Significance

The determination of the date of death from bone remains is of scientific interest but also has important legal implications since it would be of enormous help to authorities in judicial investigations. Most techniques focus on identifying postmortem interval identification in the early hours. In many times the appearance of bones in which it is necessary to clarify the possible circumstances of a possible criminal act, and knowing the potsmortem interval is one of the fundamental keys. Several methods have been described for this purpose, the most common being based on the structural and compositional modifications that take place in the bone after death. In our knowledge this is the first study that associates the protein profile of the bone with the postmortem interval.

*Graphical Abstract

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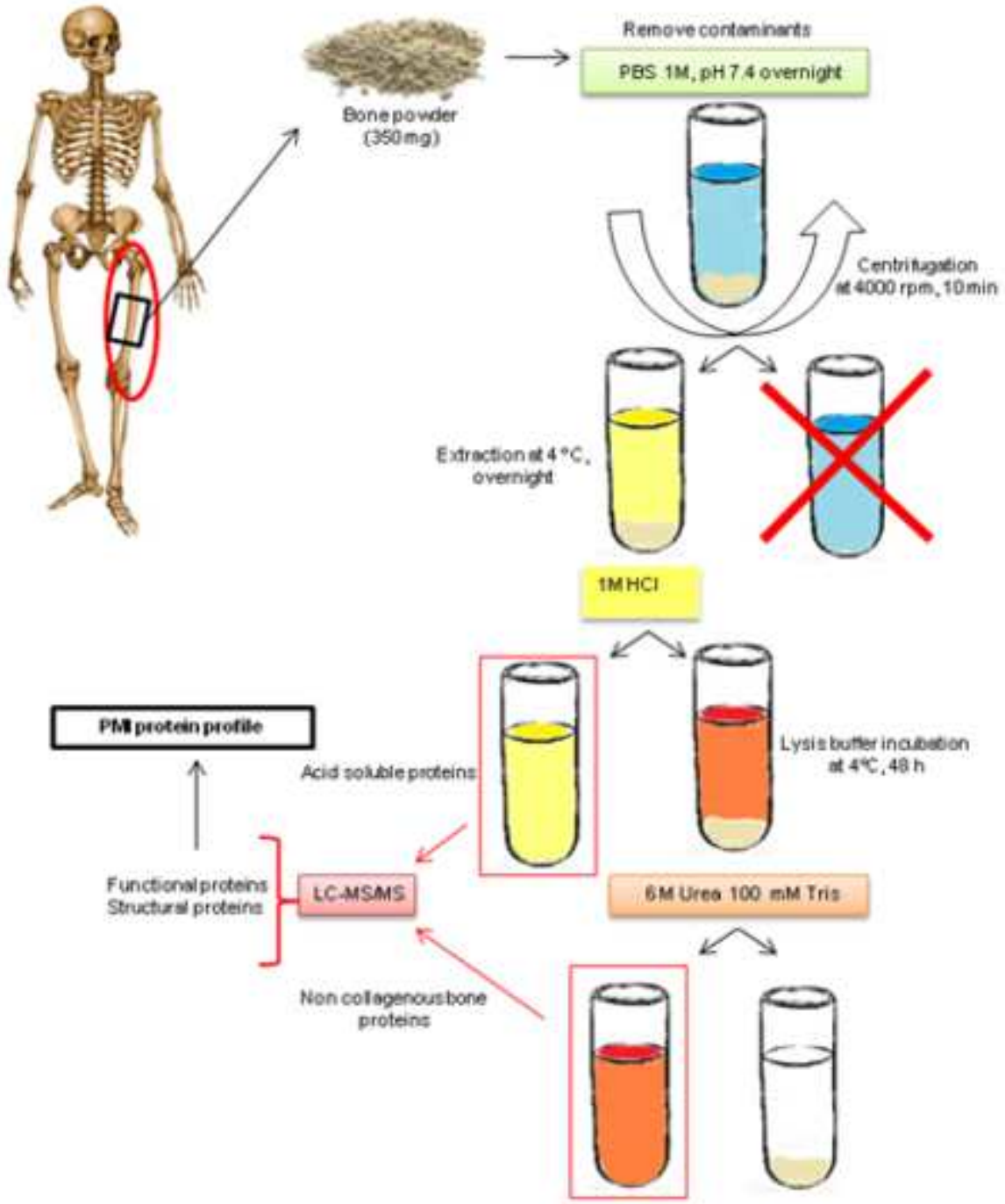

Highlights

- Postmortem interval (PMI) is the most difficult aim in Forensic Sciences.
- There aren´t many forensic techniques for estimating the date of death on bones.
- Bone is a good source of biomolecules due to its composition and structure.
- Protein profile in bone can be use a complementary technique for PMI estimation.

Association between protein profile and postmortem interval in bone remains

1. Introduction

In Forensic Sciences, proteomic techniques in bones are increasingly being applied to relate their biological age with the corresponding postmortem interval (PMI) [1]. Death is usually considered a fact, but it is actually a process that begins shortly before death and involves numerous changes in the body. There are five stages in body decomposition: Fresh, bloated, decay, post-decay, and skeletal [2], and therefore there are different methods for estimating the date of death for each of these periods [3].

Currently, the most often used techniques for establishing the PMI are temperature, postmortem muscle excitability and the variation of different chemical or biochemical substances in fluids (mainly vitreous humor, but also blood, pericardial fluid, among others). Pathologists try to minimize the error in the estimation of PMI by combining two or more approaches for each case. The most precise and most frequently used method, temperature, can only be used until 36 h postmortem (hpm) (early period), depending on individual and environmental conditions. However, in the case of skeletal human remains, all these techniques that are valid for early postmortem periods are impossible to use [3,5].

Techniques used by forensics experts to estimate PMI in the case of bone remains are based on morphological, chemical, physical and histological methods, which are increasingly accurate and involve measuring bone protein, weathering and DNA degradation [6,7]. In forensic osteology contexts, the morphological analysis of bones can be used to designate the sex, age and other individual characteristics of a victim as well as to infer the potential cause of death; however, other microstructural and biomolecular properties are increasingly used to study post-mortem changes [1,2]. There are numerous research studies studying new techniques that can be applied to bone remains such as loss of nitrogen, citrate content [8,9], immunologic activity [10],analyzing radionuclide tests [11], spectroscopy methods, luminescence [6], luminol tests [12], UV-induced fluorescence test [11,13], ultraestructural changes [3], decomposition pattern [14-16], micro-computed tomography [17] and Infrared spectroscopy and raman spectro-microscopy [18]. There are also publications that analyze various proteins in bone and in other matrixes to establish a pattern of decomposition after death and to study the profile of various disease [4, 19-32].

However, to our knowledge, there is no research that studies the protein profile in human bone remains in relation to various late postmortem intervals. Our objective, therefore, was to attempt to know whether there is a protein pro file in human bone remains that would enable a late postmortem interval ranging from 5 to 20 years postmortem to be estimated. Any findings in this respect would have important legal medical repercussions for completing investigations when bone remains are discovered.

2. Materials and methods

2.1. Bone samples

Compact bone femoral fragments (similar position in all cases) were obtained from the proximal femur of 40 cadavers (33 male and 7 female, age range 20-93, data range 5-20 years) (Table 1). The bones were removed from niches of a cemetery in Murcia, a city in the southeast of Spain, an area of very low rainfall. Winters are mild with temperatures that range between 5 and 19 °C, while summers are hot with temperatures that range between 22 and 40 °C. The cemetery is situated in a dry area and on soil which is rich in lime and gypsum. The bones had lain in niches for documented times of between 5 and 20 years. The date of the death was obtained from the cemetery registry and was later checked against civil registry documents. The cadavers, which were entirely skeletal, were buried in such a way that the bone remains did not come into contact with soil. As a result, the processes that usually occur after burial, including chemical soil factors that induce transformations of the bone mineral matrix were minimal. Taking into account that the bones were identified and authenticated using totally reliable data, no bone reference standards were used, although internal standards were used throughout in order to check the reliability and precision of the analyses.

Cases (n = 40) were distributed in three PMI categories: Group 1: 5-9 years post mortem, (n = 8); Group 2: 10-15 years post mortem (n = 12), group 3: 16 to 20 (n = 20).

2.2. Protein Extraction and Proteolysis

The protocol followed for the extraction of bone proteins from the studies is that described by Perez-Martínez et al. [24], Cleland et al. [33], Jiang et al. [34], with modifications to increase extraction performance [10] and interpretation of the results [35-38].

The bone slices were ground in a ball mill (Tehtnica Milmiz 20) to facilitate the extraction of bone organic constituents. First, 350 mg of each sample of bone powder was washed to remove contaminants with 1M phosphate buffer saline (PBS), pH 7.4 overnight at 4 °C. After washing, to improve the extraction performance any inorganic material was removed from the bone. The samples were centrifuged for 10 min at 4000 rpm in a benchtop microfuge. The supernatant was discarded and the pellets were collected for extraction at 4 ºC overnight in 1 M HCl. The resulting supernatant (acid-soluble proteins) was collected for further analysis. The pellet was washed with water and extracted for 48 h at 4 ºC in lysis buffer containing 100mM Tris and 6M Urea at pH 7.4, to extract water-insoluble non-collagenous bone proteins closely associated with hydroxyapatite crystallites in the mineralized matrix. Finally, trypsin was added (trypsin/protein 1:20-1:100), to both supernatants and the solutions were incubated at 37 ºC for 20 h. The tryptic digestions were desalted with C18 solid-phase cartridges.

2.3. Liquid chromatography tandem mass spectrometry (LC-MS/MS)

A 40 µl volume of each tryptic digestion sample was analysed by LC/MS/MS, on an Agilent 1100 series (Agilent Technologies, Santa Clara, CA, USA) coupled to a mass spectrometer (120 k resolution, full scan, positive mode, normal mass range 350–1500). Peptides mixtures in the sample were separated on a Waters Xbridge BEH300 C18 column (column dimensions 150 mm x 1 mm i.d., 5 µm), using a gradient from 0% B (0.1% formic acid in acetonitrile) to 80% B 180 min at a constant flow rate of 10µlmin^{-1}. Then, peptides were automatically selected for fragmentation by data-dependent analysis; AutoMS (Agilent Ion Trap XCT Plus mass spectrometer), product ion scans, rapid scan rate, Centroid data; scan event: 1000 count minimum signal (threshold, top 3) were acquired per cycle, dynamic exclusion was employed, and 1 repeat scan (i.e. two MS/MS scans total) was acquired in a 30s repeat duration with that precursor being excluded for the subsequent 30s activation: collision-induced dissociation (CID), 2+ default charge state, 2 m/z isolation width, 35 eV normalized collision energy, 0.25 Activation Q 10.0 ms activation time).

2.4. Data Analysis

Peptide masses obtained by LC/MS/MS were searched against the National Center for Biotechnology Information (NCBInr) *Homo sapiens* (human) database (July 2015 version) for matches to primary protein sequences using the Data Analysis for LC/MSD Trap Version 3.3 (Bruker Daltonik, GmbH, Germany) and Spectrum Mill MS Proteomics Workbench (Rev A.03.02.060B, Agilent Technologies, Santa Clara, CA, USA). The mass tolerances were 2 Da for parent masses and 1 Da for fragment masses. Peptides

were considered as positive identification using the following parameters for the "protein details": SPI > 70% for matches with score >8 for +1, >7 for +2, and >9 for +3; SPI >90% for score >6 on +1. A second autovalidation step was carried out in "peptide" mode using score criteria >13 and SPI >70%. In addition, both autovalidation steps required a forward-reverse score of >1 for +1 and +2 and >2 for +3 peptides. The validated peptides were used to identify a set of proteins from which a result file was created. A second round of searches with unvalidated peptide spectra was performed against the set of proteins in this results file, using a no-enzyme (unconstrained) search to identify possible non-specific or semitryptic peptide fragments. All database matches above the threshold score of 3 were summarized. At an estimated false-positive identification rate of <5%, a total of 275 proteins were identified in the study (Supplementary Table 1).

2.5. Analysis, selection and characterization of proteins

The proteins that can be found in the organic part of bone include different types of collagen and proteoglycans (PGs), which are regarded as structural components. Non-collagenous proteins and bone specific proteins, which might play a role during the mineralization process, or exhibit a broad array of functions including the control of cell proliferation, cell-matrix interactions, and mediation of hydroxyapatite [39]. Proteins circulating in blood vessels such as growth factors, cytokines, related to the body's defense functions, are absorbed into the bone matrix after death as a result of the cessation of circulation. Therefore, their presence is occasional and is conditioned by intrinsic factors of the person, as well as by any previous conditions and pathological processes suffered, so it would not provide great information for the estimation of the date of death. For this reason they are excluded from this study. However, the information they provide is very valuable for knowing any pathologies suffered and the preconditions of the subject [24].

The proteins included in this study are structural (collagen and proteoglycans) and functional, and those that participate in bone processes (non-collagenous proteins and bone specific proteins), since their presence is not eventual and does not depend on the intrinsic factors of the subject.

In order to facilitate interpretation of data, the proteins included in the study will be grouped into 3 groups according to the postmortem interval they characterise: group A (5-9 years postmortem), group B (5-15 years postmortem) and group C (5-20 years postmortem). To assign a functional category to each of the proteins, The Ontology GoMiner Program and STRING 10.5 was used (Supplementary table 2).

2.6. Statistical analysis

The SPSS 20.0 package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the data. The Friedman test, a non-parametric test for more than k related samples, was used to compare groups of data and the aminoacid values and molecular weight. p values less than 0.05 were considered statistically significant.

3. Results

3.1. Quantification of protein fragments in Bone Based Spectrum Mill

A total of 275 circulating, functional and structural proteins were found for the postmortem interval considered, which we have grouped into the established PMIs and then compared. It was observed 114 proteins are found for the whole PMI considered (5-20 years postmortem), among which are circulating, structural and functional proteins. In addition to the these, 7 new proteins howere identified exclusively between 5 and 15 years postmortem. And in the 5-9 PMI, in addition to the above, 154 proteins detected in longer PMIs were found (Fig. 1).

3.2. Characterization of Proteins Identified as being unique to each data group

Of the 275 total proteins, we excluded the circulating ones (n=227), leaving a total of 48 proteins (32 structural and 16 functional).

The following functional proteins persist until 20 years postmortem (group C): Protein jagged-2 (JAG2), Mediator of RNA polymerase II transcription subunit 12 (MED12), Fibroblast growth factor receptor 1 (FGFR1), cadherin-11 (CDH11) and histone H2A deubiquitinase MYSM1 (MYSM1). The functional proteins that persist until 9 years postmortem (group A) are: annexin II receptor (ANXA2R), SUN domain-containing ossification (SUCO), SOX-6 isoform 4 (SOX6), insulin-induced gene 2 protein (INSGIG2), CST complex subunit CTC1 (CTC1), Homeobox protein DLX-5 (DLX5), Transcription factor Maf (MAF), Catenin beta-1 CTNNB (CTNNB), Sulfate transporter (SLC26A2), and zinc finger CCHC domain-containing protein 12 (ZCCHC12).

The structural proteins found up to 20 years postmortem (group C) are: Collagen alpha-1(I) chain (COL1A1), Collagen alpha-2(I) (COL1A2), Collagen alpha-1(II) chain (COL2A1), Collagen alpha-1(III) chain (COL3A1), collagen alpha-2(IV) chain preproprotein (COL4A2), Collagen alpha-1 (V) (COL5A1), Collagen alpha-2(V) chain (COL5A2), collagen alpha-1(VII) chain precursor (COL7A1), Collagen alpha-1(IX) chain (COL9A1), Collagen alpha-2(IX) chain (COL9A2), Collagen alpha-2(XI) chain (COL11A2), Collagen alpha-1(XI) chain (COL11A1), alpha 1 type XXIV collagen

precursor (COL24A1), collagen alpha-1(XXVII) chain preproprotein (COL27A1), Fibrillin-1 (FBN1), Scavenger receptor cysteine-rich type 1 protein M160 (CD163L1) and Transcriptional protein SWT1 (WNT11), Peroxidasin homolog (PXDN), A disintegrin and metalloproteinase with thrombospondin motifs 17 (ADAMTS17), A disintegrin and metalloproteinase with thrombospondin motifs 18 (ADAMTS18), Pappalysin-1 (PAPPA), Laminin subunit alpha-2 (LAMA2), bone morphogenetic protein 5 preproprotein (BMP5), phosphoethanolamine/phosphocholine phosphatase isoform 1 (PHOSPO1). The structural protein persisting until 15 years postmortem (group B) is laminin (LMNA) and those that persist until 9 years postmortem (Group A) are Alpha-2-HS-glycoprotein (AHSG), Procollagen C-endopeptidase (PCOLCE), Cartilage intermediate layer protein 1 (CILP), Cartilage intermediate layer protein 1 COMP, ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPP1), amphiregulin preproprotein (AREG), Mucin-15 (MUC15), Collagen alpha-1(X) (COL10A1).

To summarize, a total of 18 proteins disappear after 9 postmortem years: (AHSG, ANXA2R, CILP, COL10A1, COMP, MUC, PCOLCE, SUCO, SOX6, INGISG2, CTC1, DLX5, MAF, CTNNB, ENPP1, SLC2GA2, AREG, ZCCHC12). For its part, Laminin (LMNA) disappears after 15 years postmortem, while 29 proteins persist throughout the 20 years postmortem studied: COL1A1, COL1A2, COL2A1, COL3A1, COL4A2, COL5A1, COL5A2, COL7A1, COL9A1, COL9A2, COL11A2, COL11A1, COL24A1, COL27A1, FBN1, CD163L1, WNT11, PXDN, ADAMTS17, ADAMTS18, PAPPA, LAMA2, BMP5, PHOSPO1, JAG2, MED12, FGFR1, CDH11 and MYSM1 (Fig. 2).

3.3. Aminoacids and Chemical analysis data profile

Analyzing the average mass of the proteins of each group of data, we observe that the proteins that remain until 20 years postmortem are those with the highest molecular weight (average mass of proteins 161239.04 Da). The proteins that characterize the interval from 5 to 15 years have an average mass of 161165.68 Da and those that belong to the most recent interval (5-9 years) have an average mass of 109600.61 Da. For a complete list of identified peptides see Supplementary Table 3.

Studies suggest that the postmortem stability of bone proteins depends on their electrical charge [39-42]. In our study each amino acid is classified as acidic (D,E), basic (R,H,K) or neutral (N,C,Q,G,S,T,Y). The total, acidic, basic and neutral amino acids decrease number, but the amino acids with the least pronounced decrease are the acids ones (Fig. 3).

When each type of aminoacid values and molecular weight were compared by Friedman test for different PMIs, statistically significant differences were found between neutral (Friedman test, P = 0.039), basic (Friedman test, P = 0.047), acid (Friedman test, P = 0.05), molecular weight (Friedman test, P = 0.05) at 5-9 years, 5-15 years, 5-20 years postmortem.

4. Discussion

Proteomics is opening up new applications in forensic sciences, which can apply different recent postmortem interval matrixes to establish the cause of death, studying patterns of decomposition or identify markers of disease. This has already been extensively studied in other branches of science such as bioarchaeology and palaeopathology on skeletal remains of various species Establishing a protein profile in bone can be a good complementary technique for PMI estimation Establishing a protein profile in bone can be a good complementary technique for PMI estimation [29,43-46], such as mammoth femurs [47], dinosarus [48-50], cattle subsfossil spcimens, or archaelogical human remains [43, 51-55], in order to evaluate the survival and protein recovery, establish relationships between species, study disease evolution [30,49,51,55-68], culture and the environment [69, 70].

In forensic sciences one of the ideal matrixes as a source of proteins in late postmortem intervals is the bone, which acts as a good reservoir of biomolecules due to its composition, since it is made of inorganic and organic fractions. The inorganic part is calcium hydroxyl apatite (HA) with additional substances such as carbonate, fluoride, sodium, magnesium, and others ions. The organic fraction (about 20-30 wt%) is composed of proteins and lipids. The mineral matrix protects proteins from degradation, especially collagen, thus allowing the recovery of large peptides from fossil bones over centuries or millennia [54]. This is due to the high density of carboxyls on the surface of the acid proteins that allows strong bonds to be created with hydroxyapatite, forming a stable whole, which acts as a black box by conserving biomolecules for long periods of time. This interaction is specific to acidic proteins, but in particular to proteins with carboxyl groups. Hydroxyapatite is a mineral whose surface of crystals presents a mosaic of rigidly fixed points of calcium and phosphate. The calcium ions present in the medium only reinforce binding by allowing the formation of additional bridges between protein carboxyl residues and the phosphate of the HA. The union of hydroxyapatite with Ca suggests that a configuration typical of protein groups that bind may be necessary for the that union [39,40,71,72]. Moreover, protein-hydroxyapatite interactions are a function of the net protein electrical charge,

either acidic or basic, although the participation of both is not the same [73,74]. Therefore, this would result in a given group of proteins persisting strongly bound to hydroxyapatite, while others disappear.

In our study, the proteins that we observed to persist for up to 20 years postmortem are mostly whose main biological function is the support and maintenance of the bone: COL1A1, COL1A2, COL2A1, COL3A1, COL4A2, COL5A1, COL5A2, COL7A1, COL9A1, COL9A2, COL11A2, COL11A1, COL24A1, COL27A1, FBN1, CD163L1, WNT11, PXDN, ADAMTS17, ADAMTS18, PAPPA, LAMA2, BMP5, PHOSPO1, JAG2, MED12, FGFR1, CDH11 and MYSM1. These proteins acquire a negative predictive value, since their absence would indicate a state older than 20 years postmortem.

The protein that only persists until 15 years postmortem, which limits its practical application, is LMNA, which is required for osteoblastogenesis and bone formation, while the proteins that define the protein profile up to 9 years postmortem with a positive predictive value (their absence allows the remains to be assigned to this interval and not a longer one) are AHSG, PCOLCE, CILP, COMP, ENPP1, AREG, MUC15, COL10A1, ANXA2R, SUCO, SOX6, INSGIG2, CTC1, DLX5, MAF, CTNNB, SLC26A2, and ZCCHC12.

Of the 48 total proteins that form this protein profile, 21 participate in biological processes of the bone, 17 have a molecular function and 20 are cellular components of the bone (Supplementary Table 2).

Therefore, it can be said that the biggest proportion of proteins (86.2%) that persist until at least 20 years postmortem are related to the support and structure of the bone, most being fibrillary, forming trimers and are part of the matrix region contributing to bone stability through strong bonds. Meanwhile, representing a very low percentage (13.8%) are those proteins related with regulatory or enzymatic processes.

In the IPMI protein profile up to 9 years postmortem CILP, COMP and COL10A1 are the only proteins that participate in extracellular matrix processes. MUC 15 plays a role in cell adhesion to the extracellular matrix and PCOLCE binds to the C-terminal propeptide of type I procollagen and enhances procollagen C-proteinase activity. Also present are proteins that intervene in different processes of specific bone cells, such as, osteoclast formation (ANXA2R), regulation of osteoblast differentiation (CTNNB1, FGFR1, AREG, SUCO), bone formation (SOX6, SLC26A2, AHSG, DLX5) and regulatory functions (INSIG2, CTC1, MAF, CTNNB1, ZCCHC12, ENPP1). Therefore, in this PMI, the predominant protein profile is that of proteins that participate in the

formation and regulation of bone processes (72.2%), and not in their structure or binding, which are a minority (27.8%).

We agree with Doherty et al. 2009 that proteins with a higher molecular mass are the most stable. These authors observed that small proteins were rapidly degraded in the cell, although he found no correlation between degradation and molecular weight, similarly to Gorbunoff [42]. Unlike Doherty et al. [42], Seo [76] noted that the more resistant proteins are the acidic ones, while the basic and neutral ones disappeared most quickly. We observed that the acidic amino acids decrease less rapidly, coinciding with Welker [75] who also noted a decrease in acidic, basic and neutral amino acids. This may be due to the fact that in an acidic environment such that existing after death, the proteins that hydrolyze least are the acid proteins. The decrease in pH can lead to the denaturing of proteins together with degradation processes, and, as indicated in another study, denatured proteins do not bind to HA [42]. It has also been observed that the lower pH blocking of carboxyls strengthens the binding of both basic and acidic proteins to HA. Thus, on basic proteins the density of carboxyls is not sufficient to permit their binding to HA once their positive charges have been abolished [74]. Also postmortem processes and bacterial action produce a degradation of proteins and therefore of amino acids [76]. According to our results, the persistence of proteins is more probably due to their structure and not so much to their electrical charge.

Unlike other authors [36,77-79], we did not find biglycan, matrix Gla protein, alkaline phosphatase, osteopontina, osteocalcina or bone sialoprotein. This may be because the kinetics of bone tissue degradation depends on the preservation, while these proteins have been found in skeletal remains of various species preserved in cold or dry climates [60,64].

In our study, individual differences in bone matrix composition and burial conditions may partially limit the application of these analyses, even though the samples analysed were randomly obtained in an arid climate. The first problem would be extrapolation of our results to skeletal remains found in very different environments from ours. For the practical application of protein analysis in the estimation of postmortem interval it would be necessary to include complementary tests to characterize the integrity of the bone and expand the number of samples and their postmortem interval.

5. Conclusion

The proteins that present a greater presence of proteins in the bone along the PMI studied are proteins that correspond to proteins with structural function in bone and with high molecular weight. The proteins that have shown less stability are functional proteins and with lower molecular weight.

The relationship between present and absent proteins can be useful for the estimation of the PMI, since we have found a statistically significant relationship between the absence of diverse proteins which will allow to be a complementary data for the estimation of the PMI.

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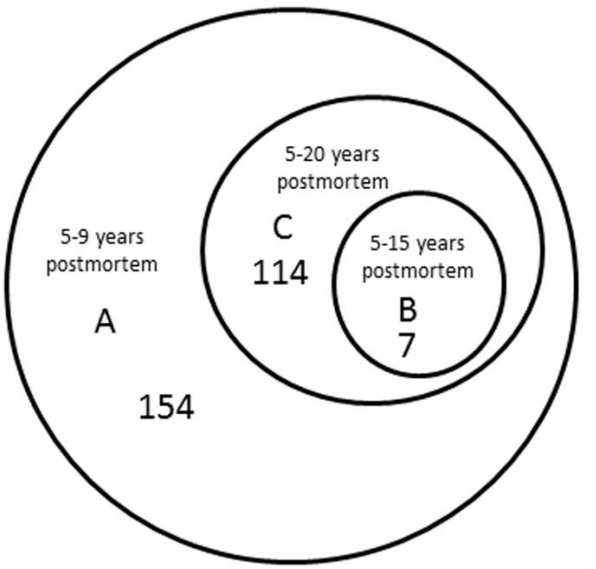

Figure 1. Diagram showing overlap of totals proteins for each post mortem interval.

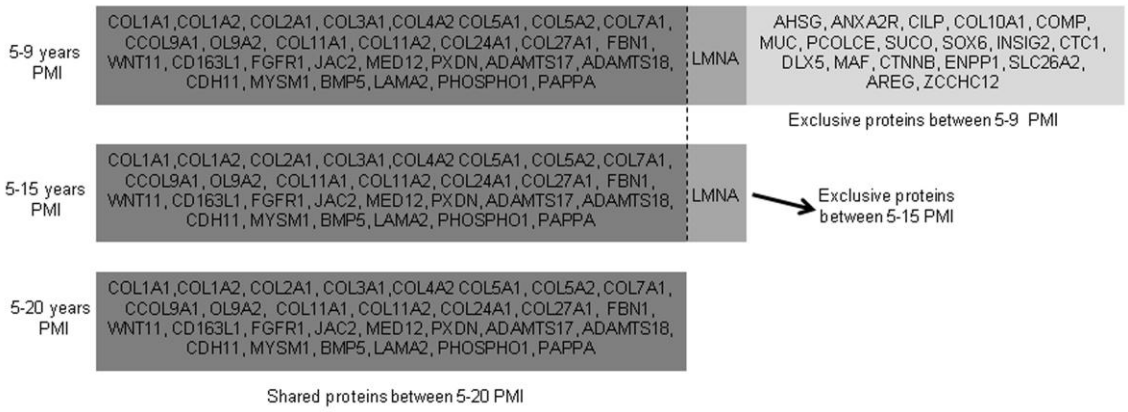

Figure 2. Diagram showing exclusive and overlapping proteins of each post mortem interval.

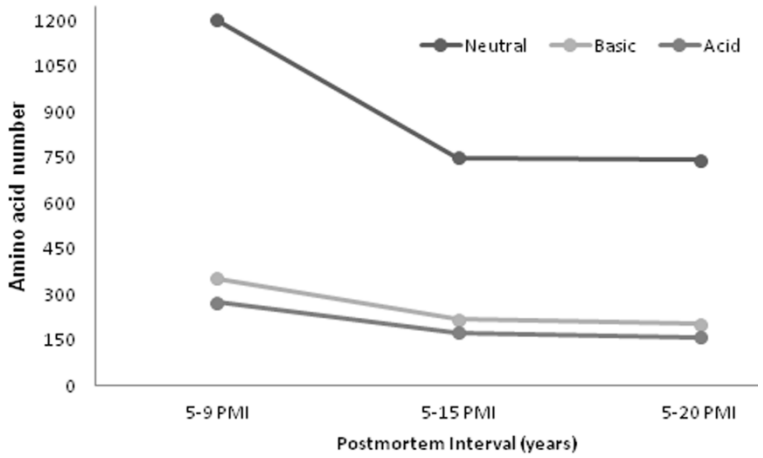

Figure 3. Trend of neutral, basic and acidic amino acids with postmortem interval.