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Forensic proteomics for the evaluation of the post-mortem decay in bones

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ABSTRACT

Current methods for evaluation the of post-mortem interval (PMI) of skeletal remains suffer from poor accuracy due to the great number of variables that affect the diagenetic process and to the lack of specific guidelines to address this issue. During decomposition, proteins can undergo cumulative decay over the time, resulting in a decrease in the range and abundance of proteins present (i.e., the proteome) in different tissues as well as in an increase of post-translational modifications occurring in these proteins. In this study, we investigate the applicability of bone proteomic analyses to simulated forensic contexts, looking for specific biomarkers that may help the estimation of PMI, as well as evaluate a previously discovered marker for the estimation of biological age. We noticed a reduction of particular plasma and muscle proteins with increasing PMIs, as well as an increased deamidation of biglycan, a protein with a role in modulating bone growth and mineralization. We also corroborated our previous results regarding the use of fetuin-A as a potential biomarker for the estimation of age-at-death, demonstrating the applicability and the great potential that proteomics may have towards forensic sciences.

Significance: The estimation of the post-mortem interval has a key role in forensic investigations, however nowadays it still suffers from poor reliability, especially when body tissues are heavily decomposed. Here we propose for the first time the application of bone proteomics to the estimation of the time elapsed since death and found several new potential biomarkers to address this, demonstrating the applicability of proteomic analyses to forensic sciences.

1. Introduction

Post-mortem interval (PMI) estimation is one of the most debated themes in forensic sciences, with new studies published regularly but remaining weaknesses in relation to their accuracy and applicability [1,2]. Although some attempts have been made to develop a universal PMI estimation formula applicable to decomposing corpses exposed to the surface as well as to buried bodies [3], this was shown to be affected by regional variables that render it ineffective on a global scale [4]. The complexity of this estimation is due to the fact that after death, bodies experience a complicated set of physical, biological and chemical changes that are subjected to great variability depending on exogenous and endogenous factors including body size, age, pathologies, traumas as well as typical environmental parameters (temperature, humidity, soil composition, etc.), burial depth (if buried) or other burial conditions (such as deposition of the body in air or indoors), accessibility of the carcass to insects and/or scavengers, among many others [5]. Furthermore, the estimation of the PMI may be even more challenging in cases of bodies found in water [6]. With reference to these scenarios, one proposed method to address this issue is the macroscopic and microscopic evaluation of the presence of adipocere in the recovered body. However, this also exhibited some weaknesses, especially when the levels of adipocere were evaluated within the bone structure of bodies with prolonged PMIs [7]. This large number of variables makes the estimation of the PMI a controversial topic, with increasing timescales further reducing its accuracy [8]. Despite the substantial number of investigations aimed at estimating the PMI of a carcass during the first stages of decomposition [8–13], the task becomes much more difficult in more advanced stages of decomposition, particularly when only skeletal remains survive.

The canonical method for the determination of the PMI from bones is based on a visual and external assessment of the progression of taphonomic processes that affect bones, and in particular on the evaluation of the extent of “subaerial bone weathering” that induces cracking and delamination of bones exposed to the open air in a time-dependent way [14]. However, rates of bone weathering are affected by

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the presence of vegetation and by environmental conditions at the locality of deposition (humidity, temperature, etc.) [14], and the evaluation of this parameter is only applicable when bones are exposed on the ground surface. Alternative methods for estimating the PMI include the study of entomological succession to evaluate the time elapsed since death [15], but severe limitations (e.g. no specific insect activity during the dry stage of decomposition [16], weakness of the method when the body is buried in the soil at different depths [15], etc.) leave demand for the development of new techniques to better address this question. Another parameter that can be used is the colour change of bones during the decomposition process, but this may be strongly influenced by several factors including the soil type in which bones were buried and by its mineral composition and the organic matter within it, as well as by the presence of adipocere and/or lipids [17] or the effects of haemolysis [17,18].

More recently, molecular taphonomic approaches have been proposed for the estimation of PMI, for example by assessing the levels of DNA preservation in bones associated with prolonged PMIs [19,20]. However, it has been found that environmental factors (e.g. humidity [19], depth of burial or soil geochemistry [20]) significantly affect the quality of DNA recovered, leaving the field open to new strategies to approach this question. It is also known that microscopic alterations of bone samples may reflect the time elapsed from death, where several recent studies have focused on the application of different visualization methods to investigate the PMI of bone remains, such as infrared (IR) microscopic imaging [21–23], micro-computed tomography analyses coupled with energy dispersive X-ray mapping [23] and fluorescence spectroscopy [24], or a combination of these with traditional approaches [25]. However, the applicability of these methodologies to forensic cases remains limited (e.g. ability to discriminate between forensic and archaeological specimens, but incapacity to accurately estimate PMIs on the order of magnitude of weeks or months), with further studies required. Other approaches have been explored to address this issue, such as the evaluation of the microbial community associated with decaying bones to differentiate between phases of skeletal decay [26,27]. However, the applicability of this method is confined to the distinction between partially skeletonized/completely skeletonized and dry remains [26] or to limited PMIs (48 days as the maximum interval) [27].

1.1. Bone composition and protein diagenesis

Bones are composed essentially of an inorganic matrix (hydroxyapatite crystals (HA), ~50–70%), an organic phase constituted mostly by proteins (~90% collagenous proteins and ~10% non-collagenous proteins and proteoglycans) and by lipids, and water [28–30]. Some non-collagenous proteins (NCPs) and proteoglycans (PGs) can strongly bind the HA matrix (e.g. osteocalcin, bone sialoprotein, osteopontin, fibronectin, decorin, biglycan and fibromodulin) due to the amphoteric nature of the HA that promotes the binding of acidic and basic proteins [31]. This interaction protects proteins from degradation and prevents their disruption after the organism's death [30,31]; for this reason, they are also found frequently in archaeological specimens [30,32,33]. Other proteins frequently found in bone samples, including archaeological remains, are abundant plasma proteins such as albumin [34,35], prothrombin [33,36], haemoglobin [37] and coagulation factors [33].

Despite the great longevity of these biomolecules in bone [38,39], the complexity of the bone proteome decreases with geological time due to the leaching of minerals and proteins to the burial environment [40,41], the latter related to the hydrolysis of peptide bonds that are thermodynamically unstable and that makes proteins to be more fragmented and damaged [42]. In particular, bone collagen hydrolyses over time, and after ~10,000–30,000 years only a little intact collagen remains (except for specimens deposited in cold and/or dry environments) [42]. Some other NCPs are thought to be more resistant to hydrolysis than collagen, and they may be successfully recovered even when only little intact collagen is still present [43]. Wadsworth and Buckley [39] used shotgun proteomics to demonstrate how the complexity of the bone proteome can become reduced in ancient bones, focusing attention on NCPs and exploring the role that the thermal history has on the degradation of these biomolecules [39]. In addition to protein hydrolysis, cumulative protein damage through deamidation is also observed [44]; in particular, this non-enzymatic post-translational modification (PTM) occurs naturally in asparagine (Asn) and glutamine (Gln) residues, which are converted respectively into aspartic acid (Asp) and glutamic acid (Glu) both *in vivo* [45,46] and post-mortem [47–49]. Promisingly there appears to be some correlation between the glutamine deamidation ratios of peptides from several different proteins in archaeological specimens and their geological age [50].

However, in contrast to our knowledge of protein decay over long periods of archaeological and geological time, little is known about the protein degradation in buried bones in shorter, forensic timescales. Although we have previously utilised proteomic analyses to look for specific biomarkers for age-at-death (AAD) estimation in a simulated forensic scenario [51], there are no studies linking the proteomic profiling of forensic bones with known PMIs. Therefore, the aims of the work presented here were to evaluate the applicability of proteomic analyses to simulated forensic burials with different PMIs in order to explore bone diagenesis from a proteomic perspective and to seek specific biomarkers that may be useful for PMI estimation of skeletal remains.

2. Material and methods

Experimental burials were conducted at the HuddersFIELD outdoor taphonomy facility (University of Huddersfield, UK), situated on grassy farmland in West Yorkshire (UK) with a soil pH ~7.2. The experiment was conducted following the guidelines of the U.K. Department for Environment, Food and Rural Affairs (DEFRA). Daily temperatures and rainfall measurements were obtained from the local Weather Station (Watson W-8681) which is situated ~5 km northwest of the burial site. The weather station collects data every 30 s and updates the website www.weatherforce.org.uk, in which daily records from 2010 to the present day are available to the public (Supplementary Figs. S1 and S2).

Four juvenile pig (*Sus scrofa*) carcasses (mean weight = 8.1 kg, approximate age at death = three to five weeks) were obtained as a commonly used analogue for humans in decomposition studies [52,53]. The pigs, which had died naturally from unknown causes, were frozen within 48 h of death and defrosted 24 h before the start of the experimental burials. We consider the PMI as set to zero on the day in which all of the carcasses were buried under ~40 cm soil, located ~90 cm away from each other to reduce the likelihood of cross-contamination. The pigs were protected by steel gabion cages that were buried inside the graves to limit the action of scavengers; each cage was also wrapped in fly netting to limit the activity of small rodents or birds on the buried bodies, while still allowing access by arthropods. Data loggers (Tinytag Plus 2, Gemini Data Loggers) with probes were used to constantly measure the temperature of the soil in contact with each of the four carcasses. Lysimeters, each under 75 psi pressure, were also placed inside the cage in contact with the soil in which the pigs were buried to allow the regular extraction of leachate from the soil surrounding the pigs. The graves were completely infilled with soil, in order to mask the burial site and to better simulate a forensic scenario. To reduce the potential damage due to the activity of scavengers, each grave was covered with a wooden palette that allowed the rainfall water to reach the soil but that prevented the area to be accessed by scavengers or by unauthorized personnel.

The four carcasses were left in the soil for different time intervals (see Table 1); after the selected time interval, the bodies were partially excavated to collect one of the two tibiae, keeping the disturbance of