3.3	3.5	7.8	8.1	Champions	Vierkant New Art	1000	Since 2010 **(2010–2016)**
15#	9.1	8	3.5	3.5	8	8.1	Champions	Vierkant New Art	1000	Since 2010 **(2010–2016)**
16#	8.2	8	4.2	4.5	8.4	8.6	Champions	Vierkant New Art	1010	Since 2010 **(2010–2016)**
17#	8.2	8	5.5	5.5	8.5	9.2	Champions	Vierkant New Art	1012	Since 2010 **(2010–2016)**
24#	12.2	12	3.2	3.5	7.8	8.1	Champions	Vierkant "classic"	1055	Since 2006 **(2006–2016)**
26#	10.2	10	3.1	3.5	7.7	8.1	Champions	Vierkant "classic"	1025	Since 2006 **(2006–2016)**

Implant-fixture					Prosthetic abutments				
Tooth	36#		37#		Tooth	36#		37#	
	Measured	Standard	Measured	Standard		Measured	Standard	Measured	Standard
Length	10.5	11	10.9	11	Diameter	5.2	4.5	5.7	4.5
Diameter	3.4	3.5	3.4	3.5	Gingival heights	2.9	3	1.5	1.5
Manufacturer	*[c] Ankylos		Ankylos		Manufacturer	Ankylos		Ankylos	
Designation	*A11*	*A11*	*A11*	*A11*	Angulations	15	15	15	15
Product no.	3101 0210				Product no.	3102 1660		3102 1650	
Probable period	2005–2008				Designation	Balance posterior abutment			

Art.-Nr.: the batch number designated as "Art.-Nr." by Champions manufacturer.
Product no.: the batch number designated as "Product no." by ANKYLOS manufacturer.

[a] Probable period: the probable period of producing and distributing the respective article of dental implant till the corpse was found (in 2016).

[b] (Champions®, Flonheim, Germany).

[c] (ANKYLOS®, Mannheim/Hanau, Germany).

DYS643, DYS393, DYS458, DYS385, DYS456, YGATAH4) were amplified using PowerPlex® Y 23 System, Promega with interpretation standards laid down in the recommendations of the Stain Commission of the German Society of Legal Medicine [23]. The biogeo-

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Table 2
CpGs (cytosine-phosphate-guanine dinucleotides) selected for age-associated methylation analysis [15] and their DNA methylation percentages.

Gene ID	CpG number	CpG ID	Position	DNA methylation (%)
ELOVL2	CpG6	–	chr6:11,044,640	78%
EDARADD	CpG1	cg09809672	chr1:236,557,683	29%
PDE4C	CpG1	–	chr19:18,343,888	36%
ASPA	CpG1	cg02228185	chr17: 3,379,567	47%

graphical assessment was done using the Y-HRD database (www.yhrd.org, Release 59 2018/Nov/01 [24]).

4. Results

4.1. Dental implants

Before identification, several attempts were made to ascertain the treating dentist by contacting the manufactures, which would subsequently lead to the recovery of the dental records for comparative identification. Although the obtained information was not complete, it provided very important clues. For instance, among all dentists/dental practices in Germany that ordered the article "Champions, Vierkant New Art" of batch number 1012 in tooth 17, no dentist in the city where the corpse was found ordered this article. However, it was ordered in the city where the later-identified treating dentist/dental practice is.

In addition, the given information demonstrated the probable period of ordering each implant article of the relevant batch number according to the mode of production, supply and style of distribution (Table 1). For example, the article of batch number 31010210 in tooth 36# and 37# (Table 1), characterized by a unique notch on the coronal edge of the implant fixture (Fig. 3), was produced and distributed only between 2005 and 2008 in the whole of Germany. Afterwards, it was updated with another new design without this notch.

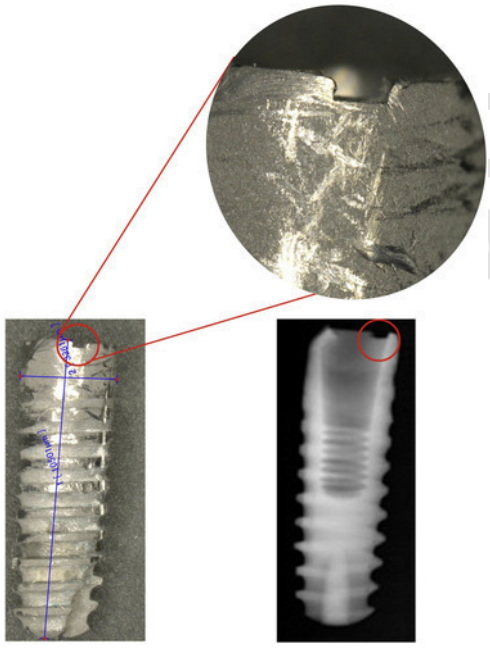

Fig. 3. Illustrates the distinct notch differentiating the used design from the next generation's design.

In the meanwhile, the man was identified by DNA genotyping. Thus, the treating dentist was identified.

Nevertheless, we requested the treating dentist for providing the dental records and the ordered batch numbers to estimate the accuracy of our implants investigations. The recovered records revealed two explainable discrepancies in implant investigations concerning the batch numbers of two implants (24# and 26#) as a result of an imprecise measuring of the relevant implant diameter and consequently an improper classification with their right analogues in the manufacture's catalogue. Apart from these two discrepancies, 9 batch numbers out of 11 could be identified (Table 3).

The accuracy of our dental investigations could be evaluated in 6 criteria as they relate to each implant fixture: length, diameter, manufacturer, designation, batch number and the probable treatment period, as well as, 6 criteria concerning prosthetic abutments of 36# and 37#: angulation, gingival height, manufacturer, designation, batch number and the probable treatment period (Table 3). In total, 66 statements could be evaluated. Positive results were obtained for all statements in all implant-fixtures with the exception of identifying the batch numbers of two implants (24# and 26#). Interestingly, positive results were obtained in 100% of all statements concerning prosthetic abutments of 36# and 37#. Overall, 62 statements of our investigation out of 66 statements of the treating dentist were matched (Table 3). Thus, the evaluation of implant investigations proved the possibility to identify the batch numbers.

Definitely, the conventional comparison between post and antemortem dental features confirmed the conclusive identification (Figs. 1 and 4).

4.2. DNA approaches

4.2.1. Age assessment

The age was calculated through substituting the percentage values of DNA methylation of CpG6 ELOVL2, CpG1 EDARADD, CpG1 PDE4C and CpG1 ASPA (Fig. S1, Table 2) in the formula of the regression model built by Smeers et al. [14].

Based on the statistical model of the weighted least squares (WLS) regression [14], the calculated age was 72.34 years. Whereas the statistical model of the ordinary least squares (OLS) regression showed that the age was 71.66 years. Furthermore, the lower and upper limits were 63.52–81.17 years [14].

4.2.2. Inference of genetic ancestry

The hypervariable regions of mtDNA sequences were compared to the revised Cambridge Reference Sequence (rCRS), as shown in Table 4. According to European Mitochondrial Population Database (EMPOP) [25], the result showed that the mitochondrial haplogroup is U5b. This haplogroup is distributed in Europe. As a rule, mitochondrial haplogroup U5 is originally found in Europe [26].

A search in Y-STR Haplotype Reference Database YHRD [24] did not lead to any match in 50692 haplotypes for PowerPlex Y 23, nor any match in 205059 haplotypes for Yfiler. In addition, no match could be displayed within 265324 Haplotypes using Minimal Haplotype (9 Y-STR systems).

5. Discussion

New avenues leading to human identification have recently become realizable due to the increased prevalence of implanted medical devices, including dental implants, in the human body [27]. When the

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Table 3
Comparison between the statements inferred by dental implants investigation referred to as "Postmortem" and the statements provided by the treating dentist referred to as "Antemortem".

Tooth	Length		Diameter		Height		Manufacturer		Designation		Art.-Nr./product no.		Probable treatment period		Estimation
	Ante-mortem	Post-mortem	Ante-mortem	Post-mortem	Ante-mortem	Post-mortem	Ante-mortem	Post-mortem	Ante-mortem	Post-mortem	Ante-mortem	Post-mortem	Ante-mortem	Post-mortem	
13#	**12**	12	**4.5**	4.5	_	8.6	Champions	Champions	Vierkant	Vierkant New Art	1060	1060	**29.01.2013**	2010–2016	**6/6**
14#	**8**	8	**3.5**	3.5	_	8.1	Champions	Champions	Vierkant	Vierkant New Art	**1000**	1000	**29.01.2013**	2010–2016	**6/6**
15#	**8**	8	**3.5**	3.5	_	8.1	Champions	Champions	Vierkant	Vierkant New Art	**1000**	1000	**10.10.2014**	2010–2016	**6/6**
16#	**8**	8	**4.5**	4.5	_	8.6	Champions	Champions	Vierkant	Vierkant New Art	**2010**	2010	**10.10.2014**	2010–2016	**6/6**
17#	**8**	8	**5.5**	5.5	_	9.2	Champions	Champions	Vierkant	Vierkant New Art	**1012**	1012	**10.10.2014**	2010–2016	**6/6**
24#	**12**	12	**3**	3.5	_	8.1	Champions	Champions	Vierkant	Vierkant „classic“	**1040**	1055	**12.02.2010**	2006–2016	**4/6**
26#	**10**	10	**3**	3.5	_	8.1	Champions	Champions	Vierkant	Vierkant „classic“	**1015**	1025	**12.02.2010**	2006–2016	**4/6**
36# *f	**11**	11	**3.5**	3.5	_	_	ANKYLOS	ANKYLOS	**A11**	A11	**3101 0210**	3101 0210	**12.02.2007**	2005–2008	**6/6**
37# *f	**11**	11	**3.5**	3.5	_	_	ANKYLOS	ANKYLOS	**A11**	A11	**3101 0210**	3101 0210	**12.02.2007**	2005–2008	**6/6**

Tooth	Angulations		Diameter		Gingival heights		Manufacturer		Designation		Art.-Nr./product no.		Probable treatment period		Estimation
	Ante-mortem	Post-mortem	Ante-mortem	Post-mortem	Ante-mortem	Post-mortem	Ante-mortem	Post-mortem	Ante-mortem	Post-mortem	Ante-mortem	Post-mortem	Ante-mortem	Post-mortem	
36#*p	**15**	15	_	4.5	**3**	3	ANKYLOS	ANKYLOS	Balance Posterior Abutment	Balance Posterior Abutment	**3102 1660**	3102 1660	**2007**	2005–2013	**6/6**
37#*p	**15**	15	_	4.5	**1.5**	1.5	ANKYLOS	ANKYLOS	Balance Posterior Abutment	Balance Posterior Abutment	**3102 1650**	3102 1650	**2007**	2005–2013	**6/6**
Total estimation															**62/66**

36# *f: the implant-fixture component of a dental implant replacing the tooth **36#**.
37# *f: the implant-fixture component of a dental implant replacing the tooth **37#**.
36# *p: the prosthetic abutments component of a dental implant replacing the tooth **36#**.
37# *p: the prosthetic abutments component of a dental implant replacing the tooth **37#**.
_: no details provided.

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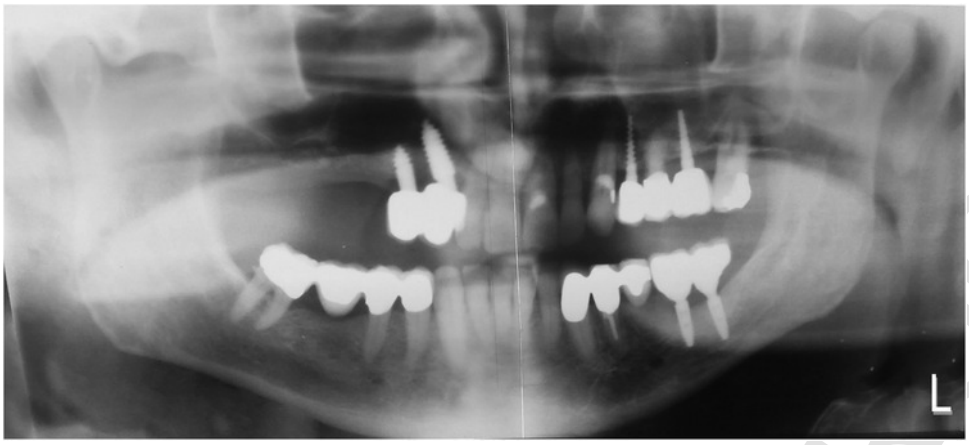

Fig. 4. Antemortem panoramic radiograph performed in October 2014.

Table 4
Haplotype sequence of the mt-DNA retrieved from unidentified corpse's sample deviating from the revised Cambridge Reference Sequence (rCRS).

Position		Anderson/ CRS[a]	The haplotype of the mt-DNA from unidentified corpse's sample
HV1	**16189**	T	**C**
	16270	C	**T**
	16192	C	**T**
HV2	**73**	A	**G**
	150	C	**T**
	263	A	**G**
	315.1	_	**C**

[a] Cambridge Reference Sequence.

missing teeth of unidentified cadavers are rehabilitated with dental implants, implant extraction, microscopic examination and metric analysis in addition to clinical and radiological examination have important implications, which facilitate the identification process. The reported literature [5,6,10] described the importance of physical and radiological features of dental implants. However, implant extraction and microscopic examination were missing. As reported in Refs. [5,6], the coronal portion of implant fixture distinguishes the various shapes of prosthetic interface and flange; the mid-body displays the general geometric shape (cylindrical, straight or tapered shaped), the presence of threads (threaded or nonthreaded), the thread geometry (square, V-shaped, buttress, reverse buttress or spiral), and the number of threads and the pitch distances between them (single, double or triple thread) [28]. The apical portion exhibits whether the apex is pointed, V-shaped, curved, or flat and provided with apical chamber, oblong hole, round hole or groove [5,6]. Furthermore, features depicted on radiographic images should not be misinterpreted as a consequence of the expected distortion resulting from implant rotation or improper projection angulations [9,10]. Microscopic examination can supplement the visual and radiological investigations with potential contributions, such as illustrating the fine microstructure of each implant, more easily and accurate measuring of different parameters, and detecting the presence of any microscopic clue about the manufacturer. Definitely, individual serial number, labelling the dental implant's body, represents a key number to trace the identity of the unidentified person [29]. It can crucially facilitate the traceability [30]. However, this scenario cannot always occur. Many implants are provided with specific information on the external coverage instead of the implant's components. Therefore, identifying the batch number, occasionally, designated as product, art or type number by different companies, represents the best alternative solution.

In this case, four factors played a crucial role in identifying the batch numbers: (i) comprehensive microscopic measurements combined with clinical and radiological examinations, (ii) implantologist consultation effectively facilitating the investigation, (iii) the search in manufactures' catalogues, (iv) verifying the results by utilizing the potential manufacturers' data.

Subsequently, the results of dental implant investigations based on the comparison between our statements, referred to as "Postmortem", and the answers from the treating dentist, referred to as "Antemortem" (Table 3), demonstrated that all post- and antemortem statements are compatible except the batch numbers of two implants (24# and 26#) as a consequence of a mistake in measuring the relevant implant diameter. Hence, these statements can be utilized in identifying the batch numbers considered as the key of traceability to the treating dentist. Interestingly, the results showed that not only the batch numbers of implant-fixtures but also the batch numbers of prosthetic abutments could be identified.

Despite the limitation of specific data able to be obtained from manufacturing companies, such as the exact numbers of production, marketing and selling, due to the trade secret [31], the prospective utility of such data could directly lead to identifying the order using simple software when multi-implants are used. Unquestionably, the combinations of different implants ordered with different batch numbers by one dentist/or clinic from one or more manufacturing companies in its/their databases can lead to an increase in the discriminative power for tracking the treating dentist. The more inserted/ordered implants by the treating dentist, the higher probability of identifying the person in question. Compared with DNA algorithm, the probability space of the treating dentists is relatively very limited due to a limited number of dentists using/ordering dental implants. This increases the discriminative power of identifying the order enormously.

The auxiliary data provided from DNA methylation and genetic ancestry inference could facilitate the investigation effectively at least by exclusion or inclusion process to limit the population pool. After being identified, it was found out that the man was 77.77 years old at the time of death. Our results showed that the actual chronological age was within the lower and upper limits of the calculated age (63.52–81.17 years) with underestimation of 5.43 years using the formula of weighted least squares (WLS) regression recommended by Smeers et al. [14]. It is well known that age assessment at death for adults using traditional methods based on dental and skeletal degenerative changes can be less accurate with higher error range [32].

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DNA methylation, despite DNA degradation, yielded acceptable deviation error and provided proper age limits. Unfortunately, Y-STR analysis revealed no match in the YHRD database, thus this could be a rare haplotype to infer a biogeographic origin. Nevertheless, the ancestry pointed out that the origin is Europe, which corresponds with the biogeographical origin inferred from mtDNA. To this end, DNA approaches reconstructing the biological profile can make a worthwhile contribution to police investigation.

In some circumstances, it is not feasible to use all of the aforementioned DNA-based approaches in every forensic institution as a consequence of the lack of required equipments and materials or exceptional conditions pertaining to mass fatality incidents. Considering the particularity of each forensic casework, the circumstantial conditions should be always taken into account by forensic practitioners to conduct the most appropriate solution of identification question.

6. Conclusion

In conclusion, this case highlights the utmost importance of dental implants in the prospective investigations for human identification. It shows the necessity of implant extraction, microscopic examination and detailed measurements used together with morphological and radiological features previously reported in literature. Furthermore, the case calls attention to the computerized data saved by dental implant manufacturers as an additional valuable reference data for dental identification. On the other side, the progressing promising DNA technologies can trigger new dimensions for police investigations by providing supplementary data about the biological profile.

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Conflict of interest

None.

CRediT authorship contribution statement

Hussam Mansour: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Jan Peter Sperhake:** Methodology, Investigation, Resources. **Bram Bekaert:** Methodology, Investigation, Validation, Supervision. **Oliver Krebs:** Methodology, Validation, Writing - original draft, Writing- Reviewing and Editing. **Peter Friedrich:** Investigation. **Andreas Fuhrmann:** Methodology, Supervision. **Klaus Püschel:** Writing - review & editing, Supervision, Project administration.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.forsciint.2019.109926.

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7.2 Supplementary material

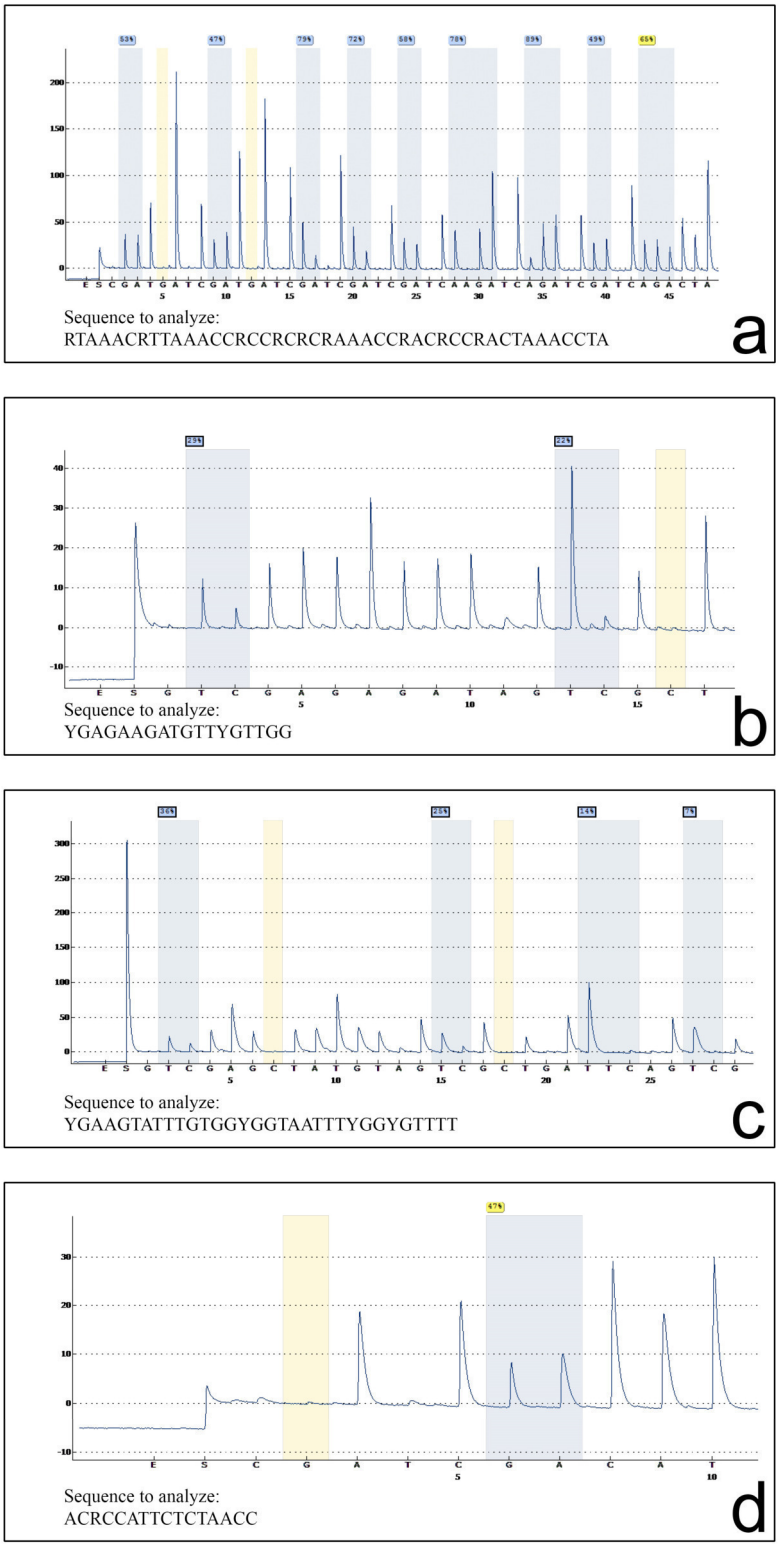

Fig. S 1 The percentage of DNA methylation at CpG sites markers within four genes: a. CpG6 in ELOVL2 gene, b. CpG1 in EDARADD gene, c. CpG1 in PDE4C gene, d. CpG1 inASPA gene

8 Publication 5: The role of Forensic Medicine and Forensic Dentistry in estimating the chronological age of living individuals in Hamburg, Germany

8.1 Publication

Mansour H, Fuhrmann A, Paradowski I, van Well E, Püschel K (2017) The role of forensic medicine and forensic dentistry in estimating the chronological age of living individuals in Hamburg, Germany. Int J Legal Med 131: 593–601. doi.:10.1007/s00414-016-1517-y.

Int J Legal Med
DOI 10.1007/s00414-016-1517-y

ORIGINAL ARTICLE

The role of forensic medicine and forensic dentistry in estimating the chronological age of living individuals in Hamburg, Germany

Hussam Mansour[1] · **Andreas Fuhrmann**[1] · **Ioana Paradowski**[1] · **Eilin Jopp van Well**[1] · **Klaus Püschel**[1]

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Abstract Age estimation represents one of the primary responsibilities of forensic medicine and forensic dentistry. It is an integral procedure aiming to estimate the chronological age of an individual, whose age is either unknown or doubtful, by means of assessing the stage of dental, skeletal, and physical development. The present publication reviews the methods and procedures used in estimating the age of young living individuals as well as the experiences of the Institute of Legal Medicine in Hamburg-Eppendorf, Germany, during the last 25 years. From 1990 to 2015, 4223 age estimations were carried out in Hamburg. During this time, forensic age estimation was requested by different concerned authorities including courts, the foreigners' registration office (Zentrale Ausländerbehörde), and the state office of education and consultation (Landesbetrieb Erziehung und Beratung). In the context of judicial proceedings, orthopantomograms, as well as X-ray examinations of both the left hand and the medial clavicular epiphyses were carried out in accordance with AGFAD recommendations. For investigations not associated with judicial proceedings, orthopantomogram examinations play a key role in the process of age estimation, due to their high diagnostic value and low radiation exposure. Since 2009, mainly unaccompanied young refugees were examined for age estimation. Orthopantomograms and clinical-physical examinations have been used as essential steps in this context to determine whether an individual is 18 years or less. Additional X-ray examinations of the left hand and the medial clavicular epiphyses have been used less frequently.

Keywords Forensic age assessment · AGFAD recommendations · Institute of Legal Medicine Hamburg-Eppendorf · Hamburg model

✉ Hussam Mansour
Hussam.Mansour@uke.de

Andreas Fuhrmann
a.fuhrmann@uke.de

Ioana Paradowski
ioana@imail.de

Eilin Jopp van Well
e.jopp@uke.de

Klaus Püschel
pueschel@uke.de

[1] Institute of Legal Medicine, University Medical Center Hamburg-Eppendorf, Butenfeld 34, 22529 Hamburg, Germany

Introduction

Currently, as a consequence of high levels of immigration into Europe in general and especially into Germany, age assessment represents one of the main challenges in forensic practice.

Already in the early 1990s, many young immigrants entered Germany after crossing the borders without identification cards or certified documents validating their age. In order to afford the appropriate care for minors and young adults, it was very necessary to assess their age according to correspondent law. For the purpose of improving the criteria of age estimation, the interdisciplinary Study Group on Forensic Age Diagnostics (Arbeitsgemeinschaft für Forensische Altersdiagnostik; AGFAD) of the German Society of Legal Medicine was established in March 2000 [1]. AGFAD organizes annual meetings seeking to improve the age assessment process by discussing scientific papers and new researches in this field. To unify and standardize the criteria, the first recommendations for age estimation in the context of judicial

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proceedings were published in 2001 [1] and updated in 2008 [2]. Recommendations for age estimation of children and young adults for cases not including criminal proceedings were published in 2004 [3]. Due to changing legal backgrounds till then, the full AGFAD-standard including X-ray imaging is now recommended for all proceedings, if a legal justification for it is provided [4].

AGFAD has been carrying out annual proficiency tests since 2001. The aim of these tests is to improve the quality of reports on age assessment and to assure the quality of experts' opinion on age [5]. The results of the proficiency tests confirm significant progress in its efforts to protect human beings under the age of 18 years. The most recent proficiency test took place in 2016 and included the participation of 31 workgroups from nine countries [4].

AGFAD recommendations in the case of legal authorization for X-ray-imaging

According to AGFAD, several possible X-ray examinations as well as physical examinations are included as a first step in the age assessment process. X-ray examinations include orthopantomograms (OPGs), X-ray examinations of the left hand and thin-slice computed tomography-scans of the medial clavicular epiphyses (CT-clavicle) [6].

The expected age-related findings from these different methods would ensure accuracy and narrow the statistical range of the age [1]. However, compared to the radiation exposure of an X-ray examination of the left hand and OPGs, a CT-clavicle is a high dose procedure and should, according to AGFAD, only be used in case of completely ossified forearm/hand provided a legal basis for ionizing examinations. When the required authorization for an X-ray examination is unavailable, the spectrum of methods will be confined to clinical physical and dental examinations [6].

In recent years, efforts have been made to raise the confidence level, to improve reliability, and to produce other alternative noninvasive methods by using nonionizing imaging methods such as magnetic resonance imaging [7–10], or ultrasound sonography [11–13].

Materials and methods

Age estimation started in Hamburg in 1990 and can be divided into three different sequential phases 1990–2000, 2001–2008, and 2009–2015.

In total, 4223 age estimations were carried out in Hamburg between 1990 and 2015. The distribution for each phase is shown in Table 1 [14, 15].

Table 1 The frequency of age estimation investigations between 1990 and 2015 at the Institute of Legal Medicine in Hamburg-Eppendorf

Period	1990–2000	2001–2008	2009–2015	1990–2015
Number of forensic age estimations	699	946	2578	4223

Methods and procedures used for estimating the age of young living individuals at the Institute of Legal Medicine in Hamburg

After receiving a request from the concerned authority, a combination of medical, dental, and anthropological investigations involving the following should be performed on the individual. AGFAD recommendations for age estimation have been considered. However, our procedures are not totally in line with the AGFAD guideline. Orthopantomogram examinations play a key role in our process of age estimation, due to their high diagnostic value and low radiation exposure in accordance with ALARA principle (as low as reasonably achievable) [16].

- First, a medical history has to be acquired, including an inquiry about current diseases, pre-existing diseases, family diseases, and medications, which in turn could affect physical development and maturation.
- A clinical-physical examination including the evaluation of vital signs such as pulse rate, blood pressure, auscultatory sounds, as well as anthropometric measures involving body height, weight, and constitutional type should be carried out.
- OPGs should be performed in order to evaluate dental development [2, 17].
- In the case of inconclusive results from the OPGs, an X-ray examination of the left hand could be used to enhance the diagnostic decision.

Usually, the two aforementioned methods (OPG and hand X-ray) are in most cases sufficient to decide with a very high probability, whether the age exceeds 18 years or not. Furthermore, if the age is younger than 18, a hand X-ray helps in determining a minimum age. On the other hand, in rare instances when the wisdom teeth are missing or the examinations reveal fairly different results, a CT-clavicle has to be carried out. In Hamburg, the focus of interest is primarily on the results from the OPGs and the hand X-ray. Nowadays, digital OPG provides a detailed panoramic view of all teeth with a relatively low radiation dose. It is estimated by expert specialists before writing the final decision with respect to the related used methods and references.

Results

The age assessment process in Hamburg has been developed according to political boundaries and scientific advances over the three mentioned periods.

Age assessment procedures from 1990 to 2000

Before 1990, the age of young unaccompanied minors, whose birth documents and identification cards were uncertified, was estimated by the staff of the Federal Office for Migration and Refugees (Landesamt für Migration und Flüchtlinge). However, this method was subjective and not comprehensive. Therefore, after 1990, efforts were made through cooperation among the Institute of Legal Medicine, Department of Maxillofacial Radiology, the Police, and the Ministry of Interior in Hamburg to define criteria as objectively as possible for age assessment.

Between 1990 and 2000, 699 age estimations were in total carried out in the context of criminal proceedings for foreign young individuals at the Institute of Legal Medicine in Hamburg [14] as shown in Table 2. At that time, about 80% of the reasons were illegal residence, false certification especially concerning personal data (age and nationality), and drug offenses [14]. Furthermore, the age investigations were requested by the concerned court. According to § 81a StPO, the requests were sent to the Institute of Legal Medicine in Hamburg, where physical examinations were carried out.

Between 1995 and 1999, 457 requests were sent to the Institute of Legal Medicine in Hamburg, while 391 examinations for age assessment were carried out. 339 (74%) requests out of 457 were hand X-rays, while 410 (90%) were OPGs. Sometimes only a physical examination without OPGs or other radiological examinations was carried out. In the northwest German Maxillofacial Clinics, the dental examinations were performed, while hand X-rays were carried out at the Department of Pediatric Radiology.

Age assessment procedures from 2001 to 2008

To enhance accuracy, OPGs were applied in 84% of all cases [15]. Table 3 shows the age estimations between 2001 and 2008. During this period, all examinations were justified in the context of criminal proceedings with §81a StPO. In accordance with court decisions, 281 X-ray examinations of the left hand, and 96 clavicular projection radiography investigations (clavicle-PR) were performed.

Owing to several procedural changes at the Police and the Foreigners' Registration Office at that time as well as the system of forensic age assessment in the Institute of Legal Medicine in Hamburg, the number of age estimations has been significantly reduced during the same aforementioned time window. Furthermore, a significant decrease has been recorded in the total numbers of asylum applications from 118,306 in 2001 to 42,908 in 2005 [18]. In 2008, only 28,018 applications were recorded, the least total number of asylum applications in Germany between 1990 and 2015 [19]. This is in turn consistent with the small number of age assessment cases (19) in the same year.

Age assessment procedures from 2009 to 2015

At the request of the Foreigners' Registration Office (Zentrale Ausländerbehörde), refugees were sent to the Institute of Legal Medicine after April 2009 to undergo an estimation of their age. The Residence Act (AufenthG) was the legal basis for the age assessment. The term physical procedure (körperliche Eingriffe) in § 49 AufenthG was the legal basis for the application of X-ray examination [20]. Since then, the physical examination and OPGs were carried out in all cases, with some cases requiring a hand X-ray (about 2.6%) and clavicle-PR examinations (11%), as shown in Table 4.

Since 2010, the State Office of Education and Consultation (Landesbetrieb Erziehung und Beratung) in Hamburg has requested age estimations for young refugees providing educational assistance (Inobhutnahme) for minors according to the social law [21]. Table 5 shows the concerned authorities asking the Institute of Legal Medicine in Hamburg-Eppendorf for the purpose of age estimation [20, 21].

Between 2009 and 2012 additional clavicle-PR investigations (29% of cases) were carried out. However, because of its relatively high radiation exposure, the number of clavicle investigations has been significantly reduced since 2013, and from 2015 they were no longer used in the context of age assessment of young refugees.

In 2015, 476,649 asylum applications have been recorded, the highest total number of asylum applications in Germany between 1990 and 2015 [19]. This in turn was consistent with the highest number of age assessment cases 769 (751 + 18 = 769) in the aforementioned period.

Table 2 The frequency of age estimation investigations between 1990 and 2000 in the Institute of Legal Medicine in Hamburg-Eppendorf [14]

Year	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	1990–2000
Number of forensic age estimations	2	9	21	73	73	51	57	101	105	77	130	699

Table 3 The frequency of age estimation investigations between 2001 and 2008 in the Institute of Legal Medicine in Hamburg-Eppendorf [15]

Year	Clinical-physical examination	OPGs	Hand X-ray	Clavicle-PR
2001	318	293	137	6
2002	248	223	58	12
2003	176	128	41	10
2004	87	76	23	18
2005	51	45	10	27
2006	26	19	6	14
2007	21	7	2	4
2008	19	7	4	5
2001–2008	946	798	281	96
Percentage	100%	84%	30%	10%

Investigations for criminal proceedings in Hamburg between 2009 and 2015

Parallel to the age estimation for refugees sent by the concerned authorities, age assessments have been performed within the context of judicial proceedings as shown in Table 6.

Age estimation of refugees from neighboring federal states

Infrequently, since 2011, additional adolescents and young refugees sent from other federal states, like Schleswig Holstein, Niedersachsen und Bremen, were examined, as well as very rare cases in the context of criminal proceedings. In addition, owing to a cooperation with the Institute of Legal Medicine Charité Berlin, 333 age estimations including 52 CT-clavicle investigations have been carried out from September 2013 to June 2016. Figure 1 shows an example

Table 4 The frequency of age estimation investigations between 2009 and 2015 in the Institute of Legal Medicine in Hamburg-Eppendorf in the context of non-judicial proceedings

Year	Clinical-physical examination	OPGs	Hand X-ray	Clavicle-PR
2009	167	167	2	69
2010	246	246	5	96
2011	219	219	1	71
2012	251	251	0	22
2013	413	413	0	2
2014	450	450	19	3
2015	751	751	39	0
2009–2015	2497	2497	66	263
Percentage	100%	100%	2.6%	11%

Table 5 The concerned authorities asking the Institute of Legal Medicine in Hamburg-Eppendorf for the purpose of age estimation

The period	Before Apr. 2009	Apr. 2009–Sep. 2010		After Sep. 2010	
		In the context of non-judicial proceedings	In the context of judicial proceedings	In the context of non-judicial proceedings	In the context of judicial proceedings
The concerned authority	The concerned courts in the context of judicial proceedings	Foreigners' Registration Office (Zentrale Ausländerbehörde).	The concerned court	State Office of Education and Consultation (Landesbetrieb Erziehung und Beratung) and other youth welfare offices (Jugendämter[a])	The concerned court

[a] Other youth welfare offices (Jugendämter) from other cities like Bremen, Flensburg, Celle, Heide, Pinneberg, and Stade

Table 6 The frequency of age estimation investigations between 2009 and 2015 in the context of criminal proceedings in the Institute of Legal Medicine in Hamburg-Eppendorf

Year	Clinical-physical examination	OPGs	Hand X-ray	Clavicle-PR
2009	5	5	0	5
2010	7	7	0	7
2011	10	10	0	4
2012	8	8	0	7
2013	18	18	0	4
2014	15	15	3	7
2015	18	18	4	10[a]
2009–2015	81	81	7	44[a]

[a] Including 3 CT-clavicle investigations in 2015

illustrating the age estimation process in the Institute of Legal Medicine in Hamburg-Eppendorf in such cases.

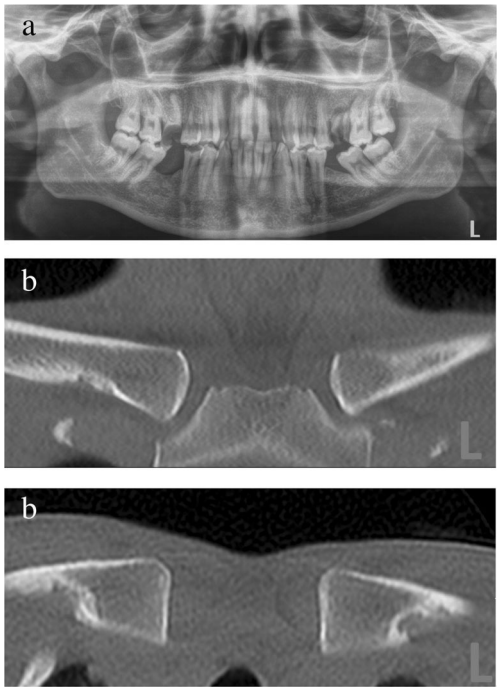

Fig. 1 The case illustrates the forensic age estimation in the Institute of Legal Medicine in Hamburg-Eppendorf in the context of judicial proceedings. After receiving a request from the concerned authority, a male person, from Egypt, was examined in February 2016. The birth date provided by the examined male was in September 1997, while the date of the committed crime was in September 2015. No evidence of current diseases, pre-existing diseases, medications, or other possible developmental disorders have been found during medical history and physical examination. **a**) OPG revealed that the mineralization of the third molars (18, 28, 38, and 48), the most important characteristic, was complete and corresponded with stage H according to Demirjian et al. [22]. According to the mineralization degree and the radiographic visibility of the root pulp of completely mineralized lower third molars, the higher minimum age corresponded with the latter markers is 17.6 [23, 24]. In addition, other age-related characteristics like alveolar resorption and general periodontal recession (about 3–4 mm) corresponded with stage (1–2) [25] and stage B [26], and completed eruption of the third molars corresponded with stage D [27, 28] as well as a mesial inclination of both right and left lower molars towards the neighbor extracted first molars have been discerned. The additional characteristics indicate that the age exceeds 18 years. **b**) Coronal and axial view of CT-clavicle revealed that the ossification of the medial clavicular epiphyses corresponded with stage 4 according to Schmeling et al. [29]. The minimum age corresponding with stage 4 exceeds 21 [29–32]. The age provided by the examined person is not compatible with X-ray findings in both OPG and CT-clavicle following the minimum age concept [33]. Therefore, the possible minimum age of the person examined exceeded 21 years not only on the date of the examination but also on the date of the committed crime

Discussion

In order to conduct the highest standard of proof, the minimum age concept should always be taken into account by comparing the minimum ages corresponding to several examinations findings and the mentioned age provided by the examined individual [33]. According to AGFAD recommendations, three different investigations aiming to assess age through hand X-ray, OPGs, and CT-clavicle could be carried out.

Hand x-ray

Skeletal age indicator is a reliable index commonly used not only in specialities of clinical medicine such as endocrinology, orthopedics, pediatrics, or clinical dentistry such as orthodontics, but also in forensic medicine and dentistry [34, 35]. Furthermore, an X-ray examination of the left hand is an effective procedure recommended by AGFAD to enhance the reliability of an estimated age [2].

For a long time, the Greulich and Pyle Atlas represented the reference of a normal skeletal maturation. The reproducibility and reliability of the Greulich and Pyle method [36], one of the most widely and frequently used methods for diagnosing skeletal maturity, has been determined in many different studies for instance Alcina et al. in Spain [37], Dantas et al. in Brazil [34], Tisè et al. [38] in Italy, Koc et al. in Turke [39], Lynnerup et al. in Denmark [40], and Zabet et al. in France [41].

Undeniably, the results of the above studies revealed different evaluations. Some authors (Koc et al.) stated that the Greulich and Pyle method is not completely applicable to Turkish boys without modifications [39]. Tisè et al. justified the error margin as a consequence of biological variability

Table 7 The effective radiation doses for different forensic diagnostic procedures [33, 63]

Forensic diagnostic procedure	Effective radiation dose (μSv)
Hand X-ray	0.1
Orthopantomogram	20
Clavicle (PR)	220
Clavicle-CT	400

during skeletal development and the inadequacy of the Atlas standards [38]. Lynnerup recommended to take the geographical difference of populations into consideration [40], while Dantas et al. found a reliability in the Greulich and Pyle method [34], and Zabet approved its applicability, but with caution especially concerning those who approach an age of 18 [41].

Orthopantomograms and dental examination

OPG evaluation is the primary method to estimate age at the Institute of Legal Medicine in Hamburg. It depends on several main criteria such as the mineralization degree and the development of the roots of the teeth, especially wisdom teeth. Furthermore, age-related dental and periodontal changes are taken into account, such as attrition, periodontal recession by estimating the alveolar bone of maxilla and mandible, the presence of fillings, the number of missing teeth, as well as retention, inclination, and elongation of the teeth.

Mineralization of the third molars: the wisdom teeth reveal very important age-related anatomical features; therefore, they can play a central role in the forensic practice of age estimation. The estimated degree of root development is the most important characteristic and depends on the applicability of Demirjian's development standards to the third molar based on the eight stages (from A to H) of dental maturity [22]. In stage H, the apices of the roots are completely closed with a uniform shape of the periodontal membrane around the root and the apex. In many different studies for example [42–46], the corresponding mean values of age with stage H exceed 18 years for both males and females, whereby such parameter has an unequivocal dependency on the upper age end of the pulled sample.

In light of this information, other age-related characteristics of the teeth and jaws are required. The combination of the root development degree with other additive age-related characteristics raises the certainty that the age exceeds 18 years. The most important additive age-related characteristics are periodontal recession, eruption status, elongation, attrition, and coronal morphological changes.

In addition, the adoption of the specific reference data to the relevant population is recommended by numerous studies when assessing the forensic age, for instance [6, 27, 43, 47–49]. Olze et al. [43] investigated in their comparative study the mineralization stages of wisdom teeth in three different ethnic populations involving South African, German, and Japanese. They found that African populations have a relatively accelerated mineralization and Caucasoid populations ranked in the middle position, while Mongoloid populations showed a comparatively delayed mineralization.

Periodontal recession: the digital OPGs can obviously represent a horizontal periodontal recession. Olze et al. [25] found that periodontal recession could provide an additional indicator in age estimation. A noticeable correlation between advancing age and increasing periodontal recession could be utilized. In several studies, the mean values of ages corresponding with 4 mm of significant periodontal recession were over 18 years, for instance Olze [25] presented an age range of 23.6 years ± 3.8, Richel [50] of 21.9 years ± 2.7, and Otte-Witte [26] of 24.6 years ± 2.7.

Eruption status and elongation: the chronological course of wisdom tooth eruption is also a helpful parameter that can be utilized for forensic age assessment [51]. Olze et al. [27, 52] estimated the age through alveolar, gingival, and complete emergence of third molars in the occlusal plane.

In numerous studies, the chronological course of eruption has been investigated in different ethnic populations, for example: a cross-sectional study for Hassanali et al. [53] between African and Asian subjects, a comparative study of Olze et al. [27] in three different ethnic populations involving black South Africans, Germans, and Japanese, a study in First Nations people of Canada [51], and a study in northern Chinese population [48].

Hassanali et al. stated that African subjects showed more advance in third molar emergence than Asian subjects [53].

Similar to dental mineralization, Olze et al. [27] found that the black South African population was the most advanced in third molar emergence followed by the German population, whereas the Japanese population was the latest.

Attrition is also an age-related indicator, occurring as a result of long tooth-to-tooth contact after the stage of reaching the occlusal plane. Thus, attrition of the wisdom teeth's surface indicates that the age is most likely more advanced. Richel found that the mean value of ages corresponding with attrition stage 1, which is defined as attrition of enamel from all cupids' teeth except wisdom teeth, to be 23.2 ± 2.3 [50].

Thin-slice computed tomography-scan of medial clavicular epiphysis

Ossification of the epiphyseal cartilage roughly begins from 16 years and continues to over 21 years of age [30, 54–56]. Therefore, the ossification degree can play a very important role in age assessment. Today, it is recommended to use CT-clavicle to assess the stages of the epiphyseal cartilage ossification instead of using Clavicle-PR [57–59]. Many studies and publications improved and facilitated the evaluation of the clavicle-investigation findings. Schmeling et al. classified the developmental stages of the medial clavicular epiphyseal cartilage [29]. Wittschieber et al. stated clarifications of the ossification stages [31, 32]. Kellinghaus et al. presented subclassification for stages 2 and 3 [60] and Mühler et al. recommended the application of thin-slice CT-scan not more than 1 mm to ensure a maximum accuracy [61]. Therefore, it becomes easier to assess the age ranging from 17 to 21 years with thin-slice CT-scanning of the clavicle.

The different ionizing imaging methods are associated with different exposure radiation doses as shown in Table 7. Today,

several studies were performed to apply non-ionizing imaging methods. Magnetic resonance tomography (MRT) could be an alternative method instead of conventional X-ray methods [62].

Legal background of using X-ray in the context of age estimation without medical indication

X-rays are primarily used in the medical field for medical or dental treatment. Furthermore, the use of X-ray in the context of forensic age estimation requires a legal basis for authorization based on § 81a StPO and consecutively in § 49 AufenthG in the past.

As of the 1st of November 2015, amendments to the SGB VIII (SGB = social code book) of the social law have become applicable for procedures within its context. Paragraph § 42 f SGB VIII approved the regulation procedures for age estimation through identification documents or equivalent inspection procedures.

In cases of doubt, on the behalf of the concerned person, his/ her representative, the concerned authorities, or the State Office of Education and Consultation (Landesbetrieb Erziehung und Beratung) in Hamburg medical investigation for age assessment has to be requested. In Hamburg, these new legal regulations have been adopted as of the 1st of November 2015. Medical investigations concerning age estimation (including radiology) with respect to legal areas, legally relevant age limit, and legal issues in Germany are today a matter of clear regulations and actions [20, 33, 64].

Conclusions

- Until now, the adopted methods of age assessment have been accepted in the scope of both social and penal law by the courts and by the concerned administrations in Hamburg.
- The necessary authorization needs to be taken into consideration. Afterwards, using a combination of different methods allows for more accuracy and narrows the probable range of age according to the expected age related findings.

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9 Summary

9.1 Zusammenfassung auf Deutsch

Zwei primär zuverlässige Methoden der Identifizierung zeigen deutlich die entscheidende Rolle der Zähne bei der Identifizierung menschlicher Überreste: konventionelle odonto-stomatologische Identifizierung und die auf Zähnen basierende DNA-Genotypisierung als außergewöhnliche DNA-Quelle, insbesondere unter ungünstigen postmortalen Umständen. In besonderen forensischen Fällen, wie z. B. bei verstreuten Skelettresten, kann die odontologische Identifizierung in Kombination mit der DNA-Analyse eine geeignete Lösung für Identifizierungsfragen darstellen, um das die Identität sicherstellende Konfidenzniveau zu erreichen.

Wenn Zähne als DNA-Quelle verwendet werden, sollten verschiedene postmortale Faktoren, wie die Leichenliegezeit (postmortales Intervall), der Zustand des Leichnams und die relevanten Umwelteinflussfaktoren, berücksichtigt werden. Diese postmortalen Faktoren weisen erhebliche Auswirkungen auf die potenzielle Ausbeute genomischer DNA aus dem Dentalgewebe auf. 10 Tage nach dem Tod ging der dentale DNA-Gehalt unter realen postmortalen Bedingungen deutlich zurück. Im Gegensatz dazu zeigten Geschlecht, Alter, Zahntyp und verschiedene Zahnwurzelregionen als prämortal untersuchte Faktoren keinen signifikanten Einfluss auf die dentale DNA-Ausbeute unter realen Bedingungen. Auf der Grundlage unserer Untersuchung sind diese prämortalen Faktoren mit großer Wahrscheinlichkeit sehr stark von der inter- und intra-individuelle Variabilität abhängig. Zwischen verschiedenen Dentalgeweben kann der Zahnzement, der durch eingebettete und gut konservierte Zementozyten in seinem Mineralmatrix gekennzeichnet ist, eine nützliche Quelle für die DNA-Genotypisierung sein, wenn andere Quellen wegen der postmortalen DNA-Degradation versagen.

Der Befund der Zahnimplantate während der postmortalen zahnärztlichen Untersuchung eines unbekannten Leichnams kann prospektive Hinweise liefern, die die Einholung der zahnärztlichen Unterlagen erleichtern. Neben der morphologischen und röntgenologischen Untersuchung der Zahnimplantate können die mikroskopische Untersuchung sowie die metrische Analyse die Artikelnummer des Herstellers effektiv identifizieren. Die Artikelnummer kann letztendlich zur Identifizierung des behandelnden Zahnarztes/ der Zahnklinik führen, der/ die den relevanten Implantatartikel bestellt hat. Es wird daher empfohlen, die von den Zahnimplantatherstellern gespeicherten Daten als zusätzliche unterstützende Quelle als wichtige Referenzdaten zu nutzen, wenn, zumindest in der Anfangsphase der polizeilichen Ermittlungen, noch keine zahnmedizinischen Unterlagen vorliegen und noch kein Treffer in der nationalen DNA-Datenbank erzielt werden kann.

Das Alter, einer der entscheidenden Aspekte der Identität, muss sowohl für lebende Personen als auch für unbekannte Toten nach wissenschaftlichen Methoden mit akzeptablen Fehlergrenzen eingeschätzt werden. In Hamburg wurde die forensische Altersdiagnostik in den 25 Jahren zwischen 1990 und 2015 durch einer körperlichen Untersuchung zusammen mit einer oder mehreren von der AGFAD empfohlenen röntgenologischen Methoden, nämlich Hand X-ray, OPG, Claviculae-PR and CT- Claviculae durchgeführt. Die in Hamburg verwendete röntgenologische Methode änderte sich entsprechend der jeweiligen Periode. Zurzeit kann die Kombination der verschiedenen Altersmarker mit der Verwendung des Mindestalterkonzepts, welches von Schmeling et al. empfohlen wird, die Entscheidung über die Alterseinschätzung verbessern und die potenziellen Fehler vermeiden, die sich auf die Einschätzung des Erreichens der relevanten Altersschwelle im Rahmen des Strafverfahrens oder außerhalb des Strafverfahrens auswirken. Dies entspricht den Anforderungen der zuständigen Behörden und der jeweiligen nationalen Gesetzgebung.

9.2 Summary in English

Two primary identifiers clearly demonstrate the crucial role of teeth in the identification process of human remains: the conventional comparative dental identification and DNA identification based on teeth as an exceptional source of DNA, especially in challenging post-mortem conditions. In some forensic scenarios, such as scattered skeletal remains, dental identification combined with DNA identification can effectively provide a proper solution for identification questions to reach the confidence level confirming the identity.

When using teeth as a source of DNA, several post-mortem factors (including post-mortem interval, post-mortem condition, and the respective surrounding environment) should be taken into account. These post-mortem factors impact the potential dental DNA yield significantly. In real post-mortem conditions, the amount of dental DNA dropped dramatically 10 days after death. In contrast, sex, age, tooth type, and different tooth root regions, investigated as ante-mortem factors, revealed no significant effect on dental DNA yield in real conditions. Based on our investigation, these ante-mortem factors are more likely to be affected by individual variation than any external variables. Histologically, among different dental tissues, cementum, characterized by cementocytes embedded and well preserved in the mineral matrix, can be a fruitful source for DNA genotyping when other sources fail as a consequence of post-mortem DNA degradation.

During the post-mortem dental examination of unknown corpses, the availability of dental implants can trigger prospective dimensions facilitating the recovery of ante-mortem dental records. Besides the morphological and radiological post-mortem examination of dental implants, microscopic examination and metric analysis can effectively identify the manufacturers' item number, by which the treating dentist or dental clinic ordering the implant article can ultimately be identified. Thus, utilizing the data saved by the dental implants' manufacturers as an additionally supportive source of important reference data is recommended, when antemortem dental records are still unobtainable and no match in the national DNA database can be obtained, at least in the early stages of the investigative process.

Age, one of the fundamental aspects of human identity, should be estimated for both living individuals and unidentified corpses according to scientific methods, with clear error margins. In Hamburg, from 1990 to 2015, forensic age assessment was performed based on physical examination along with one or more ionizing examinations recommended by AGFAD, including hand X-rays, OPG, clavicle-PR and CT-clavicle. The radiological method changed along with the respective period. Today, the combination of different age markers using minimum age concept (as suggested by Schmeling et al.) can enhance age estimation and avoid the potential errors affecting the estimation of attaining the relevant age threshold either in the context of judicial or non-judicial proceedings. This fulfills the requirements of the concerned administrations and the respective national legislation.

10 Erklärung des Eigenanteils an den Publikationen (Authorship Contribution Statement for all Publications)

1. Mansour H, Fuhrmann A, Paradowski I, van Well E, Püschel K (2017) **The role of forensic medicine and forensic dentistry in estimating the chronological age of living individuals in Hamburg, Germany.** Int J Legal Med 131: 593–601. doi.:10.1007/s00414-016-1517-y.

H. M., A. F. and K. P.	wrote the manuscript (H. M. took the lead in writing the initial draft of the paper)
H. M., A. F., E. W. and K. P.	reviewed and edited the manuscript
H. M. and A. F.	collected the data
H. M.	interpreted and discussed the results, analyzed the data
H. M.	prepared and presented the published work
All authors	provided comments on the article
K. P.	provided conceptual ideas, supervised, took responsibility for juridical issues and directed the work

2. Mansour H, Krebs O, Sperhake J, Augustin C, Koehne T, Amling M, Püschel K (2018) **Cementum as a source of DNA in challenging forensic cases.** J Forensic Leg Med 54:76–81. doi.:10.1016/j.jflm.2017.12.015.

H. M.	provided conceptual ideas and wrote the manuscript
H. M., O. K., J. S. and K. P.	reviewed and edited the manuscript
H. M., O. K. and C. A.	conceived, planned, carried out the methods concerning DNA analysis
J. S.	performed the autopsy
T. K. and M. A.	carried out the methods concerning microcomputed tomographic imaging (µCT) with help from H. M. for preparation
H. M., J. S. and M. A.	prepared and performed methods of histological and microscopic examination
H. M. and M. A.	interpreted and discussed the results, analyzed the data
H. M.	prepared and presented the published work
All authors	provided comments on the article
K. P.	provided the required resources and study materials, supervised and directed the work

3. Mansour H, Krebs O, Sperhake J, Fuhrmann A, Püschel K (2018) **Identification of scattered skeletal remains: Combined dental and DNA-based identification.** Rechtsmedizin 22:1-6. doi.:10.1007/s00194-018-0235-9

H. M.	provided conceptual ideas and wrote the manuscript with support from O.K.
H. M., O. K. and K. P.	reviewed and edited the manuscript
H. M.	conceived, planned and carried out the methods concerning DNA extraction from teeth
O. K.	conceived, planned and carried out the methods concerning DNA extraction from boons
J. S.	performed the autopsy
H. M. and A.F.	prepared and performed the methods concerning dental identification
H. M. and O. K.	analyzed data concerning DNA analyses
H. M.	analyzed data concerning dental identification
H. M. and O. K.	interpreted and discussed the general results
H. M.	prepared and presented of the published work
All authors	provided comments on the article
K. P.	supervised and directed the work

4. Mansour H, Sperhake J, Bekaert B, Krebs O, Friedrich P, Fuhrmann A, Püschel K (2019) **New aspects of dental implants and DNA technology in human identification.** Forensic Sci Int. doi: 10.1016/j.forsciint.2019.109926

H. M.	provided conceptual ideas
H. M.	mainly wrote the manuscript with support from O. K.
H. M., O. K. and K. P.	reviewed and edited the manuscript
J. S.	performed the autopsy
H. M., J. S and A. F.	conceived, planned and carried out methods concerning microscopic examination and the metric analysis of dental implants, as well as the post-mortem X-ray
H. M.	conceived, planned and performed the general dental implants investigation
B. B. and H. M.	conceived, planned and carried out the methods concerning DNA methylation
B. B. and O. K.	conceived, planned and carried out methods concerning mtDNA analysis, with help from H. M.

O. K.	conceived, planned and carried out methods concerning YSTR analysis
H. M., B. B. and O. K.	analyzed the data concerning different DNA analyses
H. M.	analyzed the data concerning dental implants investigation
H. M. and O. K.	interpreted and discussed the general results
H. M.	prepared and presented the published work
All authors	provided comments on the article
K. P.	took responsibility for communication with the police, supervised and directed the work

5. Mansour H, Krebs O, Pinnschmidt H, Griem N, Hammann-Ehrt I, Püschel K (2019) **Factors affecting dental DNA in various real post-mortem conditions.** Int J Legal Med. https://doi.org/10.1007/s00414-019-02151-9

H. M. and O. K.	provided conceptual ideas
H. M. and O. K.	wrote the manuscript with support from H. P.
H. M., O. K., H. P. and K. P.	reviewed and edited the manuscript
H. M.	planned and carried out the preparation procedures
H. M.	planned and carried out samples collection
H. M.	designed and planned the project
H. M., N. G. and I. H.	conceived, planned and carried out the methods concerning DNA quantification
H. P.	conceived, planned and carried out the statistical analyses and analytic calculations, with help from H. M.
H. P.	interpreted the results statistically
H. M., O. K. and H. P.	interpreted and discussed the general results
H. M.	prepared and presented the published work
All authors	provided their comments on the article
K. P.	took responsibility for the ethical approval of this study, supervised and directed the work

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12 Curriculum Vitae

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt

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13 Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

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