Dating death: *post mortem* interval estimation of human skeletal remains

Datando a morte: estimativa do intervalo *post mortem* de restos humanos esqueletizados

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Abstract Estimation of *post mortem* interval (PMI), particularly the late time since death, is crucial when dealing with human remains. Its establishment is an important task for forensic scientists since it has important legal implications such as identifying a decedent or prosecuting an offender. However, estimating time of death is a very complex and challenging task due to the amount of intrinsic and extrinsic factors influencing the rate and nature of body decomposition. Several methods have been used to estimate PMI, including decomposition, entomological, botanical, or, more recently, physics and biochemical methods. This paper reviews current forensic methods, focusing especially on forensic anthropological techniques for date of death

Resumo A estimativa do intervalo *post mortem* representa uma tarefa crucial quando lidamos com restos humanos, particularmente quando falamos de restos humanos esqueletizados ou em decomposição avançada. A sua estimativa constitui um trabalho importante para os cientistas forenses, já que dela advêm importantes implicações a nível legal, como a identificação ou a condenação de criminosos. Porém, datar a morte tem-se revelado uma tarefa muito complexa, pela quantidade de fatores intrínsecos e extrínsecos que podem influenciar a decomposição cadavérica. Variados métodos têm sido usados numa tentativa de estimar o intervalo *post mortem*, desde métodos clássicos baseados nos estados de decomposição a métodos entomológicos ou botânicos, e mais

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purposes. The literature is insufficient showing lack of methods to achieve an accurate and reliable PMI, meaning further investigation is required. A holistic approach, where every element is considered, is key to achieving a reliable PMI estimation.

Keywords: *Post mortem* interval; time since death; skeletonized remains; forensic anthropology; forensic taphonomy.

recentemente métodos físicos e bioquímicos. Este artigo pretende apresentar uma revisão dos métodos forenses usados para estimar o tempo decorrido desde a morte, com especial foco nas técnicas empregues em antropologia forense. Contudo, reforçamos que a literatura existente é até à data insuficiente, denotando a falta de métodos capazes de alcançar uma estimativa precisa do tempo decorrido desde a morte, sendo necessários estudos mais aprofundados nesta temática. Só uma abordagem holística, em que todos os elementos sejam considerados, poderá ser a chave para uma estimativa precisa do intervalo *post mortem*.

Palavras-chave: Intervalo *post mortem*; tempo decorrido desde a morte; restos humanos esqueletizados; antropologia forense; tafonomia forense.

1. *Post mortem* interval

Estimation of *post mortem* interval (PMI) corresponds to the time interval since death until the body is found. It imposes a challenge for forensic anthropologists, since it aims to answer one of the questions when a human body is found, "When did it happen?". An accurate estimation of time since death may prevent impunity of criminals and may determine whether or not a crime has prescribed. It may contribute to narrowing down the list of missing people and, therefore may contribute to identification.

Over the last decades, there has been an increase in the number of studies aimed at finding appropriate and accurate methods for time since death estimation. However, the attempt to establish a PMI in human remains with an advanced state of decomposition is still a challenge. Only a combination of different methods, taking into consideration the influence of intrinsic and extrinsic factors, can result in a more accurate estimation of PMI. It is also important to keep in mind that the longer the time since death, the harder it is to estimate PMI.

In a forensic context, PMI estimation begins with the macroscopic analysis of human remains, due to the appearance of *post mortem* phenomena when the vital functions cease. According to Di Maio and Molina (2021), several phenomena can contribute to time elapsed since death estimation, for example, *livor*, *rigor* and *algor mortis*, digestion of stomach contents, and potassium content in the vitreous humor or in synovial fluid, on the fresh cadaver (Henssge and Madea, 2004; 2007; Madea, 2005; Madea et al., 2019; DiMaio and Molina, 2021). After the initial period, other changes can take place and aid the estimation of the time since death, such as circumstantial factors, the body´s degree of decomposition, entomological activity, or chemical and immunological changes. These factors are more variable, since these are influenced by intrinsic factors, such as cause-of-death, sex, age of the individual, lifestyle, pathological changes, percent body fat/body mass index. Extrinsic factors such as the presence or absence of clothes, indoor or outdoor decomposition, type of soil, depth of burial, pH, fauna and flora, temperature, oxygen partial pressure, sunlight or humidity are also influential in the rate of decomposition (Buchan and Anderson, 2001; Vass, 2011; Wilson-Taylor, 2012).

This work intends to describe the state of the art of different approaches to PMI estimation of human skeletal remains or bodies in advanced state of decomposition.

2. PMI assessment during recovery of human remains (stratigraphy and circumstantial factors)

For PMI analysis, a holistic and interdisciplinary analysis must start at the scene. The presence of circumstantial factors, such as letters, newspapers, notes, or receipts, testimonies of friends, family or neighbour's about the last time they have seen the decedent, or day-to-day routines constitute an unscientific but effective method of date of death estimation. Food leftovers, dirty dishes, and its state of decomposition, can also be taken into consideration. The analysis of clothes, through the identification of brands or models and their date of manufacture, or even through their degree of degradation, can also be relevant to establish date of death intervals (Buchan and Anderson, 2001; Wilson-Taylor, 2012; Ciaffi et al., 2018; Di Maio and Molina, 2021) and have been paramount in many routine forensic cases.

In the event that human remains are buried, during their exhumation it is essential to keep the stratigraphic record, which will help to estimate the time elapsed since death. The stratigraphic method, widely used in Archaeology as a relative dating method to date sites and finds, is based on a series of principles, such as superposition (in a succession of strata, the lower ones are older and the upper ones more recent) and inclusion (the layer that includes the inclusion is newer) (Paskeyand Cisneros, 2020).

3. Body decomposition

After death, body composition and structure undergo significant changes, resulting from a complex succession of biochemical processes. The human body decomposition is dictated by destructive processes: autolysis and putrefaction. These two processes lead to cellular death, and leach enzymes, bacteria, and other microorganisms to feed on the unprotected body (Knight, 1996; Wilson-Taylor, 2012).

Autolysis results from the collapse of tissues through an aseptic chemical process, caused by intracellular enzymes, culminating in self-digestion. As a chemical process, it is accelerated by heat and delayed by cold (Clark et al., 1997; Iqbal et al., 2020).

Putrefaction rapidly follows, being closely related to temperature, humidity, and to the physical state of the body (Vass et al., 2002; Hayman and Oxenham, 2016). Putrefaction is often wrongly used as synonymous of decomposition, since it is the one that manifests itself in most cases. It results from the bacterial expansion from the gastrointestinal flora throughout the body, which causes disintegration of the molecules through oxidation and reduction reactions. It is accelerated in septicaemic individuals before death (Pinheiro, 2006).

Skeletonization occurs as soft tissues disappear through putrefactive processes. Over time, the bones and teeth themselves are also degraded, providing information about PMI, for example through the presence or absence of ligaments and the leaching rates of fats and other organic matter (Introna et al., 1999).

Occasionally, the body can undergo conservative processes, caused essentially by environmental conditions or the cadaver physical state, leading to a delay in the decomposition process and resulting in mummification, adipocere or freezing. The evaluation of these modifications can work as an auxiliary method in determining the time elapsed since death, since they tend to happen in an orderly manner (Pinheiro, 2006).

Mummification typically occurs in hot dry environments, even though it can also happen in cold regions with arid conditions (Clark et al., 1997). It refers to the process of desiccation of the soft tissues, causing significant loss of body weight. The skin becomes leathery and hard (Byard, 2020). Mummification frequently takes some time to develop, however it can differ according to influencing factors.

On the other hand, the formation of adipocere, also known as corpse wax, usually occurs in cold wet conditions, when adipose tissues decompose, by the hydrolysis of triglycerides into glycerine and fatty acids (Byard, 2016). It is a soft greyish white waxy consistence substance that becomes an armour-like solid and resistant mass over time, as the fatty acids crystallize (Fiedler and Graw, 2003). Females and obese individuals are more prone to develop adipocere due to its different fat distribution and higher fat content (Dix and Graham, 1999). It takes months or years to occur, although it is occasionally possible to develop within days, emphasizing again the variability that characterizes biological processes (Byard, 2017).

As mentioned before, the action of various extrinsic and intrinsic factors can heavily influence the decomposition process, creating "microenvironments" that lead some bodies to reveal different states of preservation at the same time (e.g. skeletonization of the head, mummification of the limbs, and adipocere in the trunk). These alterations affect the PMI estimation, due to the many possible interconnections between states.

3.1. Stages of decomposition

The decomposition process can be divided into different stages, which vary according to the authors. For example: fresh, bloated, active decay, advanced decay, and dry to skeletonization (Iqbal et al., 2020).

There are successive studies based on cadaveric decomposition. A carrion study of a baby pig was conducted by Payne (1965) recognizing six stages of decomposition for carrion exposed to arthropods and five stages of decomposition for carrion protected from arthropods. Behrensmeyer (1978) described the weathering processes on mammals' bones in Kenya and produced a timetable of weathering, based on her descriptions. Rodriguez and Bass (1983, 1985) stood out for their investigation with human cadavers, and Mann et al. (1990) conducted studies on the impact of carrion insect activity, carnivores, clothing, temperature, rainfall, body weight, trauma, burial depth and body contact surfaces on bodily decay.

Galloway and colleagues (1989) and Galloway (1997) proposed an original method developed on bodies found in the desert, mountains or indoors, providing guidelines to date death based on average decay rates indoor or in open environments. In this regard, five stages were considered within decomposition (fresh, early decomposition, advanced decomposition, skeletonized and decomposition of the skeletonized remains) and, for the first time, descriptive subcategories were listed within each stage (Hayman and Oxenham, 2016).

Fitzgerald and Oxenham (2009) developed a degree of decomposition index (DDI) based on quantifying stages of decomposition. It takes changes in the decomposition process over time into account, assuming that climate variations are already incorporated into the equation (Marhoff et al., 2016).

Janjua and Rogers (2008) and Ross and Cunningham (2011) focused their studies on estimating the PMI using skeletal human remains, an area with fewer studies and more difficulties. They have established, respectively, four and five stages of bone degradation, with different conditions.

Yet, dating time since death through stages of decomposition methods is not suitable for general application, since some decomposition conditions are specific to geographic regions where those studies were conducted. Thus, specific taphonomic data is required to accurately correlate PMI and decomposition (Marhoff et al., 2016).

3.2. The accumulated degree days

Megyesi et al. (2005) published a fundamental study where decomposition was quantified by stages, for the first time, using a scoring system based on Galloway et al. (1989) and on a total body score (TBS), where decomposition was divided into four stages (fresh, early decomposition, advanced decomposition, and skeletonization). The degree of decomposition was scored for different anatomical regions, such as the head and neck, trunk, and limbs. The scores for each region were summed to achieve TBS. For this method, a regression equation was calculated to predict accumulated degree days (ADD) from the TBS (Megyesi et al., 2005). The average daily temperatures of the place where the body was found is crucial for this method (the decomposition of the soft tissues ends when they reach 1285 ± 110ADD), since the day of death is found by the sum of those same temperatures, until reaching the ADD value. Yet, this is a disadvantage for this method, since the temperature provided by the meteorological stations is different from the temperature at the ground surface, the correct one for the application of the formula (Ferreira, 2012). This method should only be used on non-buried, submerged, or burned complete adult human remains. Several other studies predicting ADD for PMI estimation have been conducted, as for example Simmons et al. (2010), Sutherland et al. (2013), Marhoff et al. (2016), and Moffatt et al. (2016).

3.3. Decomposition in aquatic environments

In the United Kingdom, Heaton et al. (2010) aimed to increase the accuracy of *post mortem* submersion interval (PMSI) estimation. They have concluded that the duration and temperature of body's submergence in water had a significant effect on the decomposition process. A single linear regression model (TADS – total aquatic decomposition score) for predicting ADD from observed decomposition resulted from this study. However, this study is strictly related to specific geographical areas and climates (Palazzo et al., 2020). In 2017, van Daalen et al. developed another aquatic decomposition scoring (ADS) method to estimate PMSI of cadavers recovered from salt water, describing specific aquatic decomposition phenomena. Reijnen et al. (2018) investigate if van Daalen et al. (2017) ADS method could be used to estimate PMSI in bodies recovered from fresh water, since decomposition may be affected by salinity or water depth. They have concluded that this method can be accurately used in cadavers discovered in fresh water, emphasizing, however, that bodies found in this context are exposed to more diverse conditions that the ones found in salt water.

3.4. After all, how long does it take for a body to skeletonize?

Time since death can be reasonable accurately estimated through stages of decomposition. Still, the use of these stages can be problematic, since it misleads to the thought that body decay occurs in continuum definable sequences (Byard, 2017; Fitzgerald and Oxenham, 2009). Although decomposition had been considered as exclusively time dependent, it can be affected by several features such as anatomical variation, lifestyle, injuries or environmental conditions.

The major factors influencing the rate of body decay are described by Vass (2011) as being moisture, pH, oxygen partial pressure and temperature.

Temperature is widely considered the most important factor affecting decomposition. It is influenced by depth of burial, indoor or outdoor scene, humidity, elevation, seasons, air movement, presence or absence of clothes, among other factors, namely, humidity, the presence of insects and other fauna (scavenging activity), vegetation development, bacterial growth and the presence of adipocere and mummification (Vass, 2011; Ceciliason et al., 2018). Vass et al. (1992) and Megyesi et al. (2005) suggested that decomposition could be more correctly measured as dependent on accumulated temperature, described as accumulated degree days, in order to achieve a more accurate PMI estimation. Accumulated degree-days are described as the necessary heat energy units to push a biological process, combining chronological time and temperature. Concerning the decomposition process, Vass et al. (1992) established that, assuming that decomposition essentially stops at 0ºC, volatile fatty acids are no longer present in the soil after 1285 ADD, concluding that remains were mainly skeletonized by that time, since muscle and fat had decomposed. They have also highlighted that bone exposure in most of the cadaver occurs around 1200 ADD.

The existence of water close to the human remains can also enhance the decomposition due to the ability of moderating pH changes (acts like a buffer) and acts as a diluent. It is also a source of hydrogen, essential for numerous biochemical reactions (Vass, 2011). According to Mann et al. (1990) it is also positively correlated with insect activity. On the other hand, fast desiccation due to dry environments or extremely wet environments can inhibit decomposition and lead to the preservation of a cadaver (mummification or adipocere, respectively) (Carter and Tibbett, 2008).

In 2011, Vass published "The elusive universal *post mortem* interval formula". This method requires the pre-skeletonization of the remains, in an attempt to develop empirical universal formulae that are based on decomposition and environmental parameters to estimate PMI for surface and burial decomposition.

Decomposition rate may be greatly accelerated by many other factors such as the absence of clothing or bedding, open wounds, endogenous infectious, metabolic conditions or free access of fauna and flora to the cadaver (Byard, 2017). Although very little studies have been carried out concerning decomposition in indoor settings, it is known that, due to the absence of changes in temperature and humidity, and the difficult access by fauna, the decomposition of the remains is slowed down (Ceciliason et al., 2018).

The physical, chemical, and biological characteristics of the soil can also affect body decomposition, whether in case of buried corpses as well as in those left on the surface. The presence of a cadaver disturbs the ecosystem, through changes in the concentrations of chemical compounds such as ammonia, nitrates and sulphates, and variations in the succession of the communities (Carter and Tibbett, 2008).

In addition, late conservative processes such as adipocere (wet conditions and high temperatures) and mummification (dry air and high or low temperatures) turn this dating process even more difficult. While it supposedly takes months, or even years, to occur, sometimes it develops within days after death.

A particularly interesting case of super-fast skeletonization occurred in Amazonia (Brazil). According to Valente-Aguiar et al. (2021), all the muscles and viscera were consumed by action of scavenger ichthyofauna in a few hours, leading to complete skeletonization.

After all, for an accurate estimation of *post mortem* interval, is essential to consider that, regarding the decomposition process, the exception is the rule, meaning that all decaying processes are different.

3.5. Bone weathering

Soft tissue decomposition has been extensively studied in relation to time since death estimation, yet body decay is not finished with skeletonization. Bones' structural breakdown through time has also been investigated, although less information is known regarding this subject (Jaggers and Rogers, 2009). It is a process that comprises the appearance of cracking along the bone and leads to its complete loss of shape and integrity (Ross and Cunningham, 2011).

Macroscopic studies are mainly non-destructive, being able to be repeatedly performed. There are several taphonomic factors leaving strong macroscopic evidence on the skeletal remains, including deposition on or below the surface, burial soil content and pH, vegetation, scavenging fauna, or climatic conditions, like temperature and humidity, affecting bones' rate and extent of decomposition (Haglund, 1997a; 1997b).

The process in which bones are destroyed by natural weather-related phenomena is called weathering. Surface weathering can be a very useful taphonomic process in dating PMI, since bones are exposed to environmental processes like temperature, sunlight, or humidity (Janjua and Rogers, 2008). For example, uncovered skeletonized remains on the ground surface tend to undergo faster decomposition changes. On the contrary, bones buried need more time to breakdown, as the most damaging decaying agents are not acting.

Soil environment has also a significant impact on bone degradation and, consequently, on PMI estimation. The composition and soil moisture can disturb the leaching of bone chemicals, increase the loss of bone mineral and enable the ions exchange to the soil (Jaggers and Rogers, 2009). According to Behrensmeyer (1978), a soil with alkaline pH can also increase bone weathering.

Most of all, it is essential to study weathering by geographical region, as weathering rates differ with environmental changes. The length of time that a skeletonized body takes to decompose is heavily influenced by its micro-environment and context.

Bone size, shape and density are also factors to be considered in weathering rates (Henderson, 1987; Janaway, 1996; Micozzi, 1991). Still, there are evident inconsistencies in the literature relating to this topic and further research on weathering patterns is needed. However, skeletal remains from juveniles seem to breakdown easier than bones of adults, according to Behrensmeyer (1978).

4. Forensic entomology

Forensic entomology is a discipline in which biological and ecological data from arthropods are studied in the investigation of death. One of the usual applications of forensic entomology is the estimation of PMI. Forensic entomologists estimate the time it took for a corpse to become infested by insects and use that information to infer the minimum PMI (Prieto et al., 2004).

Post mortem interval can be calculated through the study of carrion fauna, using developmental data of certain species of insects, like its growth rates, arthropod community composition, dynamics and succession sequence, that are attracted by organic matter decomposition or by putrefaction derived products (Early and Goff, 1986; Tullis and Goff, 1987; Richards and Goff, 1997; Amendt et al., 2007; García-Rojo et al., 2009; Prado e Castro et al., 2011). The first method tends to be applied in initial decomposition stages, with Diptera species as the main indicators, whereas arthropod community composition and succession, mainly represented by Coleoptera species, is usually used in longer PMI (Lane, 1975; Catts and Goff, 1992; Shean et al., 1993; Prado e Castro et al., 2012). The bio-geo-climatic zone has a major impact on the species of insects present, its patterns of succession, their seasonal availability, as well as the periods of time each life stage spends on a cadaver (Prado e Castro et al., 2013; Anderson, 2019).

A comparison of fauna collected in controlled studies in similar environments with the fauna collected on a body at the time of discovery can often deliver information when no other evidence is available, allowing a minimum PMI estimation.

5. Forensic botany

Forensic botany, the analysis of plants in forensic contexts, is a recent discipline and not yet widely used. Nevertheless, botanical trace evidence has been showing its value in legal cases since the presence of plants can be a valuable source of information.

Forensic botanical traces analysis is largely based upon established knowledge of classic techniques of plant ecology, morphology, or biomolecular investigation (Caccianiga et al., 2021; Spencer, 2021). Traditional analysis includes stomach contents analysis for last meal determination and digestion time, pollen analysis, fungal and algae analysis, and DNA (Courtin and Fairgrieve, 2004; Coyle, 2004; Coyle et al., 2005).

The majority of plant studies with forensic interest have been aimed at dating death, since climatological and interspecies variations result in variations in rates and growth patterns (Caccianiga et al., 2021; Courtin and Fairgrieve, 2004). Shedding of leaves, pollination, growth of branches or even the presence of leaves, flowers or fruits, or its decomposition, may be a time indicator (Ciaffi et al., 2018).

Forensic botanists can sometimes estimate the time that a corpse had been in a present location, based on plant anatomy in situ and its stages of development. Although, a maximum time can never be established. The study of growth patterns of vegetation can also be useful in PMI estimation by establishing time windows related to broken tops, lack of chlorophyll, or the length of new shoots at the base of the plant (Coyle et al., 2001).

Palynological evidence has also been studied to establish time since death. After a careful removal from the corpse, pollen residues are identified by forensic palynologists and, according to its life cycle, it is possible to determine the period in which death occurred (Swift, 2006; Lancia et al., 2013; Hayman and Oxenham, 2016). Pollen is a natural seasonal marker, and it can be a valuable record when the season of death is uncertain. However, the airborne pollen grains depend on the local flora, the flowering season of the species, and also on meteorological conditions. Therefore, airborne pollen calendars can be a valuable resource (Montali et al., 2006).

Useful accurate methods for dating cases over years to decades fill a gap in existing techniques. Dendrochronology, which refers to a tree-ring dating, is a well-known and a useful dating method (Speer, 2010). It may be applicable in forensic contexts with plant roots that grow through or over cadavers. An estimation of the minimum *post mortem* interval can be given by counting the root rings associated with the remains (Lancia et al., 2013; Ciaffi et al., 2018; Pokines, 2018).

Bryophytes are particularly advantageous for crime scene investigations since many of them occur in all types of environments (Margiotta et al., 2015). Moss, green algae and lichens growth on skeletonised remains and associated tree roots have similarly been used to establish timeframes (Spencer, 2021).

Mycology studies have collected substantial data relevant to body decomposition, showing that certain chemo ecological groups of fungi can act as above-ground grave markers in forest ecosystems. These fungi undergo a "succession" of fruiting where one set of fungi is later replaced by another which can provide the basis for estimating the *post mortem* burial interval (Carter and Tibbett, 2003; Lancia et al., 2013).

6. Physical and biochemical methods

Over the last decades, new approaches have been developed to accurately estimate PMI in skeletonized remains, using chemical, physical, and immunological techniques. In these cases, attention is drawn to the fact that the bones to be analysed should not undergo any previous treatment, such as maceration.

According to Knight and Lauder (1967; 1969), several chemical methods have been explored, such as nitrogen content, amino-acid quantification, mineral acid reaction, benzidine reaction and bone lipids, based on its decrease on the skeleton over time. However, its application has not been useful in discriminating between bones of forensic or archaeological nature (Forbes and Nugent, 2016).

Facchini and Pettener (1977) suggested a new procedure, built on the measurement of supersonic conductivity and specific gravity of the bone. Castellano and colleagues (1978a; 1978b;1984) evaluated several biochemical characteristics of recent bones such as the amount of total lipids, triglycerides, free fatty acids, cholesterol, total proteins, zinc, manganese, phosphorus, and iron. However, due to the sensitivity of the techniques, and to environmental factors, they were deemed as unreliable and, therefore, the direction of the investigations changed.

The *post mortem* alteration of bone microstructure by the actions of fungi, bacteria and microflora was investigated by Yoshino et al. (1991) as a potential indicator of PMI, but Bell et al. (1996) observations countered the findings.

The residual serological activity (gel diffusion testing), other immunological test, as well as histological tests, were tested and abandoned, due to their susceptibility to soil contamination and high number of false positives (Forbes and Nugent, 2016).

The investigation of metabolic body fluids and biochemical methods, through the study of biomarkers using Nuclear Magnetic Resonance and Mass Spectrometry, can provide valuable information concerning PMI estimation, if the markers in question are properly identified, studied, and documented. However, although they offer advantages over conventional methods, due to the decrease of subjectivity by the observer, becoming more accurate and specific, they require a highly specialized skill set and well-equipped laboratories (Donaldson and Lamont, 2014).

Several studies of PMI estimation based on the citrate content of bone have been proposed (Schwarcz et al., 2010; Kanz et al., 2014; Wilson and Christensen, 2017). The concentration of citrate, which is present in a living human at a uniform concentration, decreases linearly in skeletal remains. It appears to be independent of temperature and rainfall and its precision of determination slightly decreases with age.

6.1. Radiocarbon and other isotopic methods

The only technique capable of providing an unambiguous result estimating PMI, although expensive and elaborated, is radiocarbon dating (Cappella et al., 2018). It is widely accepted as a method for dating skeletonized material with archaeological interest. Between 1950 and 1963, thermonuclear devices, such as the atomic bomb, produced high artificial levels of Carbon 14 in humans and other terrestrial organisms ("bomb pulse"), through the food chain. In 1963, the peak of C14 levels was reached, followed by a reduction of this levels, even though remaining above those existing previously. Thus, by comparison of the "bomb-curve" values, it is possible to distinguish individuals whose death occurred before 1950 from those who were still alive after this increase. This method is particularly valuable for the study of human remains in a high state of decomposition and it can reveal relevant information for determining the date of death of an individual (Ubelaker, 2014). Latest studies suggested the possibility of dating bones from a period after 1950 until today (modern period), using the Carbon 14 method, since C14 levels still remain above those existing previously, and are not yet stabilized (Ubelakerand Buchholz, 2005). If the remains analysed prove to be from the modern period, interpretation can be improved by analysing different types of tissues within a single skeleton, due to the known variability of its formation age and remodelling rates (Ubelaker et al., 2006; Fournier and Ross, 2013), positioning the date of death on the early rising side of the bomb-curve (before 1963) or in the falling side (after 1963). Unfortunately, there are not many studies available providing C14 values for specific skeletal tissues. However, some studies suggested that dense cortical bone, like midshaft femur diaphysis, take longer to remodel than trabecular bone, such as vertebral bodies. Thus, samples taken from these different areas, with varying rates of remodelling, should enable placement on the right side of the bomb-curve (Ubelaker et al., 2022). Nevertheless, this technique shows some difficulties related to the complexity of bone formation. It is reliant on extrinsic and intrinsic factors like environmental conditions, diet, and age at death of the individual (Ubelaker and Buchholz, 2005; Ubelaker et al., 2006).

According to Ubelaker and Parra (2011), age at death represents a feature that needs to be taken into consideration, since it influences tissue's renewal speed (Ubelaker et al., 2015), slowing down with advancing age. C14 values in bones from individuals with older ages at death show a substantial "lag time" between the actual time of death and the bomb curve year correspondent to the radiocarbon value. On the other hand, in younger individuals, the "lag time" is minimal, due to the fastest remodelling rates and recently bone formation (Ubelaker et al., 2022). In addition to Carbon 14, nuclear tests in the 1950s caused an increase in the levels of other radionuclides in the atmosphere, like tritium, caesium-137 and strontium-90. Investigations were carried out to test their usefulness in addressing this issue. However, contamination after burial by groundwater proved to be a problem, since isotopes infiltrating through the soil are absorbed by bones, affecting PMI estimation (Maclaughlin-Black et al., 1992; Forbes, 2004). Its high cost, extremely elaborate methodology and the need for equipped laboratories are also disadvantages (Ramsthaler et al., 2011).

Another major focus of interest is the study of isotopes of elements such as lead-210 (210Pb) and polonium-210 (210Po), radioactive members of uranium-238 (238U), that are widely distributed within the environment. A significant radiation exposure occurs through inhalation and by ingestion of these isotopes within food and water. As these primordial elements are not associated to nuclear explosions, uptake remains relatively constant throughout life, decaying exponentially after death (Swift, 1998; Swift et al., 2001; Forbes, 2004). Swift and collaborators (2001) evaluated the potential of 210Po and 210Pb nuclides usage to deliver an accurate method for PMI estimation. Their study used a sample of male adults, collected from a Lisbon cemetery, and demonstrated a correlation between radionuclide content and time since death. However, antemortem date were unavailable, which meant that factors known to increase 210Po during lifetime (smoking, shellfish consumption, etc.) could not be considered, leading to significant errors. Although these methods seem to provide valid information, they are expensive and must integrate diagenesis effects, cultural differences between regions, and individual variation in lead metabolism (Swift, 1998; Ramsthaler et al., 2009).

6.2. Fluorescence and chemiluminescence

UV-fluorescence has also been investigated to date time since death. According to Ramsthaler et al. (2011), UV reflection proved to be a good forensic exclusion test. When exposed to UV light, recent bone material emits fluorescence, showing a good correlation between the extent of fluorescence and the PMI. When exposed to UV radiation, the most recent bones emit a blue/whitish colour, unlike older remains that acquire a yellow/brownish or grey colour (Hoke et al., 2013;

Swaraldahab and Christensen, 2016). It is a method whose application is designed not only for a widely equipped laboratory, but also to apply in the field, since it only requires a saw and sandpaper for skeletonized remains preparation and a common UV lamp (Hoke et al., 2011). Thus, although for its own it does not present enough scientific value for a safe conclusion, it can easily be used as a presumptive test. Combining UV-induced fluorescence and 490 nm-induced fluorescence was reported by Sterzik et al. (2016) as able to distinguish recent from historical human skeletal remains, being the correlation between time since death and UV-fluorescence colour very similar to the results described in Hoke et al. (2013). UV-fluorescence was also applied by Boaks et al. (2014) to quantify the degradation of bone collagen in order to estimate PMI.

Luminol technique has also been considered as a presumptive test. The chemical reaction that occurs in the presence of Luminol is called chemiluminescence, which refers to the emission of light (Ermida et al., 2017). Luminol has a high affinity for the haemoglobin present in bones, that contains in its composition iron ions, capable of catalysing both the peroxide decomposition reaction and the oxidation of Luminol. The observed light ranges between violet and a blue background (Introna et al., 1999; Creamer et al., 2003; Barni et al., 2007; Creamer and Buck, 2009). Therefore, it would be expected a decrease in the intensity of chemiluminescence reaction with the increase of the PMI (Ramsthaler et al., 2009). One limitation of Luminol technique, as well as other blood detection tests, is its lack of specificity, making it possible to catalyse the chemiluminescence reaction with substances other than blood (Quickenden and Cooper, 2001; Creamer et al., 2005). On the other hand, the fact that it is considered a fast, inexpensive, and simple technique, along with its enormous sensitivity in a dilution scale of 1:100.000 to 1:5.000.000, have strongly contributed to its investigation and application (Ramsthaler et al., 2009).

However, the above mentioned investigations are based on a subjective component. Sarabia et al. (2018), presented an objective low-cost additional technique, using a luminometer to obtain accurate measurements of the chemiluminescence in relative light units (RLU). Through this new approach, it is possible to achieve precise quantification of data to distinguish between forensic interest remains and archaeological remains.

6.3. X-ray diffraction and spectroscopy

X-ray diffraction has been suggested as a hypothesis for many different objectives, being PMI estimation one of them (Prieto-Castelló et al., 2007). Studies indicate that the older the bone, the higher its crystallinity index and, consequently, the sharper the patterns resulting from X-ray diffraction. Based on this assumption, it is possible to develop methods for dating the time since death through X-ray diffraction (Bartsiokas and Middleton, 1992). This is an easy, quick, and economical technique that uses equipment commonly present in laboratories. Also, it values the integrity of the bones, being less invasive.

Numerous studies mention vibrational spectroscopy, particularly Fourier Transform Infrared Spectroscopy (FTIR) and Raman Spectroscopy as reliable to contribute to an accurate PMI estimation (Creagh and Cameron, 2017; Wang et al., 2017; Ortiz-Herrero et al., 2021). Vibrational spectroscopy appears to be a very suitable technique for the analysis of solid materials like bone tissue with forensic purposes, although it largely depends on taphonomic changes (Patonai et al., 2013). It allows the detection of vibrational movements of functional molecular groups and extracts spectral information from compounds directly. It is quick and cost effective, yet sensitive enough, with a simple preparation and a small amount of non-destructed samples (Baker et al., 2014; Kumar et al., 2015; Butler et al., 2016). FTIR delivers auspicious results concerning PMI estimation of powdered bones as it uses infrared radiation, which takes the molecular bonds to vibrate at different wavelengths in the IR region, producing unique vibrational patterns for each functional group (Leskovar et al., 2022). Properties of the remains such as the crystallinity index (CI), mineral-to-matrix and the carbonate-phosphate index of FTIR spectra allow the distinction between archaeological and forensic human bone fragments (Howes et al., 2012; Longato et al., 2015; Wang et al., 2017). Baptista et al. (2022) analysis spreads beyond those previously reported on estimating time since death using FTIR (CI and carbonate/phosphate ratio) since spectroscopic indices such as A-type carbonate content ratio (API), B-type carbonate content ratio (BPI), and carbonate (A + B) to carbonate B-type relationship (C/C) were interpreted. Raman Spectroscopy analysis is also sensitive to *post mortem* changes in bone on a scale of days (McLaughlin and Lednev, 2011; Delannoy et al., 2016). However, a strong fluorescence interference, which can be held by photobleaching (a destructive process), is a disadvantage to this technique. Infrared microscopic imaging techniques, such as IR reflection, ATR, and Raman microscopic imaging, were combined by Woess et al. (2017) to accomplish a more accurate PMI estimation method.

7. DNA

Few investigations have been carried out in order to estimate the PMI through the analysis of the *post mortem* level of DNA degradation. DNA molecule has been recognized to be valuable to investigate early time of death, since DNA denaturation by autolysis begins immediately after death and DNA fragmentation lasts for three days at a constant rate seemingly unaffected by environmental temperature and death mechanisms (Di Nunno et al., 1998; Zapico and Adserias-

Garriga, 2022). Estimating PMI requires a parameter that evolves constantly with a linear process, but some essential aspects are still unknown or less explored, like the definition of target organs for standardization, as well as the influence of *ante* and *post mortem* factors on DNA degradation (Tozzo et al., 2020). Moreover, very little research has been conducted for estimating later *post mortem* intervals.

Although it is no longer used today, the RFLP-DNA method, based on Southern blotting and radioactive probe detection, was the first DNA degradation method used for dating time since death (Bär et al., 1988; Perry et al., 1988).

The usage of dental pulp tissue for flow cytometric estimation of time since death was explored by Boy et al. (2003) for the first time. Although this method proved to be unreliable to estimate early PMI, it appears that DNA degradation is slowed down by factors within the surroundings of the dental pulp, which can be of greater value in studies of the later stages. Long et al. (2005), on the other hand, discovered a correlation between DNA degradation and time since death in dental pulp cells for a longer period, using the same technique.

Real-Time Quantitative Polymerase Chain Reaction methods development in the last years lead researchers to verify if this method could be applied to estimate PMI, due to their highly accurate results. A semi-quantitative multiplex PCR-based analysis was conducted by Alaeddini et al. (2011) in order to evaluate DNA degradation in human ribs. Still, this is a relatively new method on regard to PMI estimation, being too early to establish whether or not it can be valuable to accurately date time since death.

8. Final remarks

PMI estimation is one of the most challenging and complex issues assigned to forensic sciences, as it is an example of an extensively studied process but yet poorly applied to specific cases, especially involving old remains.

In figure 1, we present our proposal approach for cases when the time of death is unknown. In all, a holistic approach is the key role to achieve a reliable estimation of *post mortem* interval. As cadavers may be discovered in numerous decay conditions and circumstances, like inside buildings or cars or in gardens or streets or even floating or under water etc., the importance of a multidisciplinary team, involving professionals from various areas such as forensic anthropologists and pathologists, is crucial to achieve better results. For the same reason, ideally, the forensic pathologist and anthropologist should be able to undertake their human remains examination *in situ*, since the importance of the context should never be disregarded. However, remains are most likely used to be collected by the police. Therefore, it is mandatory that all the team members are aware of their duties and that the chain of custody is respected and no contaminations occur.

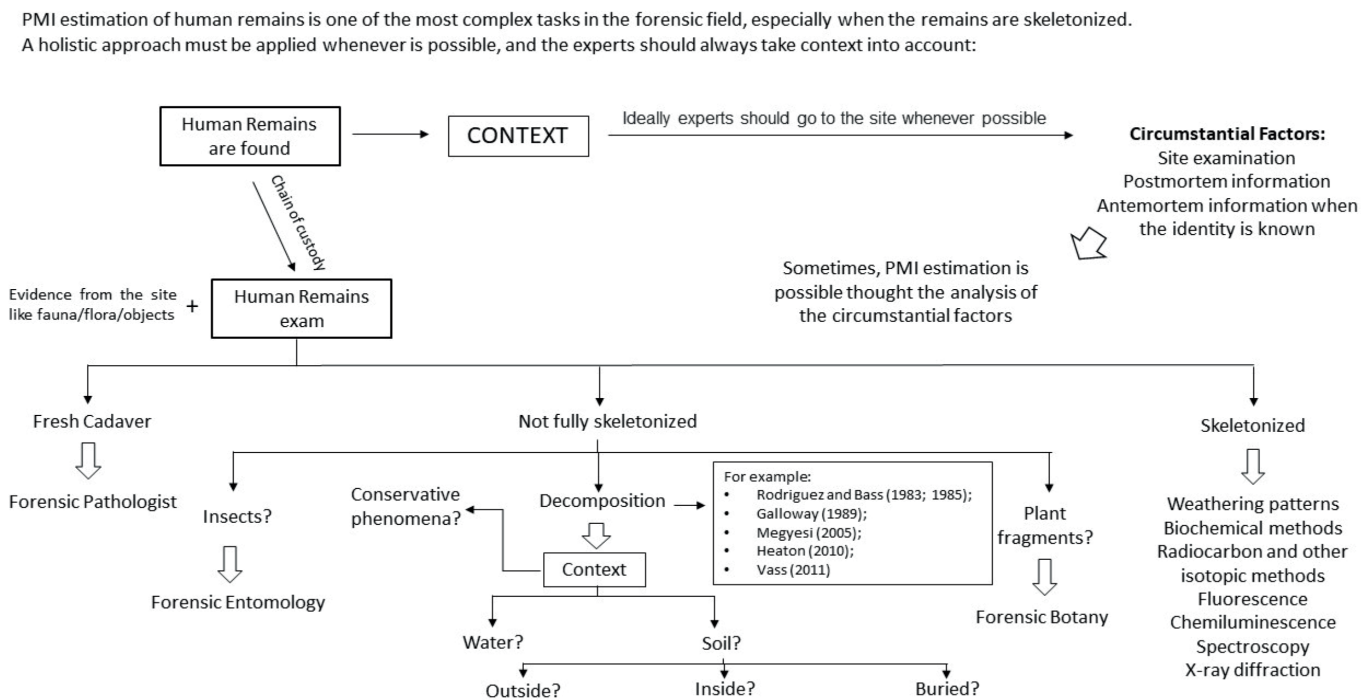

Figure 1. Decision support scheme for PMI estimation.

The decision of the methods selected for estimating PMI, in each particular case, should always be based on the state of decomposition of the human remains. As numerous environmental and intrinsic factors can affect the decomposition process, it is extremely difficult to develop reliable methods to be applied in these cases. Even though the vast literature is still increasing, and several authors have proposed promising results, the majority of these methods never gained practical relevance, since they are not reliable and precise, limiting to describe *post mortem* changes, not providing yet scientific methods to be applied in forensic cases. Thus, most of these investigations end being only of academic interest. It is necessary to make the highest number of thanatological and environmental observations to obtain accurate methods, permitting a quantification of PMI estimation of human skeletal remains. Reliable methods will only gain practical relevance with statistical description and declaration of precision, taking into consideration influencing factors. Forensic anthropologists must continue collecting data and investigating further promising accurate methods to properly estimate late PMI.

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List of abbreviations

PMI – *Post mortem* interval
DDI – Degree of decomposition index
TBS – Total body score
ADD – Accumulated degree days
TADS – Total aquatic decomposition score
ADS – Aquatic decomposition score
PMST – *Post mortem* submersion interval
RLU – Relative light units
CI – Crystallinity index
FTIR – Fourier transform infrared spectroscopy
IR – Infrared

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