University of Évora

ARCHMAT
(ERASMUS MUNDUS MASTER IN ARCHaeological MATerials Science)

Mestrado em Arqueologia e Ambiente (Erasmus Mundus –ARCHMAT)

Exploring Chalcolithic diet and mobility of humans and animals from Perdigões site

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Évora, September 2016

A tese não inclui as criticas e sugestões do Júri

ABSTRACT

Chalcolithic diet at Perdigões (Reguengos de Monsaraz) was investigated using stable carbon and nitrogen isotope analysis of human bone collagen. Strontium isotopes of faunal dental enamel were used to establish the site local range and to distinguish the presence of non-local individuals.

ATR-FTIR and EA analysis were used to determine the degree of bone diagenesis and to evaluate the reliability of the stable isotopic composition for paleodietary reconstruction.

The individuals from which paleodietary results were obtained had a diet based on C_3 terrestrial resources and some animal protein. Data from Perdigões site, was compared with the published data from other Iberian Chalcolithic populations. Site comparison revealed that diet through Iberian Peninsula have mainly maintained terrestrial dietary focus consistent with animal husbandry and farming on C_3 plants with occasional intake of freshwater or marine resources.

Strontium isotopic composition of enamel revealed that some of the individuals from Perdigões site were non-local.

Keywords: paleodiet, mobility, isotopes, Chalcolithic, diagenesis, Perdigões

Resumo

A dieta de indivíduos do período Calcolítico dos Perdigões (Reguengos de Monsaraz) foi investigada através de análise de isótopos estáveis de carbono e azoto no colagénio ósseo. Isótopos de estrôncio do esmalte dentário de fauna foram utilizados para estabelecer o sinal local dos Perdigões, permitindo distinguir a presença de alguns indivíduos e fauna não-locais.

A avaliação da diagénese óssea foi efectuada através de análises realizadas com analisador elementar e com ATR-FTIR, de forma a validar dos resultados isotópicos obtidos.

Os indivíduos para os quais foi possivel obter resultados isotópicos de carbono e azoto apresentam uma dieta baseada em recursos terrestres, plantas C_3, e alguma proteína animal. A comparação efectuada com outros sitios arqueológicos da Peninsula Ibérica revelaram que a dieta das populações é sobretudo feita à base de plantas C_3, com ingestão de alguma proteina animal e ingestão ocasional de produtos marinhos ou provenientes de água doce.

Palavras chave: dieta, mobilidade, isótopos, diagénese, Calcolítico, Perdigões

ACKNOWLEDGEMENTS

This study has been more challenging and more enjoyable than I could have imagined. I am very grateful to my principle supervisor Cristina Maria Barrocas Dias for providing me with an interesting project and for her guidance and discussions to keep me on track. A big thank you to Anne-France Maurer who took on the role as my secondary supervisor, without her expertise, recommendations and ongoing support this study would not have been completed. Special thanks to my second co-supervisor Antonio Carlos Valera for providing the material for this study and also, his helpful suggestions and comments.

The technical and analytical aspects of this thesis were supported by a large number of people within HERCULES laboratory of the University of Évora. I am grateful to Lucija Šoberl, Sérgio Martins and Pedro Barrulas for their knowledge and assistance. Big thanks to Ana Margarida Cardoso, who was very helpful in teaching how to use ATR-FTIR.

I am really grateful to Sara Ribeiro from Laboratório de Geologia Isotópica, University of Aveiro for teaching me what 'real chemistry' is and for running my samples so quickly and answering all of my questions I had about the process.

I owe a great deal of gratitude to all of my fellow ARCHMAT program colleagues. You were a wonderful group of people with their own quirks and perks and the last two years would not have been the same without all of you. There are several people I am especially indebted to: Ivona Posedi, Carla Soto, Whitney Jacobs, Drita Abazi, Milan Markovič, Guilhem Mauran, Diego Armando Badillo and Leonor Sampaio Costa, who provided company during late night dinners, interesting discussions over drinks and many adventures (which we live to tell). Big thank you to Whitney for those priceless lunch and coffee breaks and those moments where we kept each other sane.

Finally, I am exceedingly grateful to my whole family for their tireless love, encouragement and support through these adventurous two years.

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1 INTRODUCTION

Ditched enclosures have appeared at different times throughout Europe - from the Mediterranean area to Scandinavia, from Iberian Peninsula to the Black Sea (FIGURE 1). Though most enclosures in Europe were constructed during Neolithic period, some were built during the Copper Age, also known as Chalcolithic period. Each enclosure is unique, as they present a variety of architectonic solutions, locations (hill tops, slopes, low valley lands, natural amphitheaters, crests and etc.), sizes (from less than 1 ha to more than 100 ha) and contexts. Though, the most basic element that allows recognizing them is the presence of at least one ditch surrounding an otherwise open-air area. Most of them are roughly circular or oval in form and reveal cosmological connections. Ditched enclosures are important in understanding the process of social change - from Neolithic farming communities to the societies and their ritualized practices, funerary practices, finding feasting evidences and etc. (Jiménez-Jáimez, 2015)

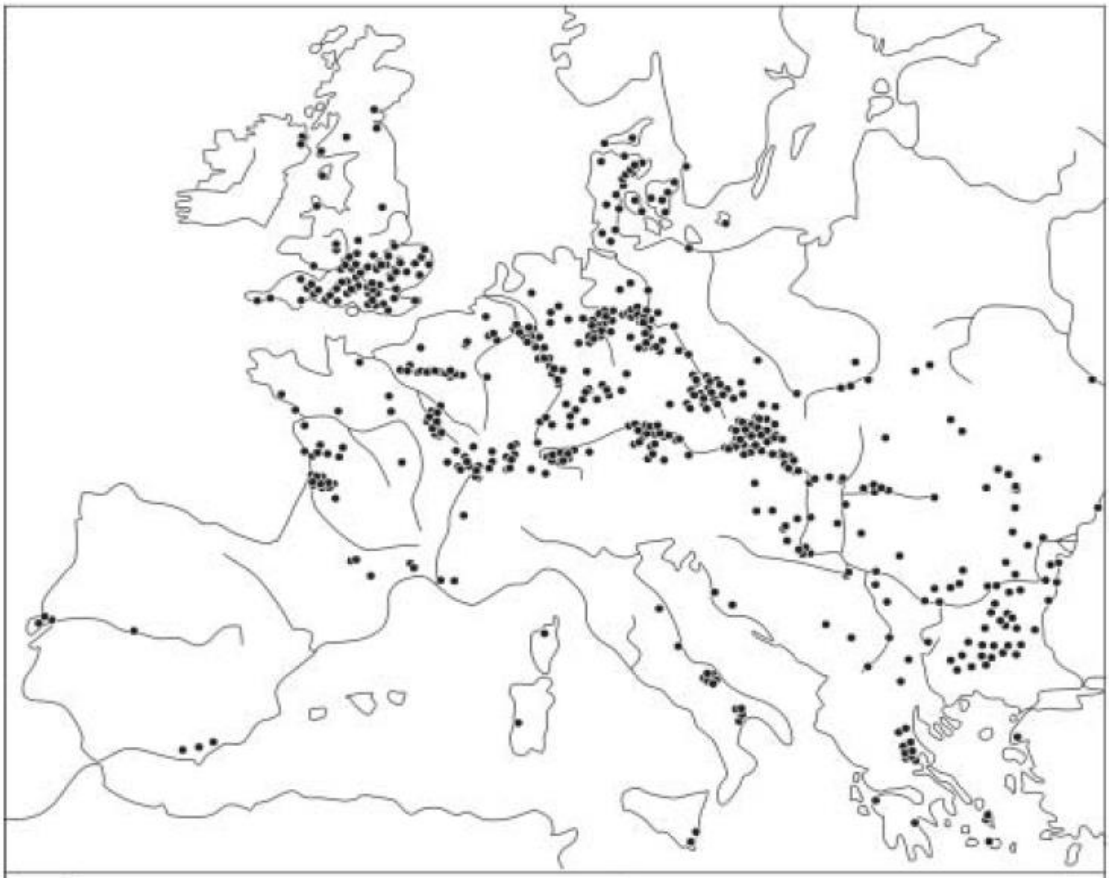

Figure 1 *Distribution of fifth to third millennia cal BC enclosures in Europe (taken from Andersen N. (1997, pp. 134–135), with additions from Darvill T. and Thomas J. (2001, p. 8) and Diaz-Del-Rio (2004, p. 108)).*

Iberian Neolithic and Chalcolithic ditched enclosures were widely unknown outside of Portugal and Spain for couple of reasons. Firstly, the knowledge of ditched sites was rather limited in those same countries (intensive practice of agriculture made it difficult to detect them by simple surveying techniques) and just recently they started to be studied (in 1975 discovery of ditch at Valencina in Spain and in 1980s Santa Vitória in Campo Maior, Évora). Secondly, the political and economic issues like dictatorship and insufficient funding have put archaeological research on the back burner (Jiménez-Jáimez, 2015). Now, after almost 40 years of surveying and excavation works done dozens of Neolithic and Chalcolithic ditched enclosures are known in the Iberian Peninsula with a high concentration in the central Meseta, Levante and the South.

Major concentration of ditched enclosures in Portugal can be found in the Alentejo region. Two largest enclosures are Porto Torrão (Beja district) and Perdigões (Évora district). Both of them dated to the second half of the 4th millennium BC reaching the end of the 3rd millennium BC (Valera, 2015). What is interesting is that these enclosures exhibit a wide range of funerary practices from different body treatments to different structures for the primary and/or secondary deposition of human remains. Very few bone isotopic analyses were completed in the Iberian Peninsula for reconstructing Prehistoric diet and the ones done are mostly from the Mesolithic or Neolithic period (in Portugal most of them were done on bone samples from Estremadura and Algarve region, because of the better bone preservation due to the limestone bedrock). Even fewer studies using strontium isotopes recorded in archaeological teeth have been conducted for mobility questions (Emslie, et al., 2015), (Waterman, et al., 2014), (Boaventura, et al., 2014), (Carvalho, et al., 2013), (Valente, et al., 2014).

Just recently stable isotope analysis of various archaeological materials started to be used in the archaeological investigations of ancient human occupation, mobility and interaction (especially in Portugal). As bone stable isotopic composition provides a direct measure of the dietary protein intake, it helps to evaluate the intensity and interaction through different food patterns, because it can distinguish consumers of C_3 from C_4 plants; meat from fish; herbivores from carnivores; legumes from non-legumes and define trophic levels. For instance, carbon and nitrogen from bone collagen demonstrates the role that marine and terrestrial resources; wild and cultivated plants played in ancient diets (Ambrose, 1993), (Ambrose, et al., 1997). It can also, reveal dietary differences in population sub-groups based on gender, age or social status. And Perdigões site is perfect for this type of analysis because it has several funeral contexts, abundance of faunal and human remains and so far, in the Alentejo hinterland only one preliminary study on Sobreira de Cima is available. And it shows a subsistence pattern based on terrestrial plants and herbivores (Carvalho, 2013).

In this study the analysis of $\delta^{13}C$ and $\delta^{15}N$ in human and animal bone collagen will help in reconstructing paleodiet practices. These results will be compared with available published data and used for further interpretation of diet at Perdigões site. The analysis of strontium isotope ratios of teeth enamel of selected animals and individuals will be used to infer about the mobility status of the site's populations. For obtaining an accurate picture of paleodiet and mobility some prescreening tests will be done on the samples for a better understanding of diagenesis (post-burial processes) that may affect the original isotopic signal.

This study will help determine diet patterns of the Chalcolithic society and see if they are consistent with local major food resources. Strontium ratios of tooth samples will help in establishing the baseline for local and foreign individuals in order to evaluate the mobility of animals and humans buried at Perdigões. Finally, the obtained results may also give insight in suggesting if the Perdigões society was influenced by large scale mobility (migrations of people between the regions) or just had a larger geographic area of food catchment and/or good regional interactions.

The results of this study will be integrated in a FCT project (PTDC/EPH-ARQ/0798/2014), dedicated to the study of mobility of humans, animals and materials in Perdigões and South Iberian enclosures, coordinated by António Carlos Valera and involving the ICArEHB centre of Algarve University, ERA Arqueologia S.A. and the HERCULES Laboratry from Évora University.

1.1 ARCHAEOLOGICAL SETTINGS OF PERDIGÕES COMPLEX

Previously it was thought that Neolithic and Chalcolithic funerary practices were restricted almost exclusively to the megalithic tombs (Valera, et al., 2014). However, as can be seen with a large and complex site like Perdigões - human remains were being deposited inside ditches, cist graves; secondary deposits were distributed in pits, *tholos*[1] tombs or open air (Valera, et al., 2014). Such diversity of funerary practices raises a lot of questions and possibilities on social change, locality, site's importance and etc. The archaeological complex of Perdigões is a Neolithic and Chalcolithic site located in Reguengos de Monsaraz near Guadiana River, Évora district, in the Alentejo hinterland, South Portugal. It is comprised of a set of ditched enclosures with 12 roughly concentric ditches defining 9 enclosures in a natural amphitheater, open to the East. Inside hundreds of pits were identified with only some of them being excavated. Though the site has been studied for almost 20 years and has a variety of

[1] It is a burial structure characterized by its false dome created by the superposition of successively smaller rings of mud bricks or stones. Often called as false-vaulted or beehive tombs. They appear around 3000 BCE in the Iberian Peninsula.

publications concerning different topics (like paleoenvironment, general synthesis of the site anthropological and material studies), there is still a lot of work left to be done. (Valera, et al. 2014; Jiménez-Jáimez, 2015) It can be said that the Neolithic and Chalcolithic phases are quite clearly distinguished with 3400-2900 BC as the Neolithic Perdigões, 2900 -2450 BC as the Chalcolithic and 2450-2150 BC as Late Chalcolithic Perdigões (FIGURE 2). Though it is still unknown if the site was continuously occupied or permanently in use, it shows growth, starting with smaller enclosures in the center and development through time, while maintaining the principles of the Late Neolithic central enclosure and topographical location (Valera, et al., 2014; Valera, 2015).

Figure 2 *Development of Perdigões site (taken from Valera, et.al, 2014, p. 21).*

Perdigões complex has a restricted visibility over the landscape due to the limits of the amphitheater, except to the east side. The location of the site, its architecture and spatial organization seems to have a cosmogenic significance - connection to the East and to the rising sun. It can be seen by looking at the sites layout, which incorporates a megalithic cromlech, chalcolithic necropolis with both of them located to the east side. Also enclosures eastern gates are orientated towards the sun's winter and summer solstices, while the western gates to the corresponding sunsets (Valera, 2012; Valera, et al., 2014; Valera, 2015). Thus, the eastern horizon of the site functioned as an annual calendar in a place where ceremonial gatherings and deposition of human remains were important.

1.1.1 Late Neolithic Perdigões funerary practice

Around 3400-2900 BC began the construction of the Perdigões 'Neolithic landscape' with a central enclosure being formed by a large ditch 6 and two small ones running parallel. It had only one gate aligned with the summer solstice. A bit was located (Valera, et al., 2014).

Partial human skeletal remains have been found in the pit graves 7 and 11, located outside the enclosure. They are the only funerary contexts in Perdigões where primary depositions have been documented with no votive artifacts. From the pit 11 three incomplete and highly fragmented individuals were exhumed. The anthropological analysis suggests that the bones belong to male non- adults - six year old and two around fifteen years with no matrilineal relationship between them (Godinho, 2008), (Silva, et al., 2015).

1.1.2 Chalcolithic Perdigões funerary practice

During the Chalcolithic period the enclosed area grew even larger with the construction of ditch 3 and 4, defining a sub-trapezoidal enclosure. In total 8 tombs were identified in the semicircular ditch, however only three of them have been excavated: two of which have been fully excavated and one (tomb III) just partially. In both cases excavated human remains were deposited in the atrium, corridor and the chamber. Tomb I and II are semi-subterranean structures, having circular chambers partially excavated into the bedrock, with walls of vertical schist slabs, a small passage (in one case made with small diorite monoliths) and a small circular or oval atrium also lined with schist slabs (Duarte, et al., 2006), (Evangelista, et al., 2013), (Valera, et al., 2014).

Both tombs are secondary in use and have more than hundred individuals each. These remains could have come from a different area within the site or from far away. Even though these monuments started to fall into ruin, when the schist slabs have collapsed, human remains were still continued to be deposited without rebuilding the structure. Probably they were used only from time to time. Contemporaneous to the tombs is a large area of cremated human remains deposition which was uncovered in the center of the enclosure, consisting of pits and a cist grave and they cut the previous Late Neolithic structure (Valera, et al., 2014). The location of funerary monuments, difference in the architecture and treatment of human remains is considerably different, again revealing that Perdigões site has a complex system of death management throughout more than thousand years of its existence. (Evangelista, et al., 2013)

1.1.2.1 Tomb I

The monument is oriented at 90° and has a circular chamber about 3.5 m in diameter, small corridor about 1.5 m long and a circular atrium around 2 m in diameter. The human remains in the chamber were associated with a rich material assemblage of bone and ivory objects, long blades, arrow heads, small pots, necklace beads and etc. The remains were more organized and concentrated along the walls. Not much can be said about the corridor, because an original context has been destroyed by the growth of olive tree. The atrium area contained only a few human remains and some votive artifacts like flint dagger, arrow heads, limestone and ceramic

pots. Some funerary assemblages, like blades, arrow points and etc. were made of exogenous raw materials which suggests long-distance network (Duarte, et al., 2006), (Valera, et al., 2014), (Evangelista, et al., 2013), (Mendonça, et al., 2016). Though, no association between individuals and artifacts can be done because of bones being in disarray and secondary in deposition.

Couple of phases of tomb use can be distinguished: construction phase, which includes the opening up and digging of the outline of the monument; a period of intensive use of the tomb (during this phase the internal chamber was no longer in use) and use in ruin phase. (Valera, et al., 2014) In the tomb both adult and non-adult remains are found. Also, there is no distinction between sexes. Though, the preliminary analysis of human remains recovered indicates an overall poor bone preservation level, and a high degree of fragmentation, the provisional minimum number of individuals (based on individual identification of teeth and their quantification by side) reaches 106. Some of the bones from the transitional area between the corridor and the chamber have traces of ochre and cinnabar. The presence of small bones recovered indicates a great care in the collection of bones from the previous place of deposition. (Valera, et al., 2014), (Evangelista, et al., 2013)

Faunal list of the species found in the tomb I is quite heterogeneous and consists of suids, red deer, domestic cattle, ovicaprids (either sheep or goat), fox and rabbit. The bone fragments analyzed do not exhibit any evidence of use for food consumption. It suggests bone use exclusive for funerary purposes (Cabaço, 2012).

1.1.2.2 Tomb II

Tomb II was oriented at 130° and constituted of a circular chamber of about 3 m in diameter partially excavated in the bedrock, with walls covered by schist slabs. Its corridor was orthostatic, 1m in length with two pillars at each side and an ellipsoidal atrium. The monument shows two general moments of use - first one restricted to the chamber and when the lateral slab started to fall, it had an emptying process (2860-2500 cal BC). Thus, the construction and the first use of tomb II was contemporaneous with that of tomb I. (Valera, et al., 2014)

The second moment was when chamber was reused after the emptying process together with atrium. This phase can be split in two episodes: one before falling of the slab that closed the entrance of the corridor. The funerary assemblage of this phase includes some pots, arrow heads, some ivory and bone objects, beads and etc. The second episode starts after the falling of the slab over the first layers of depositions in the atrium. Found materials for this moment includes gold blades, ivory button and *lunulae,* zoomorphic figurines and idols... (Valera, et al., 2014), (Valera, 2015)

Thus, as the depositions were taking place in Ditch 4, tomb II was partially emptied and reused and tomb I was nearing its end of use. Some artifacts and raw materials are foreign (like pots with weathered schist clay with a provenance from more than 5 km away) showing that Perdigões site had links to other regions of Iberia and even North Africa. (Valera, et al., 2014)

It also, can be seen that the frontier between the space of the living and the space of the dead which could be easily distinguished in the Late Neolithic period, when pits with human remains were outside the enclosure, starts to fade away during the Chalcolithic period with incorporation of pits and ditches with remains inside the enclosure.

Evaluation of wear levels at the most abundant tooth - left mandibular 1st molar suggests that a significant number of individuals were dying young at Perdigões: from 181 lower first molars present at tomb I, 45% exhibited no wear, suggesting that those individuals died around age of six or even earlier. As there are no indicators of nutritional stress on the skeletal remains, another reason might be enhanced exposure to pathogenic factors, like infectious diseases and etc. One of the causes might be animal domestication. (Evangelista, et al., 2013), (Godinho, 2008), (Duarte, et al., 2006)

1.1.2.3 Pit 16

Pit 16 is located in the center of the enclosure and is the only Chalcolithic funerary context completely studied anthropologically. Stratigraphically it had two thin sedimentation layers and a deposit of cremated human and faunal remains in conical shape, followed by four layers filled with fauna and broken pottery. The cremated human remains displayed heterogeneous color variation (meaning that bones were exposed to different temperatures and time) in different parts of skeleton. This deposition had more than 2400 bone fragments and 63 dental remains and showed a great care in bone fragment collection, because it had even tiny bones from the skeleton. The minimum number of individuals is 6 adults and 3 non-adults, though no inferences about sex were possible (Valera, et al., 2014).

1.1.2.4 Pit 40

Five meters east from pit 16 another pit and a cist were found. Pit 40 is around 2.70 m in diameter and had a primary burial of a male adult, which was surrounded by deposits of cremated human remains. And the semi-circular stone cairn contained a rectangular cist made of schist, diorite and gabbro slabs. It contained a thin layer of burned human, faunal remains and archaeological material like ivory fragments, anthropomorphic figurines, idols and etc. Pit 40 has burned bones along with few non-cremated ones and the minimum number of individuals has reached more than 150 with most of them belonging to non-adults. The obtained radiocarbon dates fits with the dates of last use of tomb I and reutilization of tomb II. (Valera, et al., 2014)

It could be said that Perdigões site was a large 'ceremonial chamber' that incorporated a progressive diversification of rituals and places involving the deposition of human remains. Because there is such a wide variety of funerary rituals and the continuous use of tombs, even when the slabs have fallen down, it brings up the theory that these tombs were used by people coming to Perdigões from time to time to bury their deceased, practice rituals and etc.

1.2 GEOLOGICAL AND PALEOENVIRONMENTAL CONTEXT OF THE SITE

The region of Reguengos de Monsaraz belongs to Ossa Morena Zone (FIGURE 3) which has the oldest rocks from the middle – upper Cambrian, while other lithological units range from the Lower Ordovician up into Lower Devonian sedimentary sequences. It is mainly composed of heterogeneous lithology including tonalites and granodiorites (dominant) and the internal area of the massif is composed of gabbro-dioritic rocks (FIGURE 4 and FIGURE 5), (Antunes, et al., 2010).

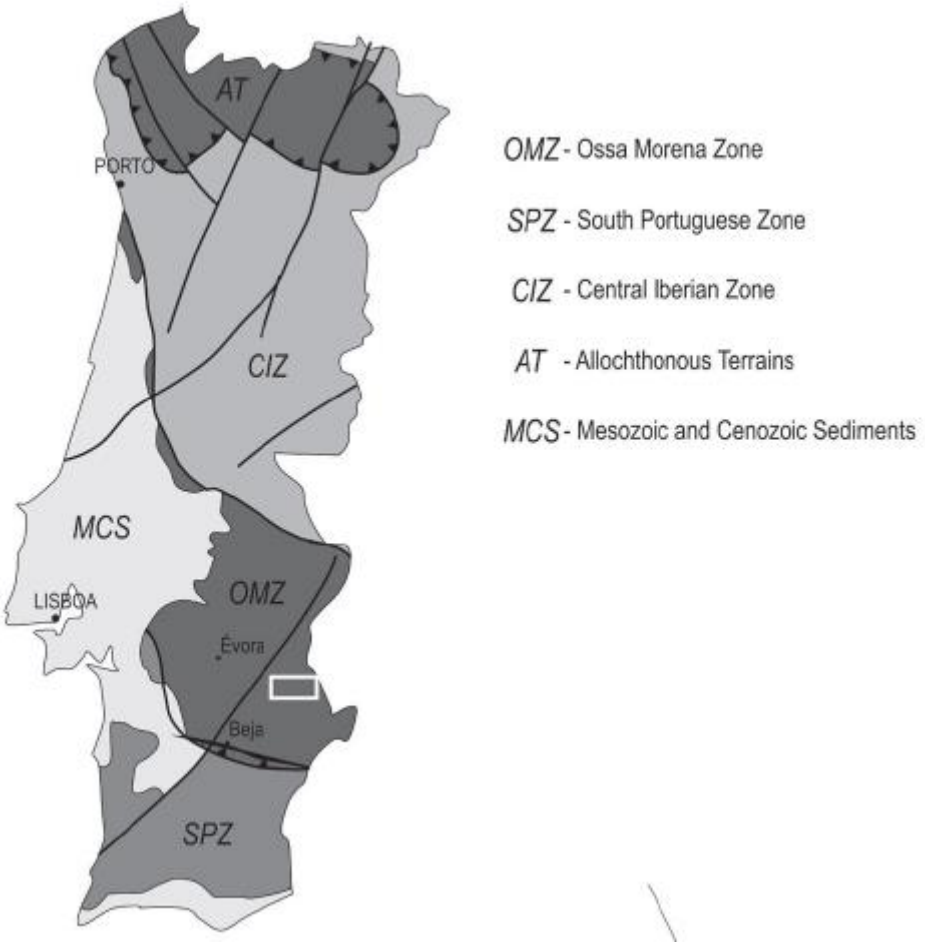

Figure 3 *Major geotectonic units of the Iberian Massif (taken from (Antunes, et al., 2010), p. 26.)*

Perdigões site is situated north-northwest of Reguengos de Monsaraz and located in elongated amphitheater between 226 and 252 m in height, with a difference of around 25 meters between its low and high points. The area where the site is located is quite special, because it has underground water resources, which is an exception for the region and is probably due to

the close proximity to Vale do Ribeira do Alamo. The peneplain is surrounded by granitoid rocks but is mainly composed of diorites. They have phaneritic texture and have plagioclase, hornblende and quartz as additional minerals. The area where the tombs were located had schist stains with laminar and no alteration. (Reis, 1998).

The area north-east from Perdigões (Motrinos, Serra das Pedras) is mostly comprised of siliceous shales, while north and south-east (Arraieiras) has a dense water network, coinciding with the passage of granitoid rocks to the schists (predominantly pelitic) of Barrancos Formation (Carvalhosa, et al., 1991).

The western and central part of the peneplain consists of medium-grained granodiorites and biotite-hornblende. Their mineralogical composition includes quartz, plagioclase, hornblende, biotite and potassium feldspar (Reis, 1998)

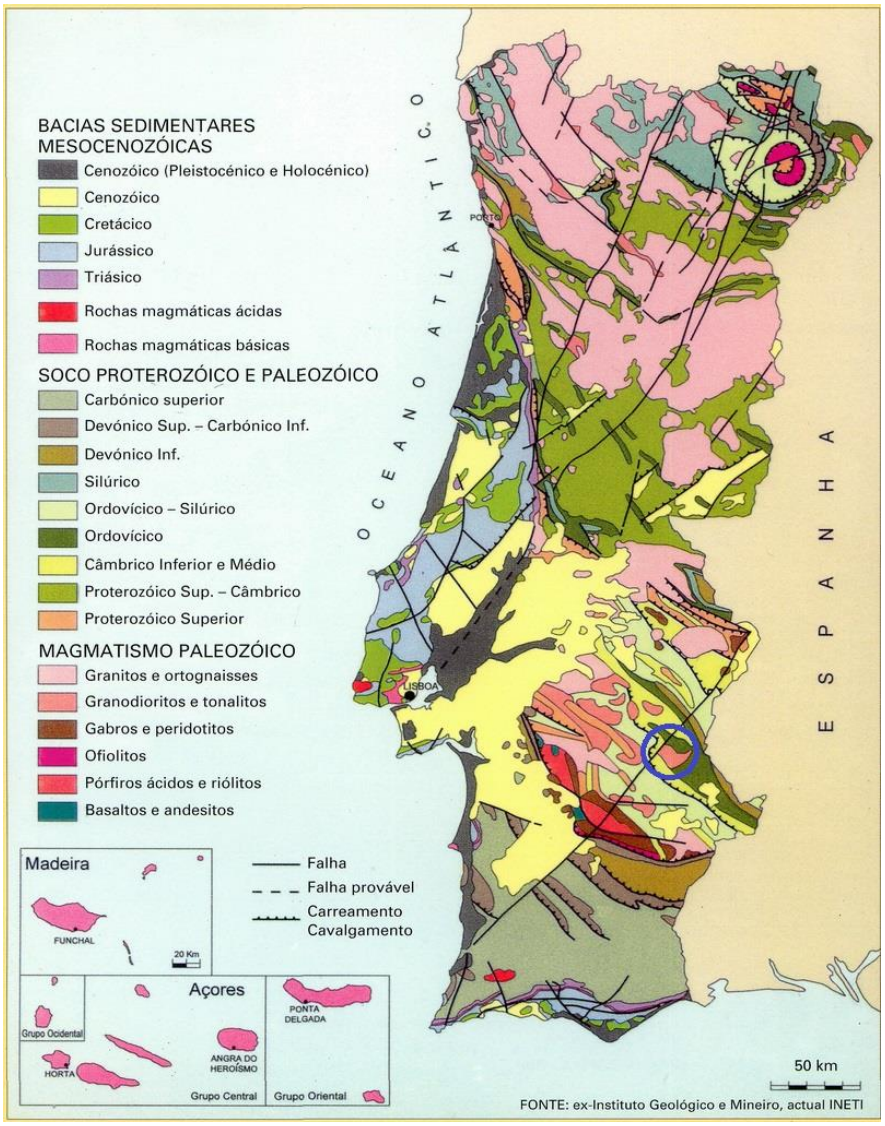

Figure 4 *Geological map of Portugal (blue circle corresponds to the location of Reguengos de Monsaraz)*

Thus, depending on the type of bedrock and its acidity, preservation level of organic materials is affected. For instance, in Portugal two regions which can contribute to the better

bone preservation due to limestone bedrock are Estremadura and Algarve (Boaventura, et al., 2014). While Alentejo region, where Perdigões is located is mainly composed of Paleozoic schist and granite rocks (Hillier, et al., 2011).

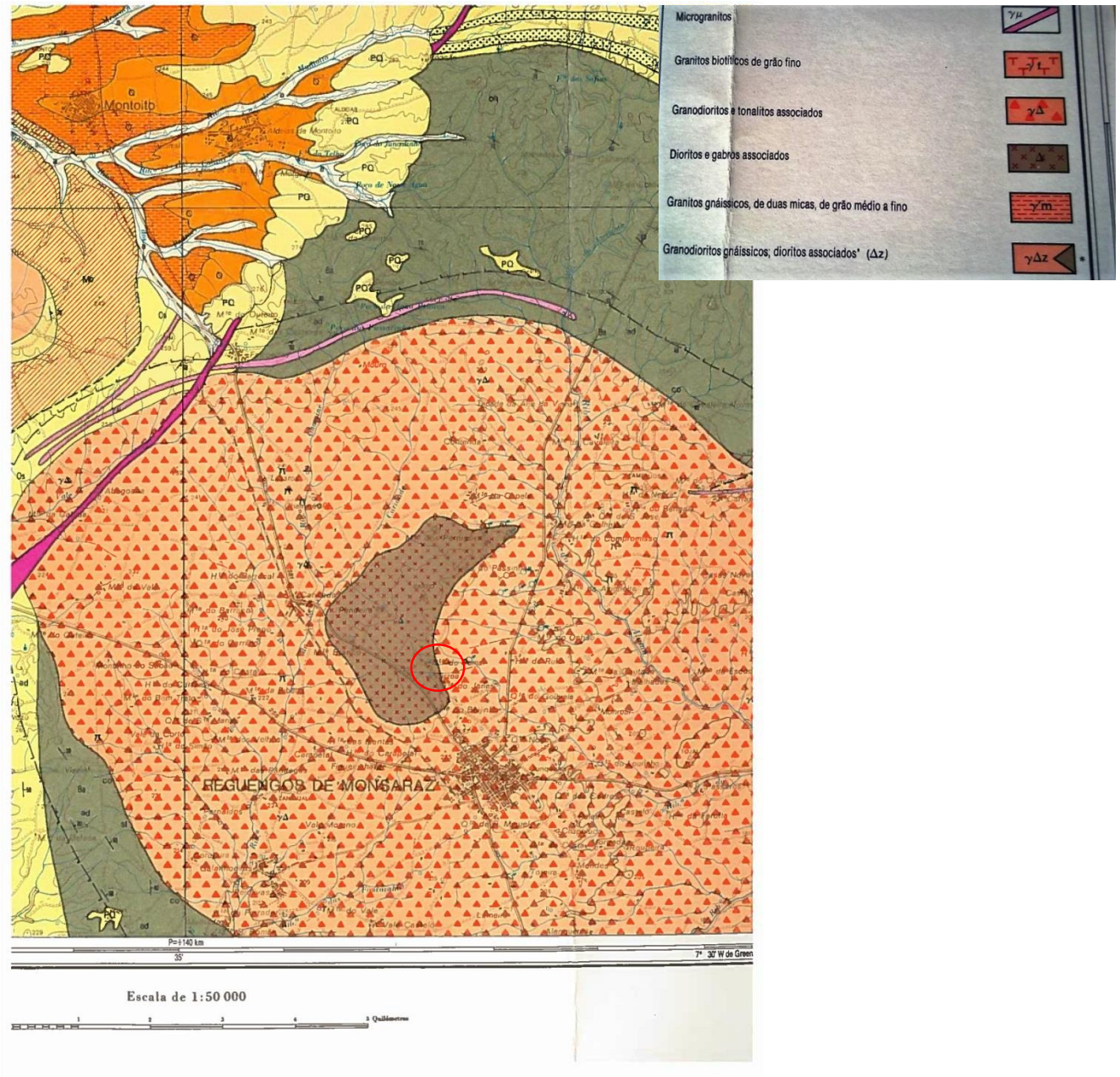

Figure 5 *Geological map of Perdigões site, Reguengos de Monsaraz, adapted from (Carvalhosa, et al., 1991).*

The paleoenvironmental assessment of 5 stratified archaeological deposits from Pit 2, Ditch 1, 6, 8 and 12 from Perdigões site has shown that microfossils and pollen have survived despite poor taphonomic conditions of arid site (sediment and pollen corrosion). Pollen spectra has revealed that the dominant species during Late Neolithic and Chalcolithic periods were oak (*Quercus sp.),* pine (*Pinaceae sp.)* and elm (*Ulmus sp.)* trees and shrub species were represented by hazel (*Corylus-avellana)* and olive (*Oleaceae*) family. Though, these species

shows a decrease during the 3rd millennium BC and an increase in *Poaceae sp.* (grasses), which shows a more open landscape. While micro-charcoal data revealed some burning activities which can be associated with the utilization of pit and ditches for domestic and/or ritual reasons (Wheeler, 2010), (Danielsen, et al., 2013). The identified species represent the plants that more or less grew locally or in the region during the period. The content of pollen from arboreal and non-arboreal plants indicates an open Mediterranean shrub land with some scattered trees of pines and oaks (Danielsen, et al., 2013).

2 BONES AND TEETH STRUCTURE AND DIAGENESIS

Human and faunal remains are like short-term time capsules. They can help investigate differences in past diet, mobility and health on the individual level because chemical composition of bones and teeth characterises dietary intake and surroundings (distinct chemical signatures of landscape can be reflected in the remains) for different time periods of human life. For instance, teeth can give information on childhood diet and habitation, while bones relates to long-term diet and residence. In very rare cases hairs and fingernails found on human remains can provide information on the short-term dietary intake and surroundings (Pate, 2008). However, chemical and physical contamination happening in the post-mortem environment can affect elements of interest. This chapter will talk about bone and teeth structure and elements from which chemical analysis on diet and mobility can be done. Also, on diagenetic processes that can alter the composition of skeletal remains thus, affecting the analysis and results.

2.1 BONE STRUCTURE AND COMPOSITION

There are two types of bone compact or cortical and spongy or cancellous bone (FIGURE 6). The compact bone is quite dense and hard and is on the outer layer of the bone, while the spongy bone forms the interior of bones. The bone is constituted of several cells, like bone-forming osteoblasts, matrix-trapped osteocytes and osteoclasts, responsible for bone resorption (Turner-Walker, 2007).

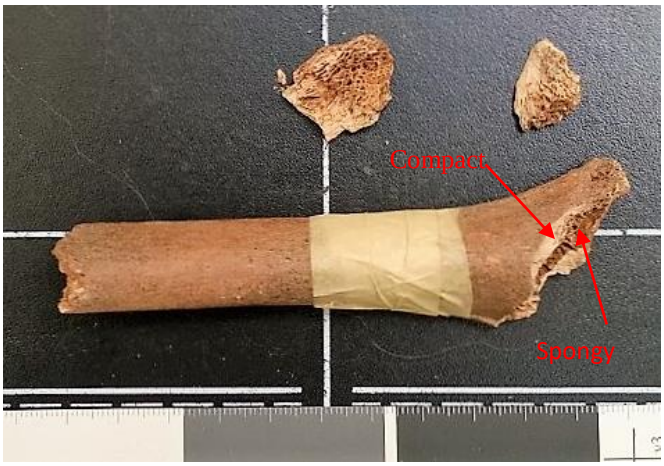

Figure 6 *Types of bone (compact in the outer layer and spongy in the interior).*

Two thirds of the bone matrix are composed of inorganic minerals in the form of calcium phosphate (also, called bioapatite), water and one third of organic matter, which is made up of

around 90% type I collagen fibrils and 10% of non-collagenous proteins (NCP) and mucopolysaccharides (Burton, 2008).

Collagen is the major insoluble fibrous protein in the extracellular matrix of the bone and is quite stable and resistant to bacterial or fungal attacks. The triple-helical structure of collagen (which is also species dependent) (see FIGURE 7) is mainly composed of three types of amino acids: glycine, proline and hydroxyproline. Multiple triple helices twist together while forming a larger molecule, which creates fibrils of collagen and then collagen fibers (Turner-Walker, 2007) (Pate, 1994). The collagen is synthesized from amino acids which were broken down from previously ingested dietary proteins of food (Pate, 1994).

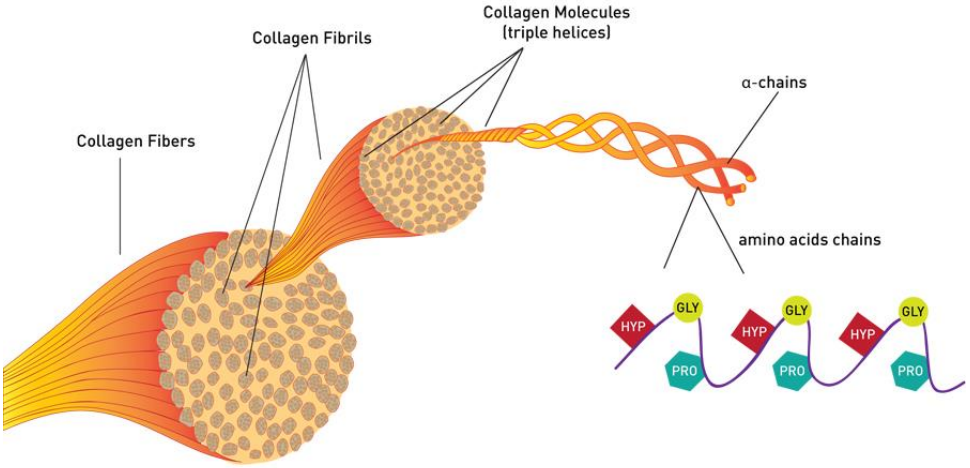

Figure 7 *Collagen structure (taken from http://www.proto-col.com/blog/2014/07/collagen/).*

Here we intend to analyse bone isotopic composition for reconstructing diet. The isotopic values vary in different skeletal tissues - analysis of bone collagen reflects the dietary protein (marine vs. terrestrial; C_3 vs. C_4 plants), while apatite reflects the whole diet. This is due to differences in tissue composition, secondary fractionation effects and synthesis from different constituents of the diet (Lee-Thorp, et al., 1989). Though to get whole picture of the individual diet all available tissues analysis would be preferable, but there are a lot of things to consider – availability of archaeological material, alteration level and etc. For instance, hydroxyapatite is more affected by diagenesis than the bone collagen, because it absorbs minerals and ions from the soil like a sponge. Thus, if collagen exhibits high levels of diagenesis, apatite will be even more affected (Weiner, 2010).

Bone renews throughout life and the process is called bone remodelling. It involves the incorporation of new mineral and organic material replacing the old one and introducing new isotopic inputs. Different skeletal components can have higher turnover rates than other parts. For instance, bone collagen‘s turnover rate is around 10 years (Pate, 1994). Of course the remodelling process and bone composition varies considerably not only with a type of bone,

but also depends on the age (it will remodel more quickly in infants than in adults) (Pate, 2008). In conclusion, bone isotopic composition relates to the last years of life of an individual.

2.2 TEETH STRUCTURE AND COMPOSITION

Teeth of vertebrates have diverse morphologies which reveal different functions used in food processing but they all have same basic design (Weiner, 2010). The mature tooth is divided into crown and root and consists of four tissue types – enamel, dentine, cementum and pulp with roots (see FIGURE 8). Enamel forms a very hard and thin layer of the tooth and is the most mineralized tissue of the human or animal body, composed of around 96 wt. % of inorganic material calcium phosphate and contains trace minerals like fluoride, magnesium, strontium, calcium and etc. The remaining 4 wt. % are composed of organic matter and water (Kohn, et al., 1999) (Wang, et al., 1994) and is way less than comparing to the weight in bone and dentin. Enamel does not have any collagen and it does not remodel after the final mineralisation, preserving the isotopic signatures during formation throughout life. As enamel is more inert and denser than dentine or bone, it makes it more resistant to post-burial contamination and a perfect material for isotopic analysis. The main cause is compact structure with little pore space because of the phosphate crystal size, which is > 1μm (Bentley, 2006). The crystals are very long and have more width and thickness compared to the crystals of bone and dentin (Weiner, 2010).

Dentin and cementum uses mineralized collagen fibril as a building block. Dentin makes up most of the tooth and though it is made of calcium phosphate ions (contains around 70 wt. %) which forms hydroxyapatite crystals, it is softer than enamel. Also, it is more porous than enamel. The remaining 30 wt. % of dentine are composed of organic matter and water (Kohn, et al., 1999). Dentine formation has two stages: pre-dentine, which is laid down under the centre of cusps and followed by formation of immature enamel and the next stage – subsequent addition of apatite crystals, which happens after the root formation and can continue throughout life but at a very slow rate. (Turner-Walker, 2007). Tooth dentine is as vulnerable to contamination as bone, because rate of degradation is reliant on the size of pores. And dentine has pores around 1μm, while phosphate crystals are smaller than 0.1 μm, enabling diagenetic strontium and other trace elements to incorporate itself in pores by secondary minerals easily (Bentley, 2006).

The third component is pulp, which is a soft tissue in the centre of the teeth and is related to dentine and contains blood vessels and nerves, though it is rarely found in archaeological teeth. While cementum covers the surface of the root that anchors the tooth (Pate, 2008). Cementum is mainly composed of type I collagen which is made of thick collagen fibrils

(known as Sharpey's fibers and reflects the tooth position in mandible socket which helps to infers seasonal changes in diet) (Weiner, 2010).

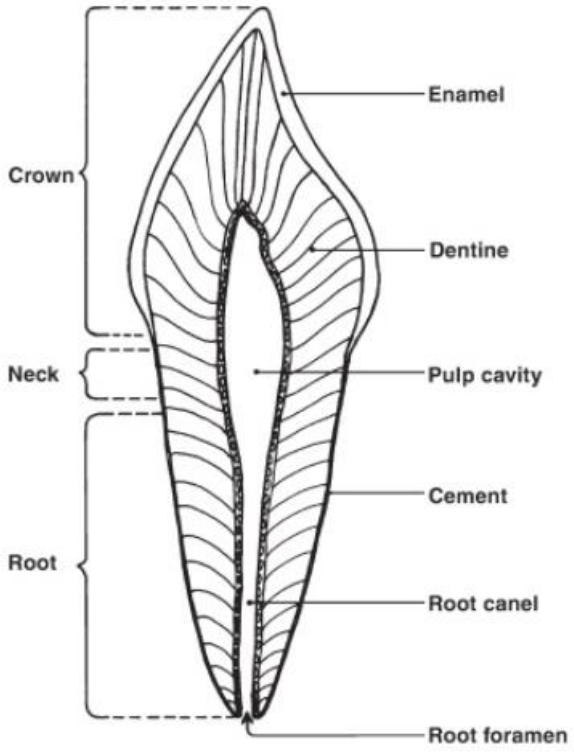

Figure 8 *Schematic representation of the major components of a human tooth (picture taken from (Ortner, et al., 2003), p. 32).*

The difference between teeth and bone is that teeth do not remodel *in vivo* (secondary dentin continuously forms during long periods of time*)*. The development of permanent teeth is divided in three parts. The first one involves incisors, canines and first molars and reaches completion between 3 and 7 years. While premolars and second molars finish formation between 4 and 8 years of age. The last phase involves third molars, which usually grows out around 7-12 years and finishes between 10-18 years of age (Hillson, 2008). Therefore, the analysis of different types of teeth is useful for investigating dietary changes during childhood. For instance, first molars have been used to study breastfeeding and weaning by Eerkens et al., 2011, Fuller et al., 2003 and Martinez et al., 2015, whereas third molars are usually analysed for diet and the differences between first and third molars can be used for mobility purposes because they can show differences of movement during childhood (Killgrove & Montgomery, 2016; XiaoSi et al., 2008; Al-Shorman & El-Khouri, 2011).

2.3 BONE AND TEETH DIAGENESIS

The post-depositional alteration should be considered when applying stable isotope analysis. Chemical post-mortem alterations of protein and mineral fractions can occur on teeth and bones; this process is usually referred as diagenesis. Different types of skeletal tissues are affected differently because of different chemical composition and structure. Diagenesis in

remains can involve exchange or adsorption of ions from the soil, dissolution, precipitation, breakdown or leaching of collagen, recrystallization, crystal growth or mineral replacement and more (Hedges, 2002). Understanding and characterizing diagenetic processes in remains are important to evaluate if and how much the original composition was altered or retained. As only, chemical data from well-preserved human and/or animal remains can be used for interpretation on subsistence and migration patterns (FIGURE 9).

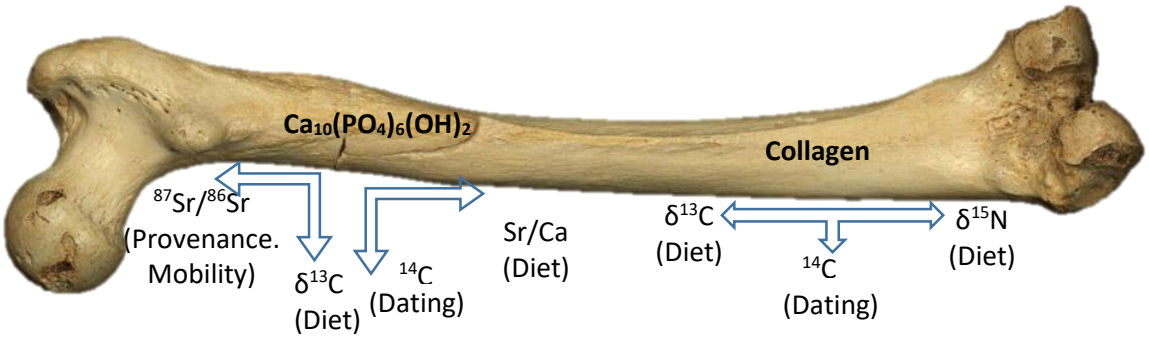

Figure 9 *Elements and isotopes from bones and teeth used for diet and mobility.*

For better understanding of diagenesis process I will briefly talk about porosity. It is quite obvious that the spongy bone is way more porous than the compact bone though both materials contain multitudes of pores of different size and as collagen is degraded and removed during diagenesis, porosity of the bone increases and allows greater surface area to interact with water in the environment. Making general porosity of deproteinated bone almost eight times more than that of the intact bone (Weiner, 2010). Thus, bone lacking in collagen becomes even more prone to diagenesis.

It is known that teeth are less susceptible to diagenesis than bones, though they still experience some alterations. In this section some examples of diagenetic occurrences in bone and teeth matrices will be mentioned. Diagenetic alteration can occur by chemical substitution between skeletal tissues and the burial environment. For example, strontium, carbon and oxygen within the surroundings can replace the original strontium, carbon and oxygen in the mineral phase of the bone (Nielsen-Marsh, et al., 2000), (van Klinken, 1999). It can happen in multiple ways: pore-filling by secondary minerals, because bone macro porosity of blood vessel canals, bone cell voids and cracks can be filled with minerals like calcite, pyrite, quartz and etc.; recrystallization by direct exchange of soil strontium or calcium with the original one found in bioapatite (Hoppe, et al., 2003). Though, changes in enamel are minimal even after very long periods of time because of its chemical composition and structure. The main alterations occurring in fossil enamel are uptake of fluoride ions and exchange of ions and/or isotopic exchange process (Weiner, 2010). While dentin diagenesis is quite similar to bone diagenesis.

Also, after the burial microorganisms and enzymes present in intestines cause autolysis and putrefaction, which further cause microorganisms to produce collagenase which break downs collagen and frees organic carbon and nitrogen for them to feed (Pate, 1994). It also causes tunnelling in the bones and crystal growth triggered by decomposition of organic material.

Another process is a chemical dissolution of the apatite by hydrolysis, depending on the groundwater levels and soil pH (bones are usually better preserved in the environment with neutral or slightly alkaline pH than in acidic soil). For instance, bones from alkaline soils are more protected from dissolution and tend to be white or cream coloured because many of metal ions are locked up as insoluble oxyhydroxides or carbonates, while bones from acidic soil tend to be brown in colour because of solubility of transition metal ions (Fe, Mn…) and humic acids. (Hedges, 2002). Hydrolysis can free ions like calcium and phosphate, which will either be leached from the bone by soil moisture and precipitation or recrystallize as soon as critical ion density is reached (Lee-Thorp, 2008), (Hedges, 2002). It can also affect the organic component - loss of collagen due to the hydrolysis of the peptide bonds of collagen. It weakens collagen's structure and causes fragmentation and leaching (Nielsen-Marsh, et al., 2000) leading to enrichment of ^{15}N values (Pate, 1994) and slight changes in $\delta^{15}N$ values.

Finally, crystallinity of archaeological bone increases by means of dissolution of the smallest crystals or dissolution and subsequent recrystallization to larger more thermodynamically stable crystals (Hedges, 2002). It happens more often when collagen or apatite have previously incurred substantial loss of matrix.

Not only there are various types of diagenesis but also it varies from site to site or even within the site depending on microbial attack, pH, hydrology, geology, temperature, humidity, redox conditions (Bentley, 2006) (Hedges, 2002) (Berna, et al., 2004) (Nielsen-Marsh, et al., 2000). However, many different chemical (IRMS, ICPMS), mineralogical (XRD, XRF), microscopic (LM, SEM, TEM) and spectroscopic (Raman, IR) techniques can be applied to assess how much altered and/or contaminated the archaeological bone sample is (Person, et al., 1995), (Wright, et al., 1996), (Brock, et al., 2012), (Tripp, et al., 2010), (Halcrow, et al., 2014).

3 INTRODUCTION TO STABLE ISOTOPE ANALYSIS

The principle of stable isotope analysis is that ingested food and water during life leaves chemical 'fingerprint' in body tissues which reflect different components of the diet. In general, bone collagen is formed from the protein portion of the diet, while bone carbonate and tooth enamel carbonate (apatite) are created from a combination of dietary protein, carbohydrates and fat. (Tykot, 2006) It also, depends on the isotopic composition of various reactants, pathway and reaction kinetics; chemical and physical conditions, relationship between underlying geology (especially in the case of strontium and sulphur isotopes) and surrounding environment, plants and soil (for carbon, nitrogen and sulphur) or ingested water if we are talking about oxygen (Sharp, 2006).

Isotope is a different form of an element that has same number of protons but differs in number of neutrons in its nucleus, which results in different atomic weight. This difference in mass affects how isotopes are incorporated into the organism tissues because the heavier isotope reacts slower in a chemical reaction. A lot of elements have couple of isotopes either stable or radioactive. The difference between them is that radioactive isotopes decay over time, whereas stable isotope ratios are fixed.

In biosynthetic chemical reactions, the isotopic compounds weighing lighter reacts faster, spending less energy. In the case of photosynthesis, the two isotopes are not incorporated equally, creating a product enriched in ^{12}C relative to the substrate, which results in changes in $^{13}C/^{12}C$ ratio (Sharp, 2006), (Tykot, 2006). This process is called isotopic fractionation. When animals ingest plants their metabolic process reverse the direction of fractionation, thus increasing the proportion of the heavier isotope (carbon, nitrogen) in the body tissue. For example, carbon and nitrogen exhibit differential fractionation during photosynthesis and nitrogen during the fixation or absorption from the soil.

The isotopic composition of an element is expressed using the delta (δ) notation, and the unit of measurement is per mil (‰). Delta values denote a measured difference made relative to an international standard. Thus, isotope ratios are defined by:[2] (Tykot, 2006)

$$\Delta\ (‰) = \left(\frac{X_{sample}}{X_{standard}} - 1\right) \text{ x } 1000$$

A positive delta value means that the ratio of the heavy to light isotope is higher in the sample than in the standard, while the negative delta value means the opposite (Sharp, 2006).

[2] X defining isotope ratio of the choice. For instance, $\delta\ ^{13}C = \left(\frac{\frac{13}{12}C\ sample}{\frac{13}{12}C\ standard} - 1\right)$ x 1000

3.1 CARBON ISOTOPE ANALYSIS

Carbon has two stable isotopes – ^{12}C, which is relatively abundant and ^{13}C, which is less common in nature (see APPENDIX C: ISOTOPE ABUNDANCE, p. **61)**. The standard reference material for carbon isotope ratios are reported relative to PDB (Vienna Peedee belemnite) and the $\delta^{13}C$ value of PDB is zero.

The carbon isotope composition of terrestrial ecosystems responds to the photosynthetic pathways and environmental responses of plants (so-called primary producers) which are at the base of food web (a network of interconnected food chains (FIGURE 10)). Plant leaves discriminate against heavier isotopes during diffusion. Therefore plants, have an increase in the ^{12}C in comparison to the atmosphere. During the process of photosynthesis, which includes carbon dioxide diffusion into leaves, dissolution and carboxylation, the discrimination against ^{13}C continues, though it depends on the photosynthetic pathway in question. As C_3 and C_4 plants shows differential discrimination against ^{13}C, they display diverse degrees of carbon isotope fractionation. For instance, plants of C_3 pathway exhibits more negative $\delta\ ^{13}C$ values in plant tissues due to discrimination due to CO_2 hydration and leaf (stomatal) diffusion and carboxylation (Sharp, 2006).

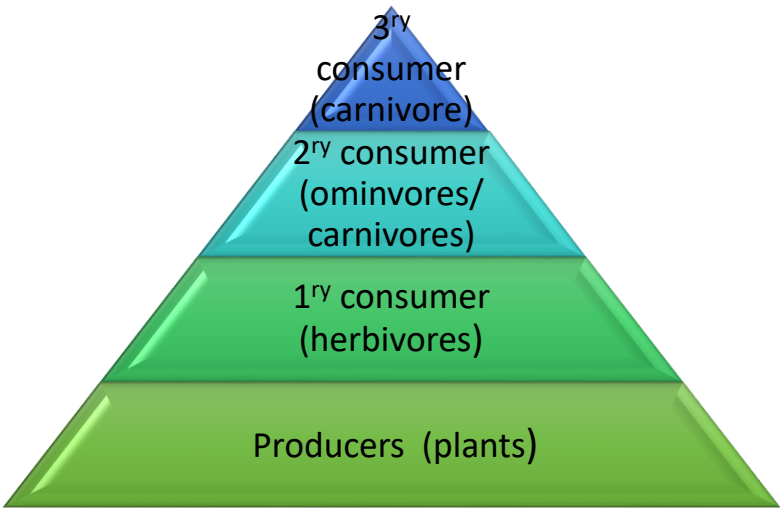

Figure 10 *Basic example of the food chain*

C_3 plants include trees, shrubs, herbaceous plants and grasses, wheat and rice, legumes and etc. (usually found in temperate and cold areas). While C_4 plants are native to tropical and savannah areas and include many grasses, sorghum, millets, maize and sugar cane. Another pathway is CAM plants (which stands for Crassculacean acid metabolism) and includes some mosses, ferns and flowering plants, like orchids, cacti, bromeliads, agaves and are classically adapted to arid conditions (Ambrose, et al., 1993). Thus, C_3 plants will have $\delta\ ^{13}C$ values averaging about -26.5‰, while C_4 plants around -12.5‰ (see FIGURE 11). Some empirical data showed that bone collagen is enriched +5‰ relative to the animals diet (Tykot, 2006).

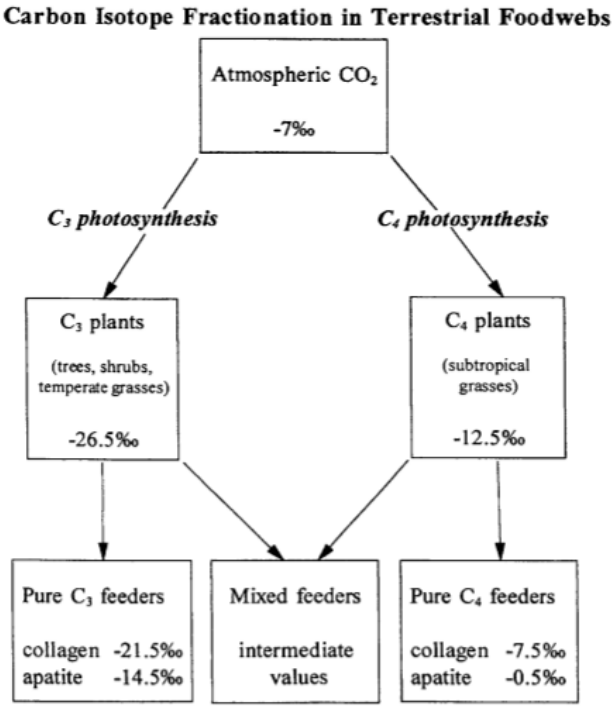

Figure 11 *Carbon Isotope Fractionation in Terrestrial Food webs, taken from (Tykot, 2006), p. 435.*

It has to be taken into consideration that the dietary input of carbon isotope of the foods consumed in the animal or human body tissue reflects the average carbon composition of its diet and also, depends on the choice of tissue which is going to be analysed.

Another thing to mention is that carbon from the marine environments is derived from several sources, which includes dissolved bicarbonate and carbonic acid and is enriched in ^{13}C relative to the atmospheric CO_2 used by terrestrial plants (Sharp, 2006). It can be said that marine plant values are closer to that of terrestrial plants of C_3 pathway.

As carbon isotope ratios, especially in C_3 plants, are affected by water availability, light intensity, temperature, nutrient availability and partial pressure of carbon dioxide - some overlaps between values may happen. Thus, it is essential to combine carbon isotope analysis with nitrogen analysis.

3.2 NITROGEN ISOTOPE ANALYSIS

Nitrogen has two stable isotopes – ^{14}N, which is relatively abundant and ^{15}N, which is less common in nature. The accepted standard for nitrogen isotope analysis is AIR – the natural abundance of ^{15}N in air being constant and is assigned a $\delta^{15}N$ value of zero by international agreement.

The vital source of nitrogen in foodwebs is atmospheric nitrogen, which is incorporated into biological systems by bacteria and soil microorganisms. They facilitate the conversion of nitrogen in three stages of its biological cycle by fixation, nitrification and denitrification. Nitrogen fixation is a process by which atmospheric nitrogen is converted in ammonia by

nitrogenase. Then ammonia or ammonium ions are incorporated into living tissues by assimilation. Next step is called nitrification, which is the transformation of ammonia to nitrate by oxidation. The final step that concludes the nitrogen cycle is denitrification – process of nitrate reduction to produce molecular N_2 by anaerobic, aerobic bacteria and some fungi. (Sharp, 2006).

Plants obtain most of their nitrogen from inorganic ammonium and nitrate in the soil or through the symbiosis with atmospheric nitrogen-fixing bacteria. The $\delta^{15}N$ values of soil are positive and generally have values of +2 to +5‰ (though some soils can have from -10 to +15‰). This is because they can be affected by factors such as temperature, water availability, salinity and so on (Sharp, 2006).

At each successive trophic level – from plants to herbivores to carnivores – the $\delta^{15}N$ values of the food source increases with a step-wise enrichment of 3-4‰ in mean $\delta^{15}N$ values. This happens in both marine and terrestrial ecosystems, though the effect is more regular and intense in marine food-web (Ambrose, 1991).

Stable nitrogen isotope analysis is helpful in quantifying trophic position and helps to reconstruct dietary preferences. While combined $\delta^{15}N$ and $\delta^{13}C$ values of bone collagen are useful in differentiating different populations and constraining individual's and community's diet.

3.3 STRONTIUM ISOTOPE ANALYSIS

Strontium is an alkaline earth element with a valence of 2^+ and has four stable, naturally occurring isotopes – ^{84}Sr, ^{86}Sr, ^{87}Sr and ^{88}Sr. With only ^{87}Sr being radiogenic and produced by radioactive β-decay of another alkali element ^{87}Rb, which has a half-life of 4.88×10^{10} years.

Strontium isotopes are used as geochemical signatures for ascribing a prehistoric skeleton to a certain geologic area depending on individual's mobility during life. Strontium isotopic signatures are incorporated into human skeleton through the food chain from soils, which have absorbed strontium and other minerals from eroding geological materials (Bentley, 2006). Because strontium is mainly released from rocks and passed into soil groundwater and plants by chemical weathering. Strontium has an ionic radius of 1.32 Å, which is slightly larger than that of calcium (1.18 Å) thus can easily substitute for calcium in minerals, while human body can absorb strontium as if it is calcium (Faure, et al., 1972).

Strontium applications rely on the principle that different rock types have different chemical composition and age will have different $^{87}Sr/^{86}Sr$ values (Bentley, et al., 2004). Thus, isotopic signatures recorded in the skeleton of an individual can match the biologically available signatures of some locations (far or from the site). Migrants can be defined by

different strontium signatures from teeth and bones or values far from the location values of where the individual was found. Though, in practice it is more trustworthy to compare each individual tooth value to a 'local' range, defined within two standard deviations from the average of $^{87}Sr/^{86}Sr$ values found in the human bones from the site (Bentley, et al., 2004).

Though the average in archaeological human bones from the site, plus or minus two standard deviations may be a useful baseline to define which individuals were local and which were non-locals – it may be not as reliable because of the post-burial contamination (Bentley, 2006). Because after the burial the skeleton's in vivo strontium signal can be overwhelmed or replaced by the groundwater strontium. Making it one of the reasons for choosing bones rather than dental enamel, which is more resistant to diagenesis (because bones will yield the local signature of the place the bone was found and can help establish the local signal). While comparing bone and dental enamel values from the same individual can show if the person was local or not, if he moved when he was young or adult. Thus, for $^{87}Sr/^{86}Sr$ analysis either the values of strontium from teeth or comparison of values between teeth and bone strontium are preferred.

In comparison to increase of carbon and nitrogen ratios in skeletal tissues due to fractionation, strontium is not affected by fractionation process but is affected by another process called bio-purification (though it mainly affects Sr/Ca ratios). Bio-purification can also affect the $^{87}Sr/^{86}Sr$ ratios due to reduction of variance. The ratio will depend on the strontium isotopic ratio of the different dietary components, the relative contributions of these components to the total diet, and concentrations of strontium and where it comes from (Bentley, 2006).

A small amount of marine strontium is transferred directly from ocean to the atmosphere and then reaches continents by precipitation and through dusts. The seawater has a standard value of 0.7092 due to the long residence time of strontium in seawater, compared to the turnover time of oceans (Bentley, 2006). Usually this value is used to see if human diet consisted of large amounts of food derived from marine environments or plant growth was influenced by sea spray.

Archaeological animal teeth are the best indicator of the prehistoric local signature, as long as the animals lived locally. It is due to narrow range of $^{87}Sr/^{86}Sr$ values that reflects an average of biologically available strontium in animal diet catchments, varying by less than 0.6% in many studies (Bentley, et al., 2004). It is best to find some faunal species that can represent the local signature of the site for a better interpretation of the results from humans and fauna.

It has to be taken into consideration that as with carbon and nitrogen rations, the values of $^{87}Sr/^{86}Sr$ reflects an average of all strontium that has been contributed to the sample.

4 MATERIALS AND METHODS

4.1 SAMPLES

Human remains of 30 individuals were chosen from Chalcolithic period Perdigões site tomb I. Because the tomb is secondary and comingled 16 mandibles and 14 right humerus were chosen for better representation of the site. Along with human samples 16 contemporary faunal samples (dog *n* = 2; pigs *n* = 3; sheep/goats *n* = 3; red deer *n* = 3; cattle *n* = 2; rabbit *n* = 3) of the site were also analyzed to ascertain the variability of baseline isotopic signatures of the site (see APPENDIX B: LIST OF SAMPLES). Animal samples were obtained mostly from maxilla or mandible elements.

One point to keep in mind is that the degree of preservation of human remains is not very good. The bones were highly fragmented due to post-depositional trampling, schist slabs falling over, leading to crushing, cracking and erosion. Also, most of them were covered in a very hard layer of concretion sediment.

Data obtained by FTIR spectroscopy in ATR mode and Elemental Analysis on bone samples were first compared in order to select samples displaying a good state of collagen preservation for further analysis by IRMS.

4.2 PRE-SCREENING TECHNIQUES FOR COLLAGEN PRESERVATION

Using prescreening techniques to identify bones suitable for isotope analysis and radiocarbon dating reduces the risk of needlessly sampling archaeological bones and also, saves time. Most popular techniques used for indicating collagen preservation level are nitrogen content (%N) of whole bone powder, collagen yields and carbon:nitrogen (C:N) atomic weight ratios; calculation of infrared splitting factor (IRSF), carbonate-to-phosphate ratio (C/P) and amide-to-phosphate ratio (Am/P) using Fourier transform infrared spectrometry (FTIR); crystallinity measurement with X-ray diffractometer (XRD) and other techniques. Some of these techniques were applied on the bone samples from Perdigões site.

4.2.1 ATR – FTIR

Because FTIR spectroscopy requires a quite complex sample preparation for analysis to overcome the disadvantages of KBr pellets and liquid cells a lot of IR measurements are performed in attenuated total reflectance mode. All types of sample can be placed as powder on the ATR crystal. The infrared beam enters the ATR crystal usually at an angle of 45° degrees. The internal reflectance creates an evanescent wave which protrudes only a few microns beyond the crystal surface and into the sample (Griffiths, et al., 2007). For a high

quality spectrum sample has to be homogenous and a good contact between the sample and crystal is required. Though, it should be noted that ATR is more like a surface technique. The analysis of the sample takes just few minutes.

4.2.2 Sample preparation by ATR-FTIR

29 human and 13 faunal bone samples were examined using ATR-FTIR. Samples were ground by hand using an agate mortar and pestle to a fine powder. Infrared spectra were collected in 100 scans using Bruker Alpha spectrometer coupled with a single-reflection diamond ATR module, in the 4000-375 cm^{-1} wavenumber range, with a spectral resolution of 4 cm^{-1}. All spectra were acquired in the absorbance mode and were recorded, analyzed and normalized using OPUS/Mentor software (version 6.5). For each spectrum, the baseline was corrected, and peak absorbance measured at approximately 565, 603, 1030, 1035, 1415 and 1640 cm^{-1} for the calculation of the Infrared Splitting Factor (IRSF), relative carbonate content (C/P) and relative collagen content (Am/P) (see FIGURE 12).

The IR splitting factor is one of the most widely used parameters to estimate the diagenetic alteration and it represents crystal structure of the bone. Modern fresh bone values have been reported to be in between 2.5 and 3.25 and have a small-sized crystals and irregular lattice structure. Thus, the archaeological bone that should have enough collagen would exhibit values lower than 3.3 and those with higher values than 3.4 would be diagenetically altered (Beasley, et al., 2014). Collagen can be slowly and progressively leached mainly by bacterial collagenases that are responsible for collagen degradation by reducing it to peptides which easily leaches away into the ground. Which results to voids being filled through the precipitation of apatite crystallites and leading to crystal growth (Pate, 1994), (Hollund, et al., 2013).

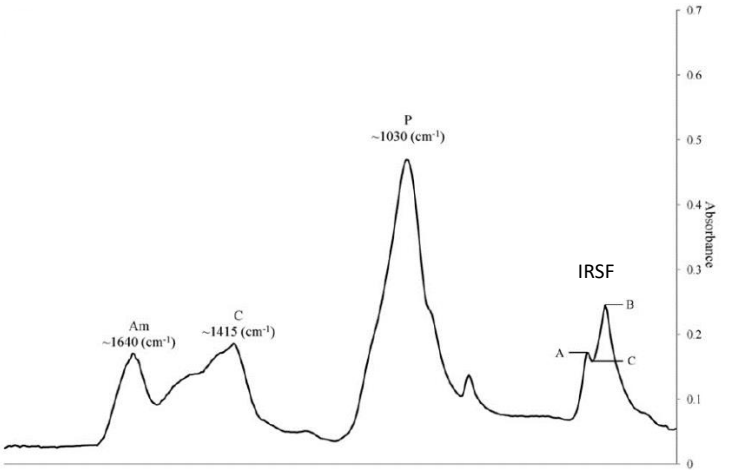

Figure 12 *Infrared absorption spectra of modern bone sample produced by FTIR-ATR (corrected), adapted from (HOLLUND, et al., 2013), p. 513.*

The IRSF value is given by the sum of the absorbance values of the two phosphate peaks at wave-numbers 565 (v_4PO_4) and 603 cm^{-1} (v_4PO_4) and then divided by the height of the

valley between them, following the protocol described by Weiner and Bar-Yosef (1990). Also, crystallinity can be linked to the mean crystal length. The increase in length reflects changes in the crystal growth or loss of the smaller crystals (FIGURE 13). The relationship can be expressed using this equation (Trueman, et al., 2004):

Mean crystal length (nm) = (IRSF – 0.822)/0.048

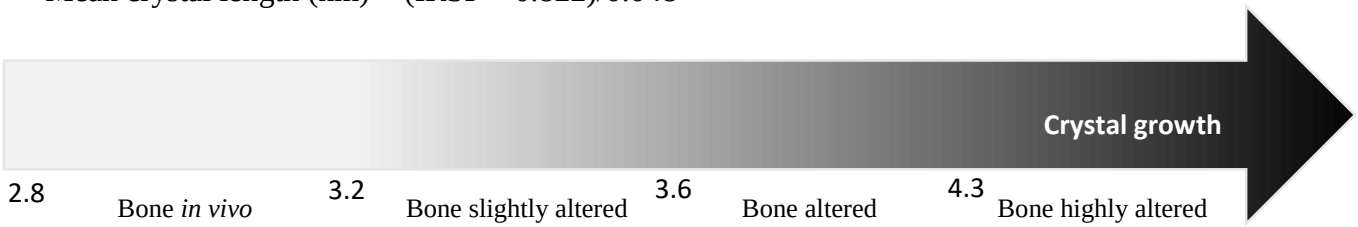

Figure 13 *Evaluation of the alteration stage of organic matter in bone by IRSF*

Relative carbonate content (C/P) ratio reflects carbonate to phosphate content in a bone sample and is measured by the peak heights at wave-numbers of approximately 1035 (ν_3 PO_4) and 1415 cm^{-1} (ν_3 CO_3). The phosphate peak at 1035 cm^{-1} is used because it is the main absorbance peak and isn't as affected by peak splitting as the one used for IRSF calculation (Beasley, et al., 2014) (Wright, et al., 1996). For modern bone the C/P value using KBr pellet preparation mode was reported to be around 0.23-0.34. Diagenetically impinged bone then will have elevated (due to the presence of calcites) or depleted (due to the loss of carbonates) values comparing to the modern bone (Beasley, et al., 2014).

Relative collagen content (Am/P) was measured by the intensity ratio of the amide I peak at 1640 cm^{-1} versus the phosphate peak at 1030 cm^{-1} (Trueman, et al., 2008). There is no literature reference on Am/P values on modern or archaeological bones, except mentioning that it has a wide range of values (Hollund, et al., 2013).

Attenuated total reflectance FTIR is a rapid technique with a minimal sample preparation and a powerful tool in assessing the level of diagenesis in bone samples. The combination of FTIR-ATR with other techniques, like XRD to test crystallinity levels or EA to check the nitrogen levels can be useful in understanding degradation of the bone or pre-screen bone samples for isotope analysis and dating.

4.3 IRMS

Isotope ratio mass spectrometry is a technique which has a wide application field from medicine, food authentication to archaeology and forensic science. It accurately and precisely measures variations in the abundance of isotopic ratios of carbon, hydrogen, nitrogen, oxygen and sulfur. The ratios of these isotopes are always measured relative to an isotopic standard to avoid any bias or systematic error in the measurements.

IRMS instrument is composed of five main sections, which are a sample introduction system, an electron ionization source, a magnetic sector analyzer, a Faraday-collector detector array and a computer controlled data acquisition system (Muccio, et al., 2009).

The most common interface to introduce samples to IRMS is elemental analyzer (FIGURE 14). Making EA-IRMS a technique for bulk measurement, which provides representative data of the average isotopic signal of the entire sample. It is especially used in archaeological science for carbon and nitrogen isotopic ratios of collagen for providing insights into dietary changes through time.

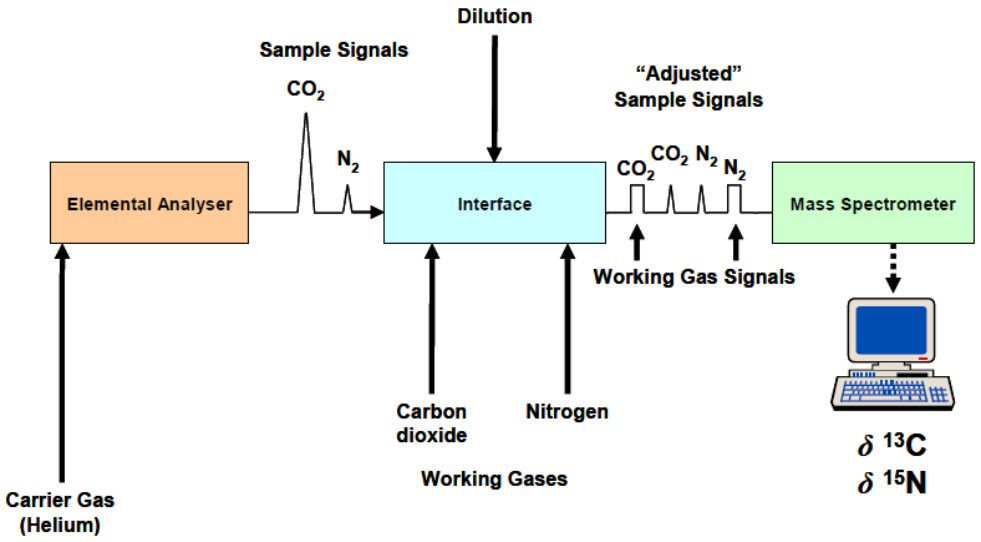

Figure 14 *Simplified schematic drawing of an EA-IRMS setup for the determination of $\delta^{13}C$ and $\delta^{15}N$, taken from (Carter, et al., 2011), p. 3.*

4.3.1 Sample preparation for collagen analysis

Because not all of the samples exhibited enough or good collagen (according to the results from ATR-FTIR and EA, see chapter 5: RESULTS AND DISCUSSION for further details) only a handful of samples were prepared for further analysis by EA-IRMS (total *n* = 16, out of them human *n* = 7; fauna *n* = 9, representing 35% of all samples for this study).

A small chunk of bone samples (~500 mg of compact bone and ~600 mg for those with lower N% levels) were cut and surface-cleaned using a diamond-tipped dremel (Dremel model 225). Samples were weighed in order to calculate collagen yield.

The protocol used for collagen extraction was a method created by Longin (1971) with some modifications done by DeNiro and Epstein (1981). This modified protocol was used to prepare collagen samples for stable carbon and nitrogen isotope analysis. Though each laboratory exhibits some variations of collagen preparation.

Weighed bones were broken into pieces and transferred into labelled plastic centrifuge tubes. The tubes were then filled with ~10 ml of 0.5M hydrochloric acid (HCl) solution for demineralization. Then vortex mixer was used to mix the acid with bones. The samples were

left to sit for several hours at room temperature and put overnight in the refrigerator. This process (vortex, half a day at room temperature, and overnight in the refrigerator) was repeated each day with a weekly acid change until the bone took on a soft consistency, which took about two weeks.

Once the mineral component had been removed the pseudomorphs were centrifuged (at 5.0 RPM x 1000 for 5 minutes) five times in milli-Q water to remove any residual HCl until the pH was neutral. Following this, around 10 ml of 0.125M of sodium hydroxide (NaOH) solution was added to each centrifuge tube and the tubes were left to sit for 20 hours in room temperature in order to remove humic acids and non-collagenous organic residues. The pseudomorphs were then centrifuged four times in milli-Q water at 5000 rpm for 5 minutes to remove the NaOH.

In order to change the pH to a more acidic one for microorganism growth prevention tubes were filled with ~10 ml of 0.01M HCl solution and put in a drying oven at 70°C for 48 hours to gelatinize with regular mixing using vortex. Samples were ready for ultra-filtration, which was done by filtering the samples through Ezee-Filter™ separators (Elkay) to remove any insoluble residues. Labelled collagen vials were then filled with the gelatinized collagen and covered with parafilm, then put in liquid nitrogen until it froze. Finally, frozen vials with collagen were freeze-dried for 48 hours (the illustrated process of collagen extraction can be seen in the APPENDIX D). Upon removal from the freeze-dryer the samples were immediately weighed and collagen yields calculated[3]. The samples were put in the Desiccator with silica gel to keep the moisture out and to wait for further analysis.

4.3.2 Sample analysis by EA-IRMS

Approximately 0.75 mg of lyophilized collagen was weighed into tin capsules. Due to some technical issues further analysis was performed at the Stable Isotopes and Instrumental Analysis Facility (SIIAF) in the Plant Biology Department, Science Faculty of University of Lisbon (FCUL). Analysis of carbon and nitrogen isotopes were performed using a Sercon 20 Hydra IRMS coupled to EuroEA elemental analyzer EA3000. Carbon and nitrogen results are reported using the delta notation relative to internationally accepted standards PDB and AIR respectively. Based on a replicate analyses of international (IAEA: Casein B2155, Sorghum Flour and IAEA N1) and in-house laboratory standards (Casein Iso), measurement errors are less than ±0.8 ‰ for both nitrogen and carbon.

[3] $Collagen\ yield$ (%) = (($collagen\ weight\ of\ the\ sample$)/($bone\ weight\ of\ the\ sample$))∗100%

4.3.3 % N content by EA

Another criteria for prescreening bones to check collagen preservation in addition to XRD or FTIR techniques is the nitrogen percent content of the whole bone using elemental analyzer.

As collagen deteriorates, %N content also decreases due to the relative decrease in amino acid content, and this relationship allows for a prediction of sample integrity. Though it does not distinguish between collagen and non-collagenous protein or nitrogen coming from the soil (Brock, et al., 2012). Thus, it is better to couple this method with some other technique to get better results.

Fresh modern bone contains around 3.5 to 4.5 % of nitrogen and makes up approximately 90% of collagen and the remaining amount is of non-collagenous proteins. Analysis done by Brock et al. (2013) showed that the threshold of 0.7 % N can be used with a 72% success rate for determining if the samples will yield sufficient amount of collagen (at least 1%). Though some samples yielding ~0.5–0.7% N can be considered individually if the data of the sample is very important.

4.3.4 Sample preparation for % N content

Bone surface was cleaned from 16 faunal and 30 human samples using the drill. Samples of whole bone were ground by hand using an agate mortar and pestle to a fine powder. Around 2.5 mg of bone powder was weighted into tin capsules and the %N content was measured using Thermo Scientific Flash HT Plus elemental analyzer. Samples analyzed by this elemental analyzer constituted of faunal (n = 16) and human (n = 16) bones. The in-house standards used were L-alanine and aspartic acid.

Due to some technical issues, which occurred later the final batch of 14 bone samples from human humerus were done by a different elemental analyzer and the reference material used was acetanilide.

4.4 TIMS

Thermal ionization mass spectrometry (TIMS) is a technique that was developed mainly for geological analysis and is used for geochronology and determination of rare-earth elements studies. This technique requires an extensive chemical pre-treatment of the sample, usually involving ion exchange separation as to avoid isobaric interferences (Copeland, et al., 2008).

TIMS is also commonly used for measuring strontium ratios in teeth. It involves removal of about 5-20 mg of tooth enamel, acid digestion, and isolation of strontium, usually done by ion exchange method. This method is quite precise and accurate but a bit destructive. However, if the enamel is removed carefully, samples do not suffer much damage and can be even used for museum exhibitions (FIGURE 15).

4.4.1 Sample preparation

Sampling concentrated on permanent third molars (M_3), which mineralizes between ages 7 and 18 and in few cases on second molars (when the third molar was unavailable or too damaged for sampling). The strontium isotope analysis were carried out on 19 samples of which 6 belonged to fauna from Chalcolithic times and 8 from Neolithic times, while 5 samples were of Chalcolithic humans. Samples of tooth enamel were collected and powdered using a drill with a diamond tip, or chipped off and ground in an agate mortar. Samples were treated to remove any secondary carbonates that might have introduce diagenetic strontium with 1M of acetic acid buffered with Li-acetate solution for 12 hours, because evidence suggests that exposure to strong acids (≤ 1.0 N) can cause bones to recrystallize (Hoppe, et al., 2003). Samples were rinsed four times with milli-Q water and during each rinse they were vortexed and centrifuged for 5 minutes at 5000 rpm. Afterwards, they were put to dry overnight in the oven at 70 °C. The complete pretreatment led to some sample loss.

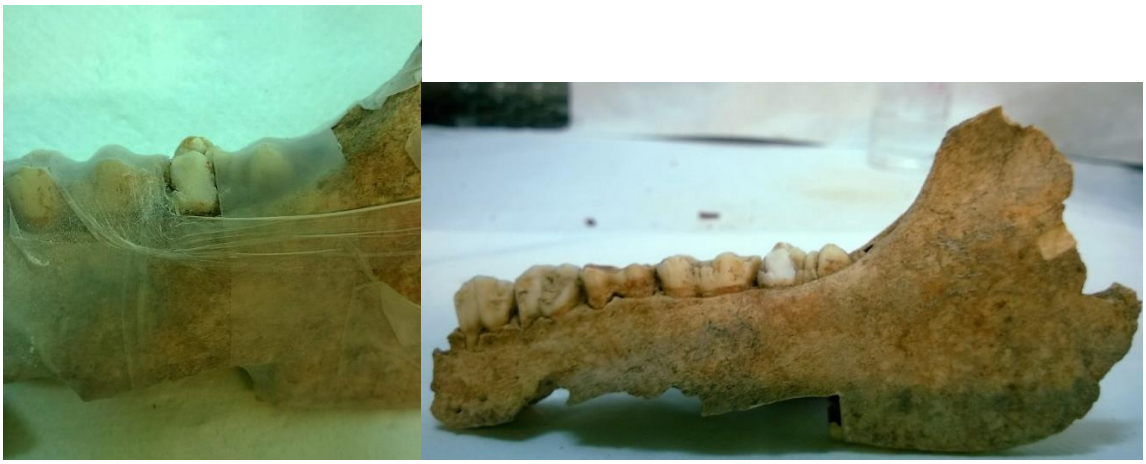

Figure 15 *Removal of enamel (picture on the left - before removal, picture on the right – after).*

Further preparation and analysis were done under clean lab conditions at the Laboratory of Isotope Geology of the University of Aveiro, Portugal (LGI-UA). About 6 – 15 mg of powdered samples were weighed and dissolved in 1 ml of 14 N HNO_3 in Teflon tubes and left overnight on a hotplate at 120° C to dissolve any remaining secondary carbonates.

Ion chromatography was used to isolate strontium and separate it from rubidium and calcium. Columns were made from modified 1 ml Plastic (PE) Pasteur pipettes. The top of the pipette bulb was cut off to leave the reservoir and the length of the column was shortened to obtain the required volume. A PE frit was added in the bottom to retain the resin. These columns prior to use were soaked in a diluted HCl and rinsed with deionized water. Around two hundred microliters of Sr-Resin (50–100 μm, Triskem) was deposited into each column. The cleaning of the resin was done first using 3 ml of Milli-Q water, followed by 2 ml 4N HNO_3 to ensure that there were no contamination inside the columns.

Samples were then dissolved in 0.1 ml of HNO_3 and loaded to the assigned columns using pipette. Elements are eluted from the column roughly in the following order: Fe, Na, Mg, K, Rb, Ca and Sr. Thus, it requires increasing volumes of eluent to release successive elements. To accomplish that, each resin loaded with sample was washed three times with 0.1 ml of 4N HNO_3 eluent and one time with 2 ml of 4N HNO_3 to remove matrix elements, including isobaric rubidium. Rubidium must be eliminated from strontium because it can cause a direct isobaric interference onto ^{87}Sr. Of course, small levels of it in an otherwise clean sample is not a problem due to rubidium being burned before the collection of strontium data begins inside TIMS. However, if the sample also, has calcium in the strontium fraction it can prevent the burn-off of rubidium causing a major interference problem.

The strontium fraction was collected inside the Teflon tubes using 2 ml of Mili-Q water. The purified Sr solution was then evaporated on the hotplate at ~120° C and stored dried to await further analysis. Along with the samples was prepared a blank (^{93}Sr), which was treated as an independent sample and was used for sample correction.

Samples were redissolved with a tiny drop of phosphoric acid (H_3PO_4), and loaded on a single tantalum filament before the insertion into the vacuum system. Phosphoric acid is used because it destroys organic residues mixed with the sample (like ion-exchange resin) and glues the sample to the filament (Dickin, 2005). These purified samples were analyzed using a VG SECTOR 54 multi-collector thermal ionization mass spectrometer with a 1.5-2V ^{88}Sr beam for 50-100 cycles and an internal precision less than 20 ppm (1-sigma). During mass-spectrometric analysis, the filament current was raised by means of a stabilized power supply to yield a certain temperature at which simultaneous volatilization and ionization of strontium sample occurred.

$^{87}Sr/^{86}Sr$ ratio was achieved using the exponential law correction relative to $^{86}Sr/^{88}Sr$ = 0.1194. External reproducibility of the NBS-987 Sr standard since installation has been maintained at 0.710255 ± 0.000004 (5 ppm, conf. lim. 95%, n=169).

5 RESULTS AND DISCUSSION

5.1 PRE-SCREENING OF COLLAGEN

5.1.1 Diagenesis evaluation by ATR-FTIR

The IRSF, C/P and Am/P values based on ATR technique has a clear distinction between faunal and human bone samples. The three indices calculated from ATR spectra was used to assess bone diagenesis parameters, based on the absorbance ratios. Baselines were drawn manually by connecting valleys between the main peaks from approximately 490 to 1700 cm^{-1}. The results are listed in TABLE 7(faunal samples) and TABLE 8(human samples).

All human samples display higher IRSF values (from 3.6 to 4.7) comparing to the faunal samples (from 3.2 to 3.7), except one HPerd-10, whose value falls down the range of faunal samples with a value of 3.6. European rabbit and red deer yields the lowest SF values while goat/sheep – highest (more graphs can be seen in APPENDIX E: SUPPLEMENTARY TABLES AND GRAPHS – FIGURE 30, FIGURE 31 and FIGURE 32). This probably can be explained by the bone morphology and size. Wild animals like red deer and European rabbit have sturdier bones than the domesticated sheep/goat, because it is linked to the domestic animal confinement, lack of exercise and poorer diet and nutrition which can lead to osteoporosis, relative porosity of bones and etc. Also, animal bones have a greater density relative to size and are less porous and thicker than bones of humans (Gilbert, 1989).

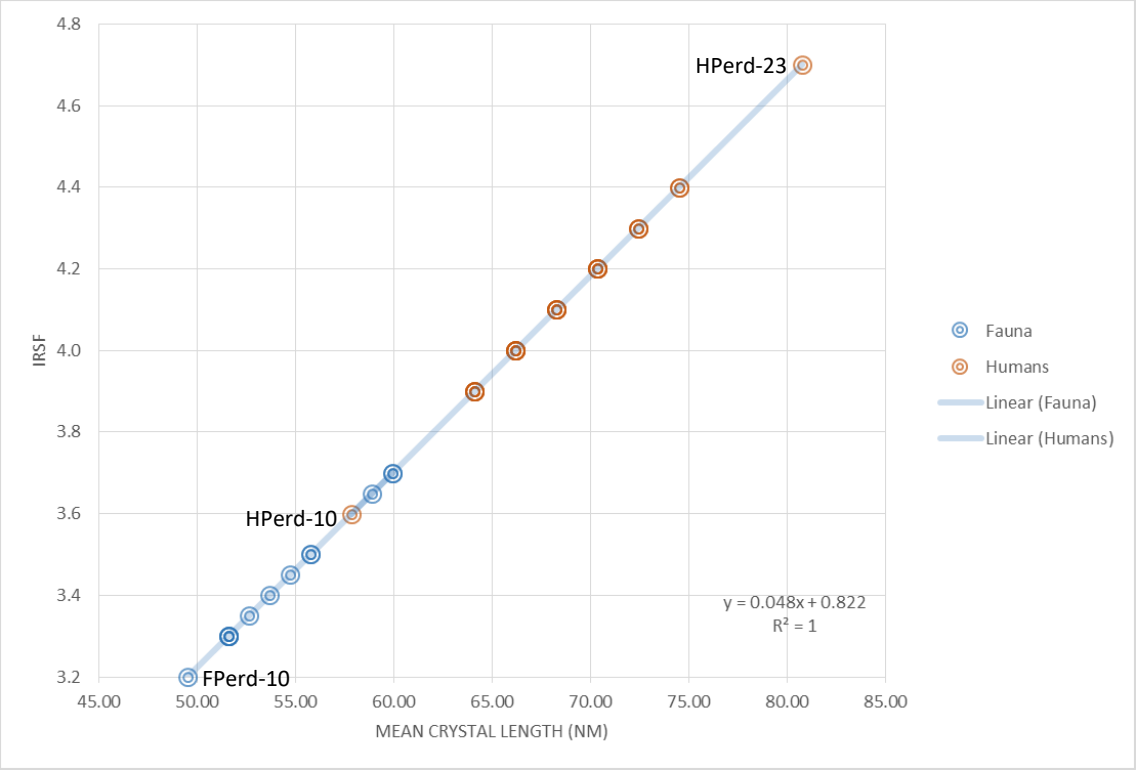

Figure 16 *Relationship between IRSF and Mean crystal length of faunal and human samples*

As mentioned previously an increase in IRSF can indicate an increase in crystal size and order, which generally can be coupled with a loss of carbonate, which can be seen in C/P values (Hollund, et al., 2013). In the FIGURE 16 a close relationship between IRSF and mean crystal length (which uses IRSF as an indirect measure and the formula is in chapter 4.2.2) can be seen (Pearson's correlation P <0.00001; R = 0.9994). If faunal crystal length varies from ~49 to 60 nm, then human crystals from ~58 to 81 nm. Again with HPerd-10 having the smallest crystals out of all human samples. According to Beasley, et al. (2014), diagenetically altered bone will have higher IRSF values than 3.3 and depleted or elevated amount of carbonate, less than 0.23 or more than 0.34. All human samples from Perdigões site Tomb I shows way higher IRSF values and depleted C/P values (FIGURE 17). The exception are samples HPerd-8 and HPerd-16 which have C/P values of 0.26 and 0.32, however their IRSF values are 4.1 and 4.0 respectivelys. Only one sample from fauna FPerd-10 has both values which don't show any signs of diagenesis. Interesting thing to note, is that domesticated animal bones are more affected by the diagenesis than the wild species, which can ascertain Gilbert's (1989) theory, that microstructural effects might reflect biochemical reactions brought by the domestication process.

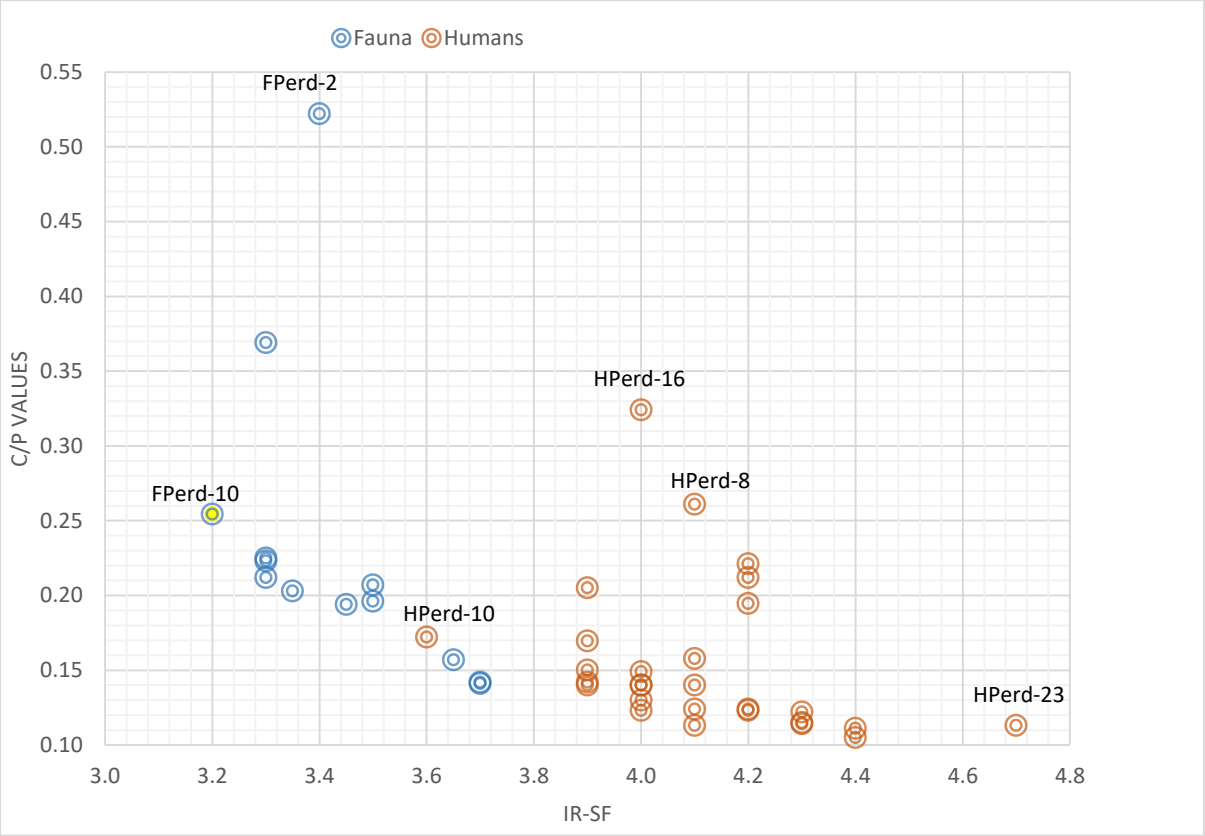

Figure 17 *IRSF and C/P values comparison for both faunal and human samples*

As there is no actual reference of Am/P values (relative collagen content), it can only be said that it ranges widely. Fauna is from 0.028 to 0.109 with the average of 0.055 and human

bone values differ from 0.016 to 0.055, with an average of 0.025. This shows that human values are lower than fauna ones, which is quite common in warmer Mediterranean climate where bone collagen is less preserved (Weiner, 2010) and another factor is that animal and human bones have different macro and micro structure, which affects preservation. HPerd-10 is the least altered out of all human samples by all calculated indices. As can be seen by the FIGURE 18 the results of Am/P exhibit a strong negative correlation (Pearson's R = -0.8085; P < 0.00001) with the SF. While C/P and Am/P have a positive correlation (Pearson's R= 0.333); P < 0.033384), but the relationship between the variables are quite weak. Likely the collagen loss was replaced by phosphates and other minerals entering into the bone pores.

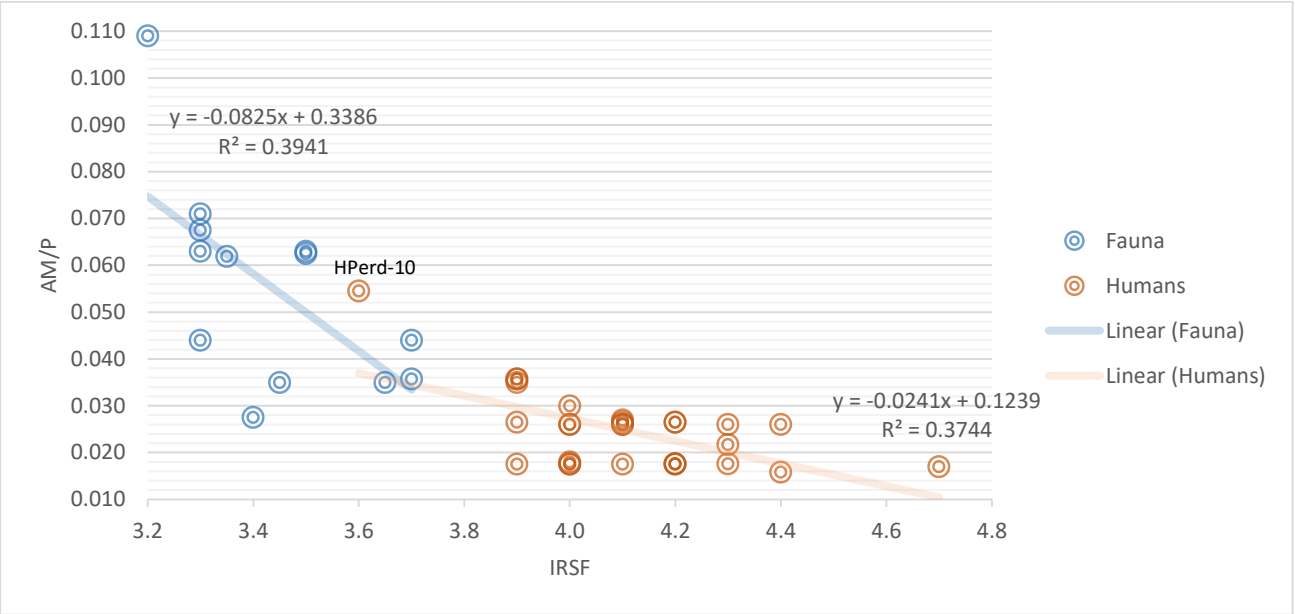

Figure 18 *Relationship between IRSF and Am/P values*

Taking into the consideration the calculated indices from the ATR spectra it can be said that all bones are quite poorly preserved and have underwent some diagenetical alteration (see FIGURE 19) with faunal bones being only slightly altered, while human bones vary from altered to highly altered (FIGURE 13).

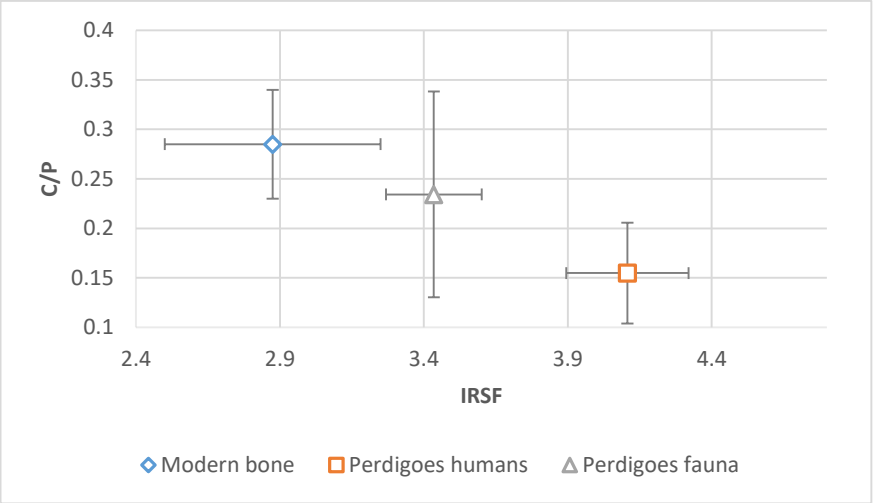

Figure 19 *Comparison of average values of modern fresh bone vs. fossil bones from Perdigões site*

5.1.2 Diagenesis evaluation by %N content results

Results from 32 analyzed samples are presented in the TABLE 1 (p. 38), TABLE 2 (p.38-39) and FIGURE 20. Samples of fauna (from 0.5 to 1.8 % with an average of 1.1 %) exhibit higher nitrogen values than the human samples (from 0 to 1.3 % with an average of 0.24%) with the exception of HPerd-10 with a value of 1.3.

According to Brock et al. (2013) experiments samples with a threshold of 0.7% N should contain sufficient amounts of collagen, while those in between 0.5-0.7% values should be chosen with care. In the Figure 20 we can see that if we consider a threshold of 0.7% almost all faunal samples (with FPerd-2, FPerd-5 and FPerd-6 falling into the 0.5-0.7% threshold) would pass the evaluation, while only one human sample would be included. Values above 1% of nitrogen content belong to red deer, European rabbit, one cattle and one pig.

While all the human samples (with the exception of HPerd-10) fall below 0.5 %, meaning that these samples would not yield sufficient amounts of collagen for analysis of diet.
Unfortunately, nitrogen content results of human mandibles (n=16) could not be compared to those from human humerus (n=14) because the elemental analyzer used for their analysis (see Section 4. Materials and Methods) was not as sensitive and none of the samples have yielded any nitrogen. Thus, only the results from ATR-FTIR analysis and the collagen yield (%) values of 14 humerus were considered in finding out which samples will have sufficient and good quality collagen for further analysis.

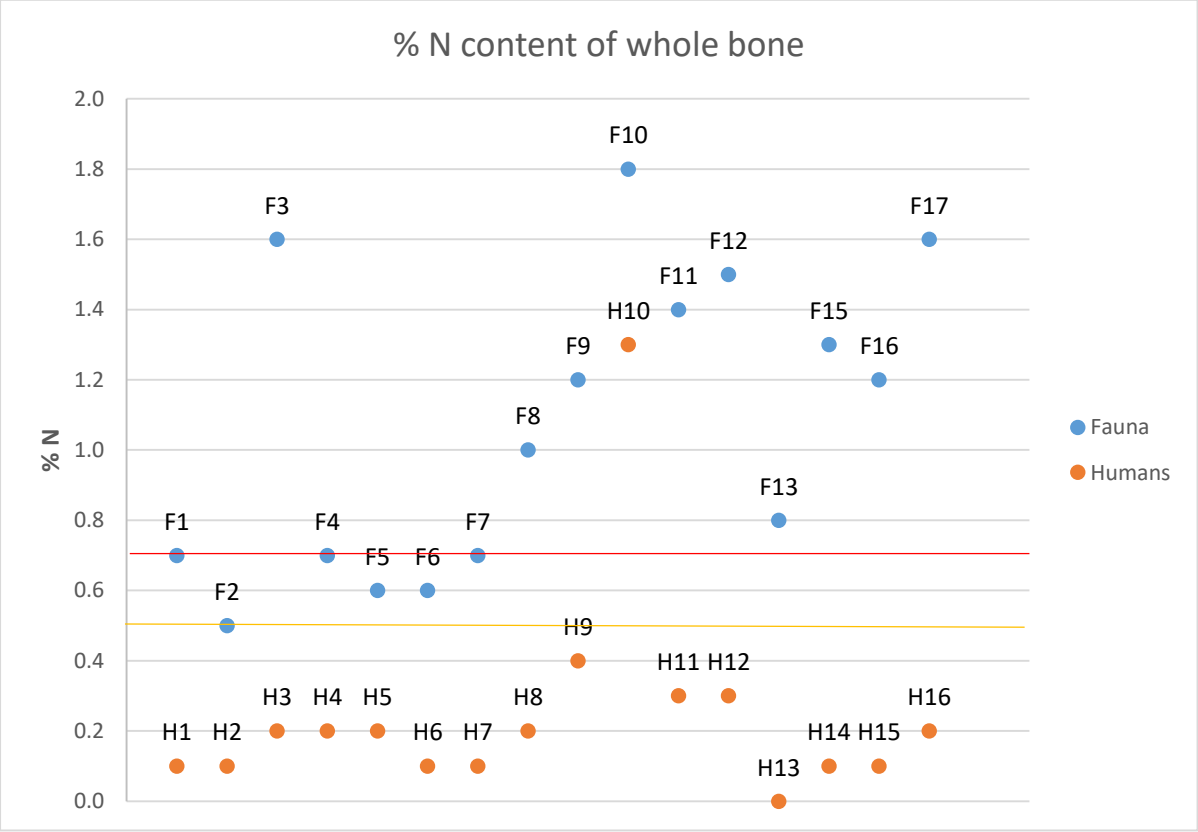

Figure 20 *%N content of whole (raw) bone powder analysis by EA. Samples above the red line are considered to contain enough collagen. Samples below yellow line will not yield sufficient amounts of collagen.*

One detail for future analysis should be considered - if measurements of whole bone samples by EA are done not only for % N content but also, % C content, then the calculated C/N ratios could provide not only an indication of general state of preservation of the bone, but also the extent to which deamination of amino acids has taken place or even how much exogenous carbon-containing compound have contaminated the sample (Brock, et al., 2012). Of course, comparison of the results with some other technique (XRD, XRF, and FTIR) is a welcome possibility.

5.1.3 Comparison of ATR-FTIR and %N content results

Results from ATR-FTIR and EA analysis have been compared to see if they correlate with each other. A major advantage is that both techniques did not require extensive sample preparation and used only tiny amounts of whole bone powder.

All of ATR-FTIR results (IRSF, C/P and Am/P) were compared against % N content using scatter plots (FIGURE 21, FIGURE 22, FIGURE 23). From these figures a difference between human and faunal bone samples can be seen – animal bones are less diagenetically altered in comparison to human ones. For instance, red line in the figure 20 shows that bone samples with lower IRSF values are less diagenetically altered, while the yellow arrow shows that samples with higher %N content supposed to have higher amounts of collagen. Thus, samples in the lower square on the right corner (all samples from fauna and one of human) should yield sufficient amounts of collagen after the pre-treatment with acid procedure is done.

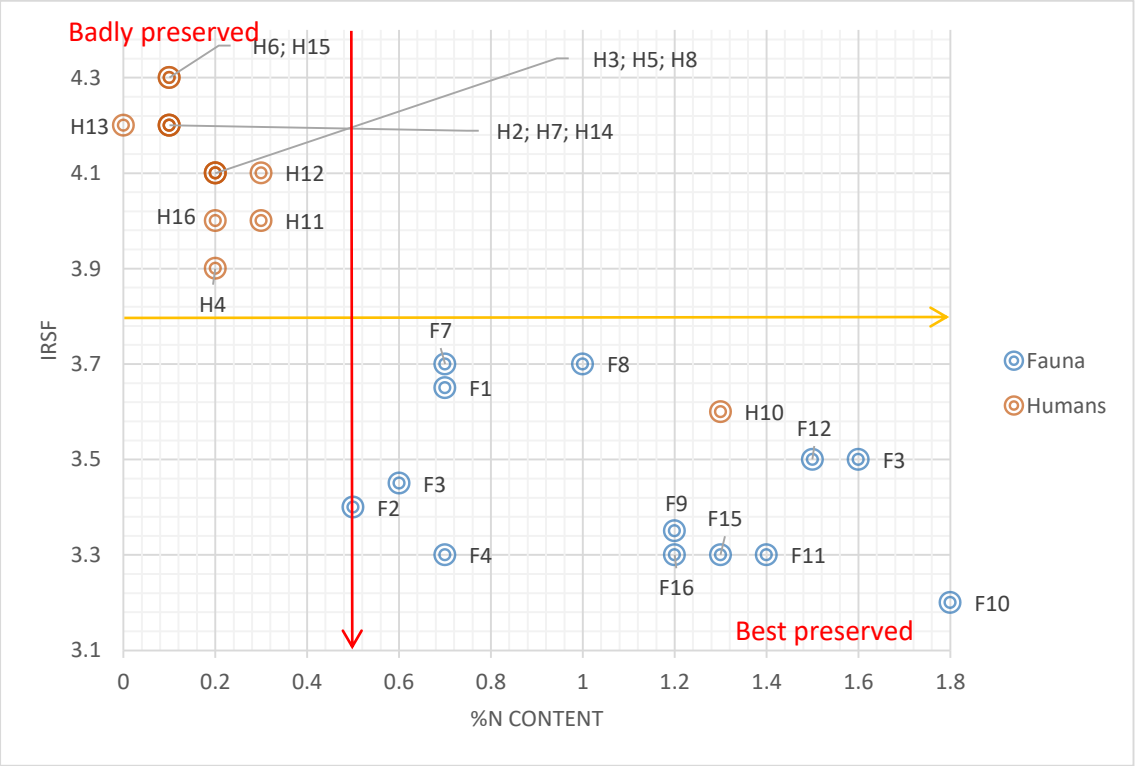

Figure 21 *Comparison of IRSF and %N content results of fauna and humans. Red and yellow lines showing preservation levels.*

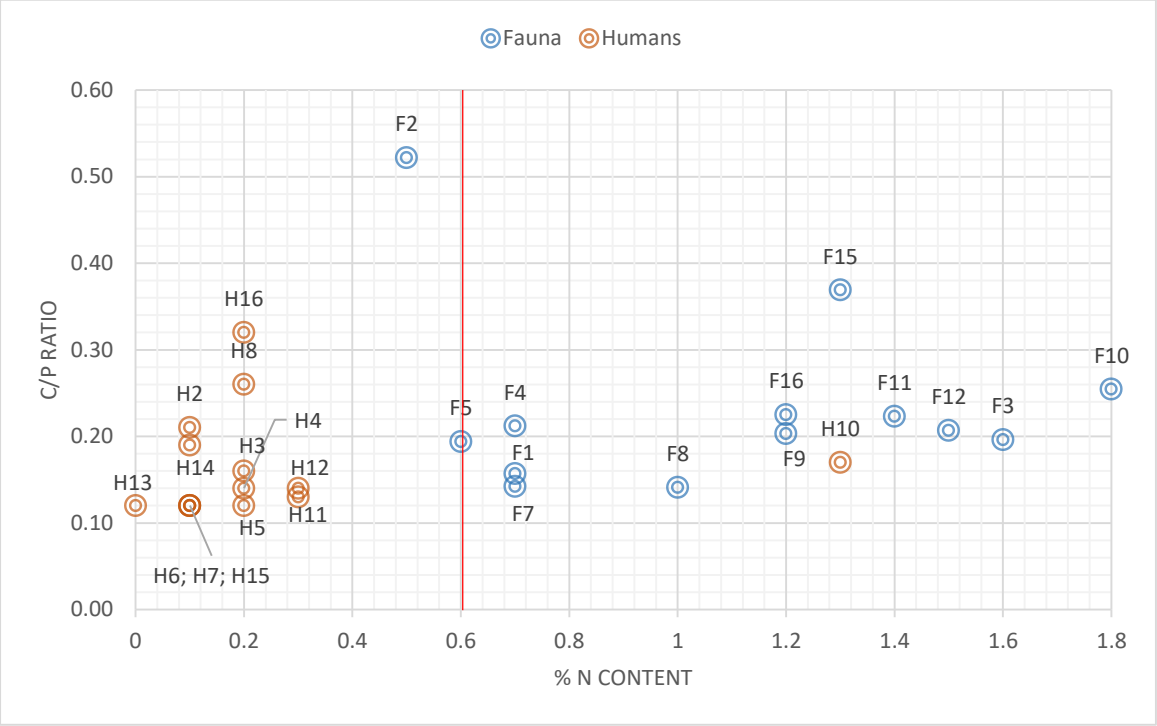

Figure 22 *Relative carbonate content by ATR-FTIR vs. % N content by EA.*

Two samples from fauna group stands out as being the highest and the lowest in all results: FPerd-10 (with Am/P ratio of 0.11, C/P ratio of 0.25, SF with 3.2 and %N content of 1.8) and FPerd-2 (with Am/P ratio of 0.03, C/P ratio of 0.52, SF with 3.4 and %N content of 0.5). Despite the fact that red deer sample (FPerd-10) is in better preservation state than sample from the dog (FPerd-2), both samples can be considered as able to yield enough collagen, red deer will definitely have not only sufficient amount for collagen analysis but is the least diagenetically altered, so the results from it can be interpreted with ease. The two samples from the human group with the widest gap between values are HPerd-10 (Am/P ratio of 0.05, C/P ratio of 0.17, SF with 3.6 and %N content of 1.3) and HPerd-13 (Am/P ratio of 0.01, C/P ratio of 0.12, SF with 4.2 and %N content of 0). Making HPerd-10 the only sample from all human mandibles (*n*=16) analysed able to yield enough good quality collagen. While all other 'possible' samples from mandibles have been removed from further analysis.

ATR-FTIR showed that some of the samples displayed lower relative collagen values (Am/P) than expected according to their nitrogen content analysis by EA (FIGURE 23) – some fauna samples have higher values in nitrogen content comparing with human ones but lower or same values as human in relative collagen content. By definition collagen presence requires nitrogen, so the values should correlate. As both analysis were done on whole (raw) bone with just surface cleaning, higher values of nitrogen detected could arise from soil contaminants or from degraded collagen proteins (Brock, et al., 2012). Despite this discrepancies both analysis

complement each other and can be used together or separately coupled with other techniques to prescreen bones for diet or dating analysis.

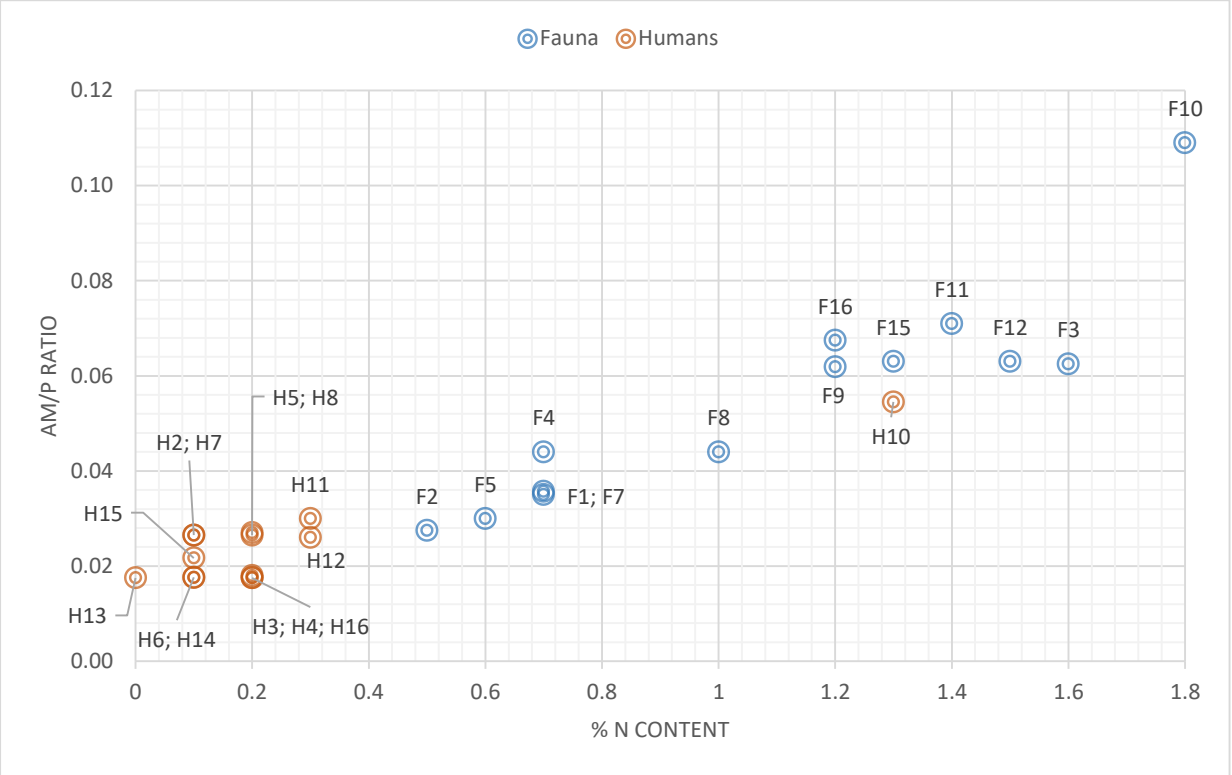

Figure 23 *Relative collagen content by ATR-FTIR vs. % N content by EA.*

5.2 COLLAGEN QUALITY

Collagen yields varied between 0.2 and 2.6% (average 1.1±0.2%, n = 15) for human remains and 4.3 and 7.0% (average 5.8±0.3, n = 9) for the faunal remains. Out of 15 human samples only 7 samples (those with higher collagen yield) were chosen for further analysis (TABLE 2). Bone samples of fauna exhibiting sufficient amounts of %N content of whole bone and/or yielding enough collagen (see TABLE 1) were selected for further analysis by IRMS.

Most of the faunal samples and couple of humans met the established quality criteria for collagen preservation from the archaeological remains. Nitrogen contents of human remains ranged between 0.2 and 13.7% (average 4.7±2.4%) and from 4.5 to 14.7 (average 11.1±1.3%) for faunal remains. Carbon contents varied from 0.8 to 39.7% (average 13.7±6.8%) for human remains and from 13.1 to 41.5% (average 31.4±3.6%) for faunal bones. Atomic C/N ratios ranged between 3.4 and 5.0 for human samples, while faunal ones between 3.3 and 3.4. Based on these results four human samples (HPerd-17, HPerd-18, HPerd-24 and HPerd-30) were removed from data interpretation on subsistence patterns due to carbon and nitrogen content values and atomic C/N ratios being above or below the expected range (TABLE 2, in red). If C/N ratios are higher than 3.5 (the recommended C/N values are between 2.9 and 3.6) then the $\delta^{13}C$ may point to the source of excess carbon contamination from humic acids found in soils and

thus can produce more negative carbon isotopic values (van Klinken, 1999). Thus, values from four human of the previously selected seven samples are not considered in this study.

Table 1 *Results of nitrogen content of whole bone by EA and IRMS results of collagen from fauna*

Assigned N°	% N bone	Collagen yield, %	%C	%N	C/N	$\delta^{13}C$ ‰ vs PDB	$\delta^{15}N$ ‰ vs AIR
FPerd-1	0.7	5.36	15.0	5.3	3.3	-19.2	9.1
FPerd-2	0.5	not analysed	-	-	-	-	-
FPerd-3	1.6	6.23	36.8	13.2	3.3	-20.1	5.2
FPerd-4	0.7	not analysed	-	-	-	-	-
FPerd-5	0.6	not analysed	-	-	-	-	-
FPerd-6	0.6	5.59	13.1	4.5	3.4	-20.2	4.4
FPerd-7	0.7	not analysed	-	-	-	-	-
FPerd-8	1	not analysed	-	-	-	-	-
FPerd-9	1.2	6.41	33.8	12.1	3.3	-19.9	4.8
FPerd-10	1.8	5.33	41.2	14.6	3.3	-19.9	4.4
FPerd-11	1.4	6.35	28.6	9.9	3.4	-19.5	4.4
FPerd-12	1.5	6.99	41.5	14.7	3.3	-19.9	6.2
FPerd-13	0.8	4.31	31.7	10.9	3.4	-14.7	8.6
FPerd-15	1.3	not analysed	-	-	-	-	-
FPerd-16	1.2	5.26	41.2	14.7	3.3	-21.9	3.7
FPerd-17	1.6	not analysed	-	-	-	-	-
Mean value	1.1	5.76	31.4	11.1	3.3	-19.5	5.6
St. Dev	0.42661	0.80860	10.84831	3.90925	0.05203	1.94036	1.94798
St. Error	0.1	0.27	3.6	1.3	0.0	0.6	0.6

5.2.1 Dietary reconstruction of Perdigões fauna

Stable isotope ratios of all faunal remains vary between -21.9‰ (*O.cunniculus*) and -14.7‰ (*Bos taurus)* in $\delta^{13}C$ and 3.7‰ (*O. cunniculus*) and 9.1‰ (*Canis familiaris*) in $\delta^{15}N$. Based on the collagen data, the sampled fauna obtained the majority of their protein from a food web based on C3 plants (FIGURE 24). This is not surprising because Europe has a relatively narrow variations in $^{13}C/^{12}C$ ratios at the beginning of the food chain, and marine plant and sea-food consumption (especially in the case of fauna) is also, not widespread (van Klinken, et al., 2002). Also, marine plant consumption in Perdigões was highly unlikely due to the site's geographical location (inland). Millet, a C_4 pathway plant, used for human and animal consumption, starts to appear in Europe in the Bronze Age, though not during Chalcolithic.

Table 2 *Results of nitrogen content of whole bone by EA and IRMS results of collagen from humans. Values in red from EA analysis of %N content were not considered due to technical issues. Results in red obtained by IRMS were not included due to possible contamination. Mean value, standard deviation and standard error calculated excluding those values.*

Assigned N°	% N bone	Collagen yield, %	%C	%N	C/N	$\delta^{13}C$ ‰ vs PDB	$\delta^{15}N$ ‰ vs AIR
HPerd-1	0.1	not analysed	-	-	-	-	-
HPerd-2	0.1	not analysed	-	-	-	-	-
HPerd-3	0.2	not analysed	-	-	-	-	-
HPerd-4	0.2	not analysed	-	-	-	-	-
HPerd-5	0.2	not analysed	-	-	-	-	-
HPerd-6	0.1	not analysed	-	-	-	-	-

HPerd-7	0.1	not analysed	-	-	-	-	-
HPerd-8	0.2	not analysed	-	-	-	-	-
HPerd-9	0.4	not analysed	-	-	-	-	-
HPerd-10	1.3	2.57	39.7	13.7	3.4	-19.6	8.5
HPerd-11	0.3	not analysed	-	-	-	-	-
HPerd-12	0.3	not analysed	-	-	-	-	-
HPerd-13	0	not analysed	-	-	-	-	-
HPerd-14	0.1	not analysed	-	-	-	-	-
HPerd-15	0.1	not analysed	-	-	-	-	-
HPerd-16	0.2	not analysed	-	-	-	-	-
HPerd-17	0	2.15	0.8	0.2	4.6	-30.9	-7.6
HPerd-18	0	1.25	1.8	0.5	4.5	-23.7	5.7
HPerd-20	0	0.19	-	-	-	-	-
HPerd-21	0	0.55	-	-	-	-	-
HPerd-23	0	1.21	38.8	13.4	3.4	-19.8	8.9
HPerd-24	0	1.59	1.2	0.3	5.0	-29.0	5.8
HPerd-26	0	2.51	12.8	4.3	3.4	-19.4	10.0
HPerd-27	0	0.76	-	-	-	-	-
HPerd-28	0	0.20	-	-	-	-	-
HPerd-29	0	0.80	-	-	-	-	-
HPerd-30	0	1.72	1.0	0.2	4.7	-29.0	-1.2
HPerd-32	0	0.20	-	-	-	-	-
HPerd-33	0	0.19	-	-	-	-	-
HPerd-34	0	0.72	-	-	-	-	-
Mean value	0.2	1.11	30.4	10.5	3.4	-19.6	9.1
St. Dev.	0.29882	0.83830	15.24108	5.31453	0.03969	0.23755	0.77943
St. Error	0.1	0.22	8.8	3.1	0.0	0.1	0.5

5.2.2 Dietary reconstruction of Perdigões inhabitants

Overall, δ^{13}C values of humans range from -19.8 to -19.4‰ (average -19.6±0.1‰, n = 3) and δ^{15}N values from 8.5 to 10‰ (average 9.1±0.5‰, n = 3).

To reveal better information on the protein sources the differences between the faunal and human values were calculated. The human bone collagen δ^{13}C ratios are, on average, only 0.9‰ above the combined average of cattle, sheep/goat, pigs (domestic animals) and 0.2‰ below the average collagen carbon isotope ratios of red deer (wild animal). The human bone collagen δ^{15}N ratios are, on average, 3‰ above the combined average of cattle, sheep/goat and pigs and 4.6‰ above the average collagen nitrogen isotope ratios of red deer. Typical differences of one's trophic level are 1-2‰ for carbon and 3-5‰ for nitrogen (Lee-Thorp, 2008), (van Klinken, et al., 2002). Thus, the nitrogen values fall into the expected range of trophic level, while the carbon falls a bit short on expected trophic level. This may be due to cattle's (FPerd-13, Bos taurus) unexpectedly low carbon values, maybe it was a juvenile or the sample is contaminated (it would be advisable to run the sample a second time or increase new faunal samples in the study). It was chosen to remove the FPerd-13 sample from further

calculations and interpretations as it is an obvious outlier. And with this, the trophic level of carbon (humans versus domestic animals) normalizes.

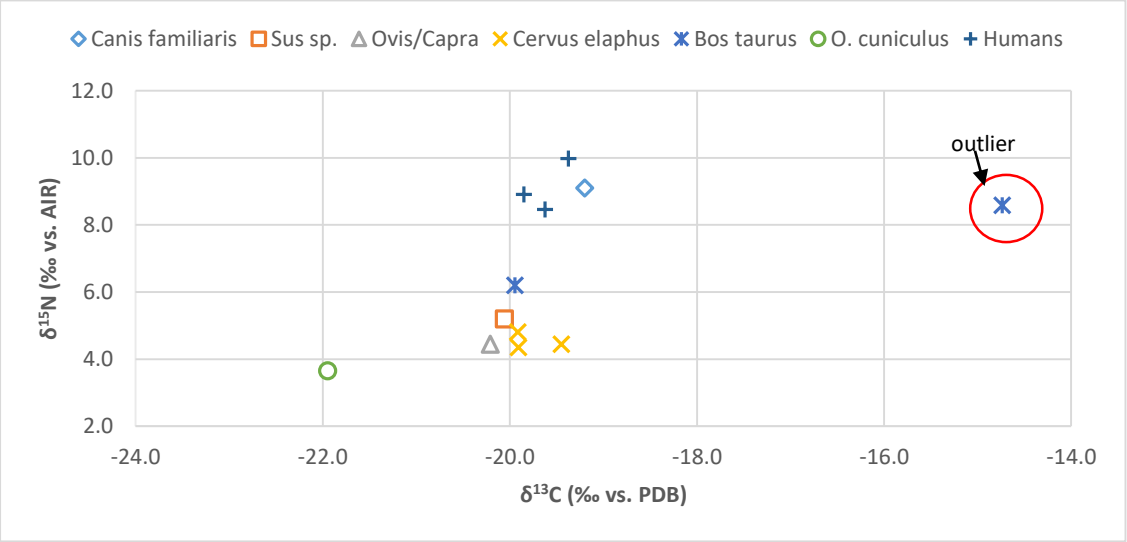

Figure 24 *Stable isotope composition of human and faunal bone collagen samples of Chalcolithic period.*

The only analysed dog (Figure 24) remains showed that it was likely provisioned with scraps from human meals and thus, shares an isotopically similar diet with contemporaneous humans. The similar result were observed in the study *"Dogs as Analogs in Stable Isotope-Based Human Paleodietary Reconstructions: A Review and Considerations for Future Use*" by Guiry (2012), where he used dogs as human proxies.

As there was previously a small study done on Perdigões site human remains by Emslie et al. (2015) - those results and additional data on Late Neolithic fauna from the site were included in FIGURE 25 for comparison.

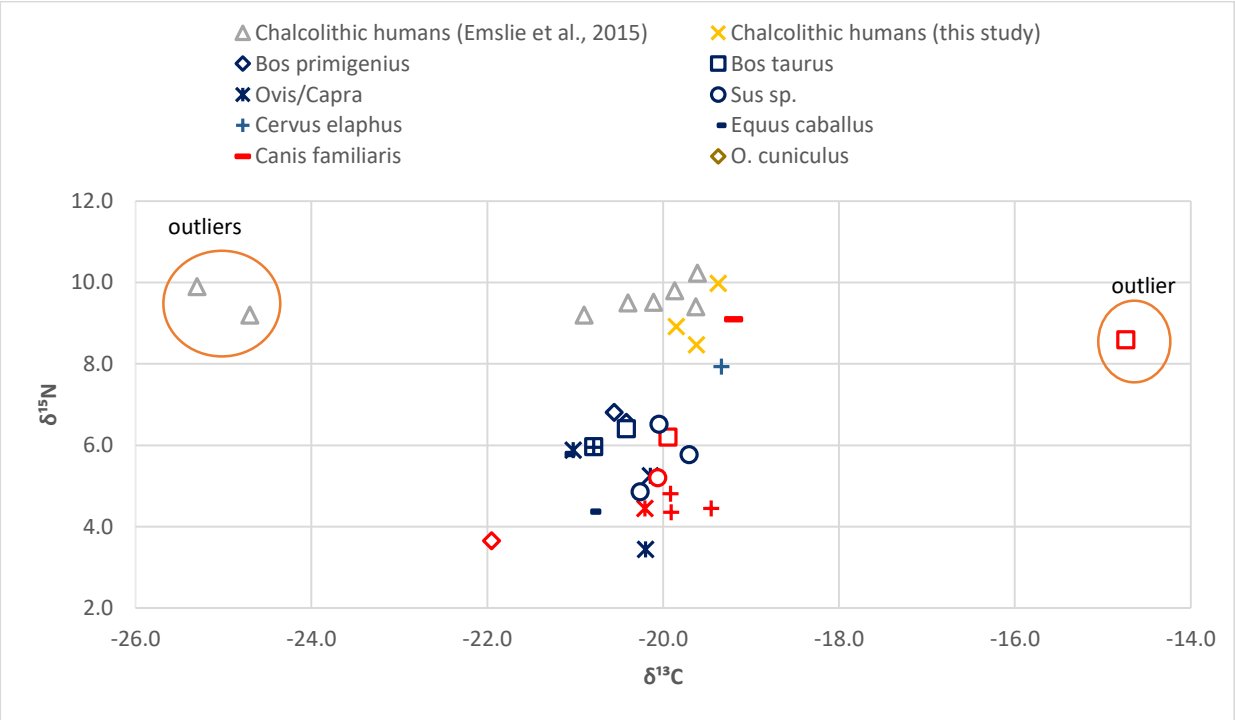

Figure 25 *Stable isotope composition of human and faunal collagen samples from Late Neolithic and Chalcolithic period (additional data from Emslie at al. (2015)). Red colour for Chalcolithic fauna, blue – Neolithic fauna.*

As can be seen, humans from tomb II are exhibiting similar values to those from tomb I, except two samples whose carbon isotope values are -24.7 and -25.3‰ (with C:N ratios of 3.2 and 3.0 respectively (Emslie, et al., 2015)). As there was no additional information on these two strange samples it would be best to exclude them (probably these two samples are outliers) and maybe do more tests on other collagen preservation criteria.

Fauna from Neolithic period do not differ much from those of Chalcolithic period. Except red deer (*Cervus elaphus*) which differs in collagen nitrogen isotope values by almost 3‰. Maybe this individual was a juvenile, as weaning enriches ^{15}N values (Martinez, et al., 2015) relative to those of adults of same species. Or it can be attributed to aridity – the more arid, the higher $\delta^{15}N$ values (Ambrose, 1991). Another, reason can be variations in $\delta^{15}N$ values of the plants the red deer eats (as plant nitrogen values depend on the source of nitrogen that they use: nitrogen directly from the soil or by micro-organism fixed one).

These isotope results indicate that the analysed Perdigões individuals had a preference for predominantly terrestrial-based subsistence pattern based on C3 plants and domestic animals like pigs, sheep/goats and cattle with maybe small amounts of wild animals.

5.2.3 Comparison with other sites from Iberian Peninsula

Twelve sites from which the data was obtained for subsistence comparison ranged in time from Late Neolithic to Late Chalcolithic (i.e. between 3500 and 2200 BC). Sites were from Spain or Portugal and varied in burial types. Site locations can be seen in FIGURE 35.

Valencina-Castilleja is located in 6 km from the city of Seville, Spain and is a Chalcolithic period site, dating 3000-1710 cal BC. The area in which site is located is very rich in natural resources with marine and lake environments near important forestry. The monument is a collective *tholos* with a double chamber and has a MNI of 24 individuals. The human mean $\delta^{13}C$ value is -19.5±0.6‰ and the mean value of $\delta^{15}N$ is 9.1±0.4‰. These values places humans in a 3.3‰ higher trophic level than the herbivores with no trace of marine protein consumption in their diet, though it does not exclude the occasional consumption of freshwater protein (Fontanals-Coll, et al., 2015).

Cova de la Pastora is a limestone cave with an altitude of about 860 m above the sea level, located in the municipality of Alcoy, Alicante (Spain). It spans Late Neolithic and reaches Late Bronze Age. Stable nitrogen isotope values range from 7.5 to 10.6‰ and -19.6 to -19.0‰ for carbon values. Though the sample data of collagen isotopes used for comparison from this site belongs only to Late Neolithic and Chalcolithic.

Avenc dels Dos Forats or Cova del Monedero is located in the municipality of Carcaixent, Valencia (Spain). It is a Late Neolithic to Copper Age cave. The human mean $\delta^{13}C$ value is -19.1±0.0‰ and the mean value of $\delta^{15}N$ is 10.2±0.3‰. (Fontanals-Coll, et al., 2015).

Bolores is a rock-cut tomb which was primarily used between 2800-2600 cal BC for burying adults, juvenile and children. It is located in the municipality of Torres Vedras, in the district of Lisbon. The site has primary and secondary burials with a minimum number of individuals reaching 36. Thirteen individuals were analysed with only 10 yielding viable collagen data. The values ranged from -20.8 to -19.5‰ in $\delta^{13}C$ and between 7.4 and 9.8‰ in $\delta^{15}N$ in bone collagen. (Lillios, et al., 2014).

Feteira II is a burial cave located near Lourinhã in the Estremadura region of Portugal and is dated between ca. 3700-2900 BC with a MNI=68. The average value of $\delta^{13}C$ is -20.2±0.3‰ and 8.2±0.6‰ in $\delta^{15}N$ values. (Waterman, et al., 2014).

Paimogo I is a *tholos* monument, located west of Feteira II, near the Atlantic coast and is dated between ca. 3300-2500 BC with a MNI of 413. Paimogo I exhibits a slightly larger range of carbon isotope in collagen in comparison to Feteira II. The average of $\delta^{15}N$ is 8.5±0.8‰, while the δ13C is -20.2±0.5‰ (Waterman, et al., 2014).

Zambujal is a walled fortified settlement located near the city of Torres Vedras in the Estremadura region, Portugal and is dated to ca. 2800-1800 BC. The site has an estuarine environment. The average $\delta^{13}C$ value is -20.4±0.4‰ and the mean value of $\delta^{15}N$ is 9.3±1.2‰ (Waterman, et al., 2016).

Cova da Moura – a natural cave burial site from Middle Neolithic to Copper Age period, considered as one of the richest burial sites in the region, having the average $\delta^{13}C$ values of -19.9±0.7 and 9.4±0.9‰ of $\delta^{15}N$ (*n*=10).

Other sites located in the Estremadura region are Borracheira (tholos monument), Lapa da Rainha II (natural cave burial site) and Cabeço da Arruda I (artificial cave). All of these monuments are dated to Late Neolithic and Chalcolithic period (Waterman, et al., 2016).

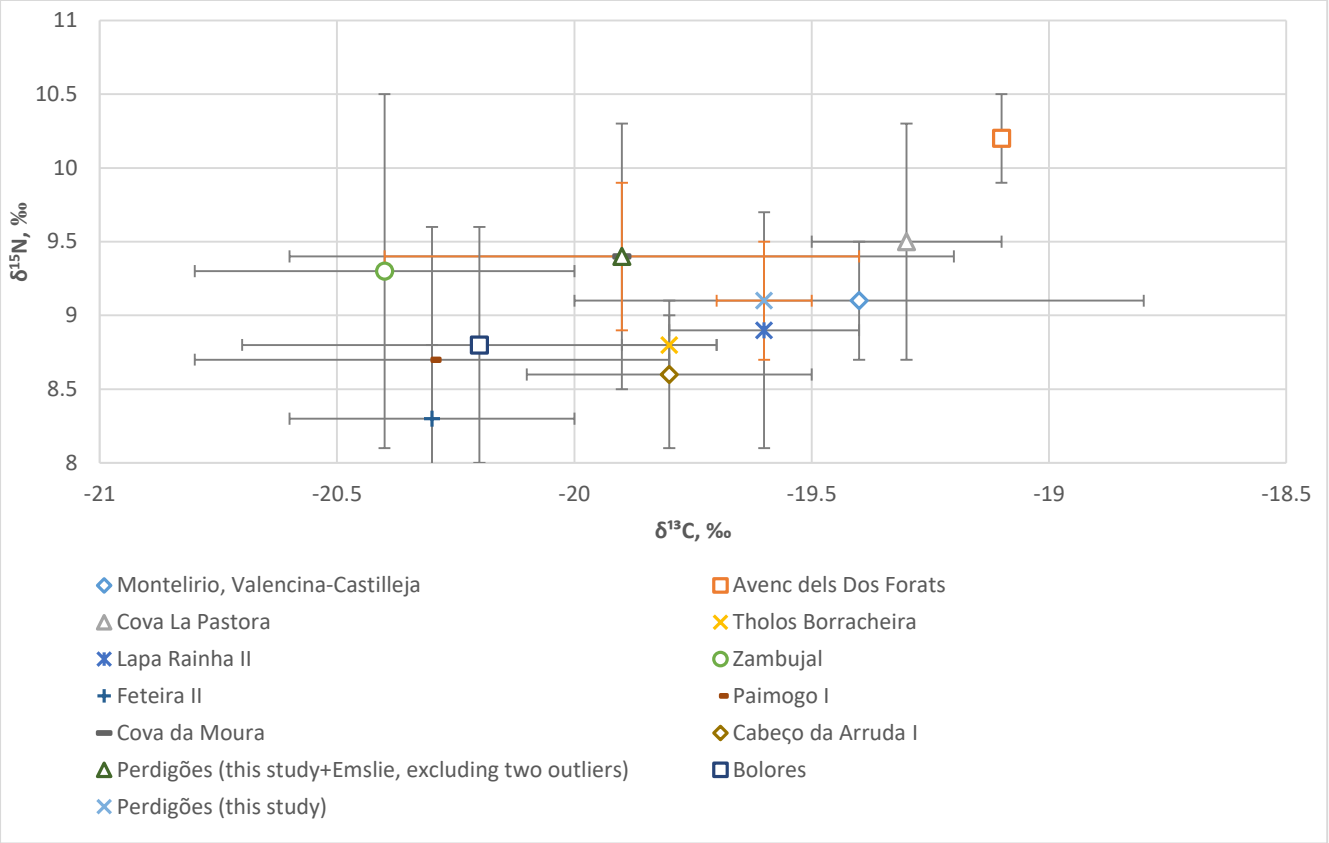

Figure 26 *Plot of δ¹³C and δ¹⁵N average value, with error bars, from Late Neolithic and Chalcolithic period human (adults) remains from the Iberian Peninsula. For more information see* ***Table 9*** *from the appendix.*

All the sites compared present a mainly C_3 terrestrial diet that shows no significant isotope evidence for aquatic resource consumption with maybe some freshwater resource consumption for those sites which are near rivers or lakes. According to Tykot (2006), a pure C_3 consumer would have a $\delta^{13}C$ value of approximately -22‰ in collagen, while the individuals from sites form Iberian Peninsula exhibit $\delta^{13}C$ value ranges from approximately -21.0 to -19.0‰. These slight differences in carbon values can be attributed to the differences in the soils, growth conditions of the plants, types and parts of the plant consumed and even on animals consumed.

Sites from Spain (FIGURE 26) are more concentrated to the right side (values range from -19.4 to -19.1‰), while sites from Portugal are a bit more spread out ($\delta^{13}C$ values range from -20.8 to -19.6‰). Perdigões site has the closest isotope values to the site of Zambujal (one is located in Alentejo region and the other in Estremadura), which shows that during Late Neolithic and Chalcolithic period different regions similar subsistence patterns. Though, it would be interesting to compare both regions with stable isotope values of sites from Algarve region (full of marine resource possibilities).

Chalcolithic Perdigões calculated average of this and Emslie's study together (excluding the two outliers from the calculation) shows a more negative average than the average from only this study, this might be due to the sample number (n=9 and n=3 respectively). Comparing the Perdigões human remains (of this study and Emslie's combined) values with those from

the other sites, it looks like Perdigões (*n*=9) has similar values to those of Cova da Moura (*n*=10) in Estremadura region. Though, Cova da Moura shows a wider range in both $\delta^{13}C$ and $\delta^{15}N$ values (the number of individuals analysed from both sites is similar but the variability of the range is bigger in Cova da Moura site). If comparing the samples analysed from only this study (*n*=3), it has similar values to those of Lapa da Rainha (*n*=3), with only nitrogen values being slightly higher than from Lapa da Rainha II individuals. All of these small differences in variations can be attributed, as previously mentioned, to region differences in terms of soils, rain, and likely nutrients, leading to differences in isotopic composition of plants and animals consumed.

Usually in isotopic markers of the diet, a standard error of ±0.3‰ or less points towards a population with a very homogenous diet (Lillios, et al., 2014). Sites of Borracheira, Avenc del Dos Forats have those standard errors in both nitrogen and carbon values, while Cova de La Pastora, Lapa da Rainha II, Feteira II and Cabeço da Arruda I have them in carbon isotope values.

It is difficult to say something more about the samples only from this study because the sample number is quite small due to the poor preservation. Overall, it can be said that populations from these sites practised a similar type of agriculture and animal husbandry and the environmental variations between regions and sites have effect on the diet of the fauna and, accordingly, lead to slight dietary variations between those human communities.

5.3 MOBILITY STUDY

For this study, dental remains from 3 humans (Chalcolithic period) and 14 animals (8 from Late Neolithic period and 6 from Chalcolithic period) were selected from Perdigões site. Most of the samples were taken from third molar. In addition bone samples from two human mandibles (HPerd-5 and HPerd-10) from which dental enamel was also extracted, were added to check the difference between strontium ratios in enamel and bones and if they correspond to local or non-local signatures. The results from all the sampled fauna and humans are presented in TABLE 3 and

FIGURE **27**.

The total isotopic range of strontium data obtained from all samples involved in this study is extensive (from 0.711062 to 0.716339). The average $^{87}Sr/^{86}Sr$ value in animal enamel from Neolithic period is 0.714171± 0.001634, from Chalcolithic period is 0.714842±0.001939, while human is 0.713427± 0.000486.

Table 3 *$^{87}Sr/^{86}Sr$ ratios for fauna and humans (HPerd-14 sample was used as a duplicate)*

N°	Structure	U.E.	Assigned N°	Period	Species	Skeletal part	$^{87}Sr/^{86}Sr$	Error (2σ)

10	Hypogeum	235	FPerd-1 (N)	Neolithic	Sus sp.	teeth	0.712111	0.000021
4	Hypogeum	238	FPerd-2 (N)	Neolithic	Ovis/Capra	teeth	0.714810	0.000024
12	Fossa 49	310	FPerd-3 (N)	Neolithic	Cervus elaphus	teeth	0.715000	0.000020
8	Hypogeum	238	FPerd-4 (N)	Neolithic	Bos taurus	teeth	0.716078	0.000024
	Hypogeum	235	FPerd-5 (N)	Neolithic	Sus sp.	teeth	0.713091	0.000021
7	Fossa 48	309	FPerd-6 (N)	Neolithic	Bos taurus	Teeth	0.711974	0.000019
5	Hypogeum	182	FPerd-7 (N)	Neolithic	Ovis/Capra	teeth	0.714203	0.000024
13	Hypogeum	220	FPerd-8 (N)	Neolithic	Cervus elaphus	teeth	0.716098	0.000017
2	Fosso 7	91	FPerd-1	Chalcolithic	Canis familiaris	teeth	0.711062	0.000020
5	Fosso 7	85	FPerd-4	Chalcolithic	Sus sp.	teeth	0.714832	0.000023
7	Fosso 7	93	FPerd-6	Chalcolithic	Ovis/Capra	teeth	0.715374	0.000023
12	-	333	FPerd-11	Chalcolithic	Cervus elaphus	teeth	0.716192	0.000020
13	-	340	FPerd-12	Chalcolithic	Bos taurus	teeth	0.715251	0.000020
1	Fosso 45	279	FPerd-0	Chalcolithic	Canis familiaris	Teeth	0.716339	0.000017
253	Sepulcro 1	172	HPerd-5 (t)	Chalcolithic	Human	teeth	0.713334	0.000024
253	Sepulcro 1	172	HPerd-5 (b)	Chalcolithic	Human	bone	0.713820	0.000024
D6#13	Sepulcro 1	96	HPerd-10 (t)	Chalcolithic	Human	teeth	0.713818	0.000026
D6#13	Sepulcro 1	96	HPerd-10 (b)	Chalcolithic	Human	bone	0.713879	0.000024
D3#2a	Sepulcro 1	91	HPerd-14 (1)	Chalcolithic	Human	teeth	0.712817	0.000020
D3#2a	Sepulcro 1	91	HPerd-14 (2)	Chalcolithic	Human	teeth	0.712891	0.000020

Tooth enamel from animals was used to define a local range, the difficult part of this task is to choose which animal lived locally. According to Bentley et al. (2004), cattle, sheep and goats are more likely to be ranged away from the site, while deer may have lived locally but also may have been hunted and bought down from some other place. He hypothesized that pigs or dogs were the most likely species to live locally. However, probably the best way to the problem – is assuming that the specie, which presents the smallest range in $^{87}Sr/^{86}Sr$ range is the one that can be used as the local range (though there might be a slight chance that all of the sampled fauna came from a non-local area; thus, in future, it would be better to sample larger numbers of current fauna and plants to ascertain the baseline correctness). Accordingly, strontium isotopic values of dogs cannot be used for baseline, because they exhibit the most extensive range (from 0.711062 to 0.716339). So, at least one or even both dogs were likely not local. The average strontium ratio value from cattle teeth is 0.714434±0.002171, from caprine teeth enamel is 0.714796±0.000586, from pig teeth 0.713345±0.001378 and from deer teeth is 0.715764±0.000663. The standard deviation in the sheep/goat enamel values is less than half that of cattle or pig, with red deer values being not so much different from sheep/goat. Thus, sheep/goat, and maybe red deer, values can help in establishing the baseline range of 0.7142 - 0.7162. With this range all Chalcolithic (except both dogs) and Neolithic (except FPerd-1 (N), FPerd-5 (N) and FPerd-6 (N)) animals should be considered local and none of the humans' analysed falls in the local range (see FIGURE 27).

We could infer from the data that HPerd-5, HPerd-10 and HPerd-14 show signs of mobility. For instance, HPerd-5 bone and tooth values of 87Sr/86Sr slightly differ (0.7138 and 0.7133 accordingly) showing the possibility that, when younger, the analysed individual lived

in a different place than when he was an adult. Also, values of HPerd-10 (0.7138) from bones and teeth are very similar to that of HPerd-5 bone, which can be explained by the fact that they could be coming from the same place or from places with a similar geological substrate (when they were adults they most likely lived in either the same place or in places with similar geology due to similar bone values). HPerd-14 shows different value from his two other counterparts ($^{87}Sr/^{86}Sr$ of 0.7128) which can be interpreted as him living somewhere else. The carbon and nitrogen isotope analysis were done only on HPerd-10 and the values obtained didn't showed any differences in dietary preferences from other humans. While the fauna which was cross-referenced for Sr, C and N analysis did not show any particular variations in dietary patterns. Though, it would be interesting to check the $^{87}Sr/^{86}Sr$ ratio of FPerd-13 (Bos taurus) which had a very different carbon isotope values.

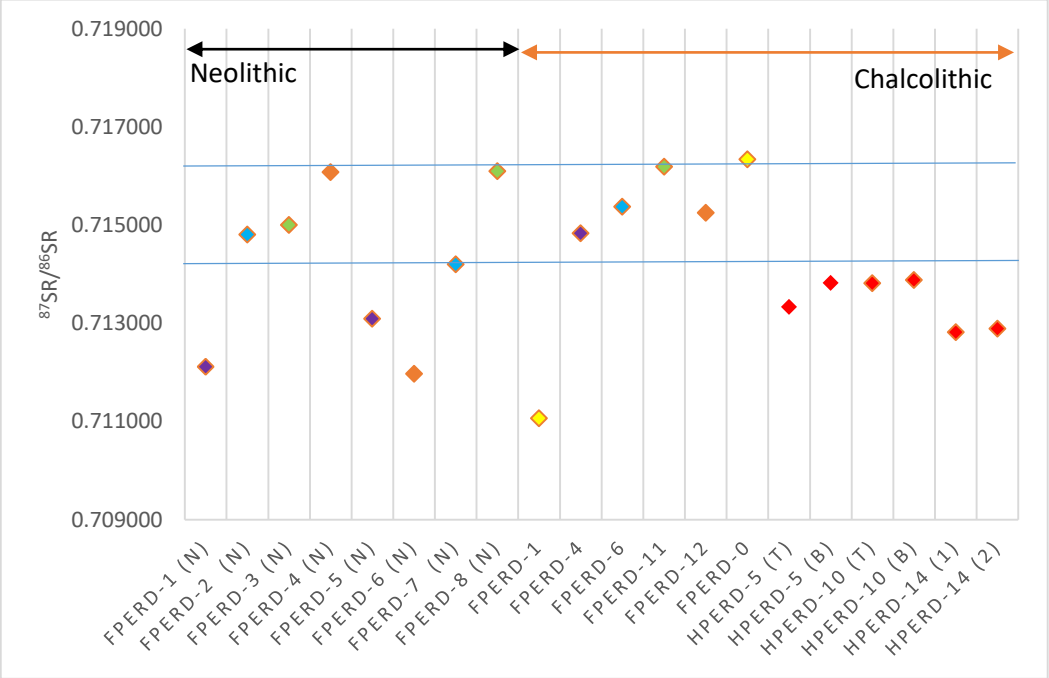

Figure 27 *Scatter plot of $^{87}Sr/^{86}Sr$ ratios for fauna and humans (different colours indicates different species: purple – Sus.sp; blue – Ovis/Capra; green – Cervus elaphus; orange – Bos Taurus, yellow – Canis familiaris; red – human (bone and teeth samples)). The blue lines shows the range of established baseline from fauna.*

There was a study done by Hillier et al. (2010) in which authors compared samples from Carcavelos, Estria (Estremadura region) and Perdigões sites. It showed that Perdigões had more radiogenic values than other sites and that it is quite expected because of site's geology (Paleozoic schist and granite rocks, characterizing the region and giving higher $^{87}Sr/^{86}Sr$ ratios), making the established baseline of bioavailable strontium from fauna of Perdigões site (*Bos taurus, Ovis/Capra, Sus sp., Cervus elaphus)* with the range of 0.7148 - 0.7182 (Hillier, et al., 2010). As the established baseline of local range and interpretation of Perdigões can differ - this time the results were plotted against the one's from Hillier's study (because it only includes fauna from the Chalcolithic period and should correlate to strontium levels of Chalcolithic

humans) as can be seen in FIGURE 28 and FIGURE 29. Only half of the fauna (results obtained from this study) from the Neolithic period (FPerd-2, FPerd-3, FPerd-4 and FPerd-8) fall between the proposed local ranges of Hillier's study. The fauna from Chalcolithic period, except FPerd-1 (*Canis familiaris*), correspond to the local baseline determined by Hillier et al. Comparing Hillier's (0.7148 – 0.7182) with the range established in this study (0.7142 – 0.7162), we can say that the range established now is narrower. Taking in consideration both local ranges - one dog (FPerd-1) with quite low strontium value falls below both baselines, which leads to conclusion that this dog is definitely non-local. While all the humans (except three adults from Hillier's study) from Perdigões are all below the so-called local range of both baselines.

As there was no available anthropological information on their sex, age and etc. further interpretation is not possible. However, the $^{87}Sr/^{86}Sr$ values measured in this study are not close to those from Estremadura region. In the future it would be important to compare Perdigões strontium data with strontium ranges of Algarve and North Portugal and even within Alentejo region.

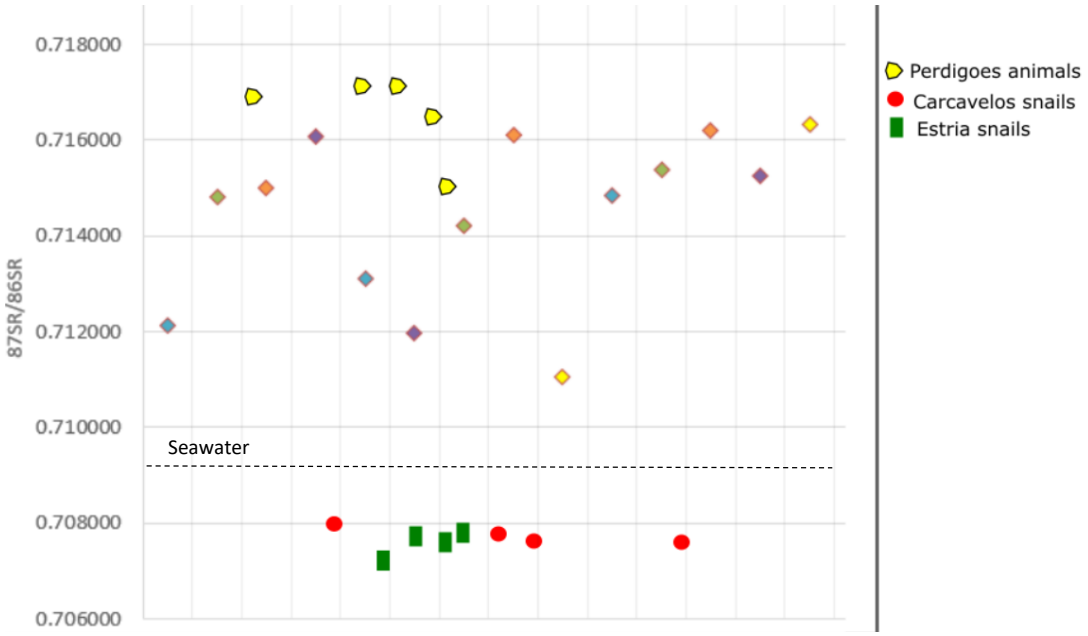

Figure 28 *Plots of values obtained from fauna from this study comparing with data from Hillier et al. (2010) poster (Carcavelos, Estria and Perdigões sites). Diamond shapes are Sr results from this study, different colour defining different species.*

A study done on Late Antiquity population of Monte da Cegonha (southern Portugal, Alentejo region) analysed modern vegetation samples and obtained strontium values ranged from 0.7083 to 0.7125 (Saragoça, et al., 2016). As can be seen the values were quite diverse within the same environment. And if we compare the modern plant values obtained from Saragoça et al. study to the Perdigões values of this study (as they belong to the same geographical region – Alentejo) it can be seen that all of the human samples do not fall in the established range, while three fauna samples (FPerd-1 (N), *Sus sp.*; FPerd-6 (N), *Bos taurus* and FPerd-1, *Canis familiaris*) might be from the same or very similar geological substrate of

the Monte da Cegonha established baseline. These results demonstrate how variable the strontium data can be even in a relatively close places within the Alentejo region. This means that the non-local individuals from Perdigões can come from a different region, which does not need to be as far away as the Estremadura.

Of course all the data obtained is difficult to interpret as long as there is no thorough local signal established by analysing some modern plants from Perdigões site itself (this might be difficult because modern plants may be impacted by modern agriculture due to the winery near the field) and nearby surroundings.

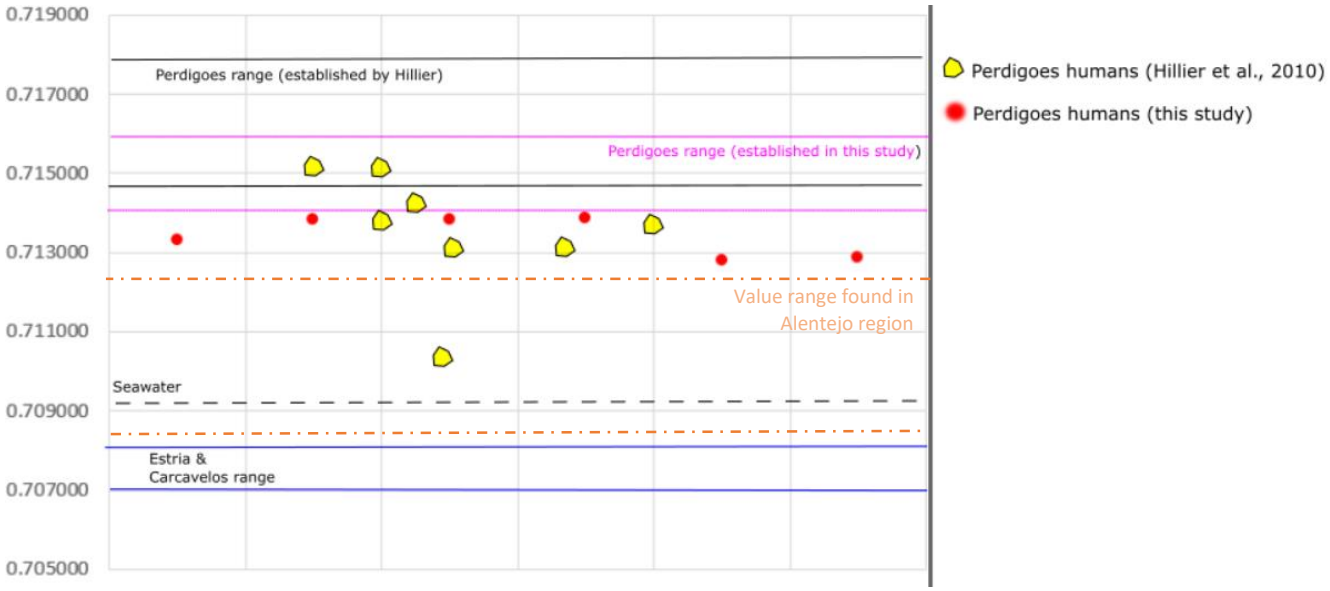

Figure 29 *Plots of values obtained from human from this study comparing with data from Hillier et al. (2010) poster. Peach lines show the values obtained of modern plants in Alentejo region from Saragoça et al. (2016) study.*

As only couple of human remains and animals were analysed for strontium isotopic analyses from such extensive site as Perdigões not a lot can be said, though one firm conclusion can be drawn and it is that the lower $^{87}Sr/^{86}Sr$ isotope ratios exhibited by the migrants identified suggest that people were migrating (if the local baseline established is the right one). Though, whether the individuals moved to live close by the site and died there or their remains were taken to the final resting place is still uncertain.

6 CONCLUSIONS

The first aim of this thesis was to pre-screen the samples and choose the best preserved for bone collagen analysis. The pre-screening was done by ATR-FTIR (calculating splitting factor, relative carbonate and relative collagen contents of each sample) and EA (measuring the percent nitrogen content of whole bone). The obtained results were compared and it showed that they can be divided into two big groups. First group comprised by all faunal samples and one human and exhibited low levels of diagenesis. The second group contained only humans and showed a high level of diagenetic alteration. Two of animal samples (FPerd-2 and FPerd-5) had borderline values thus, it was decided not to analyse them to optimize the results and be sure that the values obtained on subsistence pattern are done on good quality collagen.

It would be advisable to do more research with ATR-FTIR, with larger amounts of whole bone samples to establish a baseline data for non-diagenetically altered bones to validate the range of IRSF, C/P but especially for Am/P (it does not have range for diagenetic bone values) because the established values of ranges were established on FTIR and not ATR-FTIR and they have small variations in the results (Beasley, et al., 2014).

The second aim was to investigate paleodiet during the Chalcolithic period in the Perdigões site (tomb I). It was shown that the typical diet pattern was terrestrial-based on C_3 plants and domestic animals just like as in most of the sites compared. Each site exhibited small variations in $\delta^{13}C$ and $\delta^{15}N$ values. As collagen alone does not detect significant dietary variations (like ability to quantify proportions of animal protein in the diet and whole diet) because collagen reflects only protein component of the diet, it would be good to couple it with apatite measurements. Despite its higher risk of contamination, apatite allows a better assessment of whole diet and especially component (lipids) not presented in collagen. The apatite analysis can be done on samples which yielded high collagen yield (%) and C/N ratios.

The third aim was to establish strontium baseline of fauna and to reconstruct couple of individual's patterns of mobility. Strontium isotope ratios ($^{87}Sr/^{86}Sr$) in dental enamel were used to establish the Perdigões local range and distinguish migrant individuals. The sampling showed that while most of the fauna was of local range, all of the three individuals fall below the established local range and were most probably migrants. Further sampling of fauna (especially small one, like rabbits) firstly, from Perdigões site and then going wider (within Alentejo region and whole Portugal) is a requirement, in order to obtain a $^{87}Sr/^{86}Sr$ map of the region and Portugal as a whole. This would allow tracing the prehistoric human mobility in the site and whole Portugal.

Finally, it should be stressed that only a small number from burials of tomb I were analysed in this study and the site itself is very big with lots of tombs and individuals buried, meaning that even the inter site comparison could yield different results of diagenetic levels, subsistence patterns and mobility. Thus, it is quite difficult to draw firm conclusions from these results about whole site in general. They do, however suggest that Chalcolithic individuals from Perdigões site tomb I maintained terrestrial dietary focus with animal husbandry and farming on C_3 pathway plants while some individuals were definitely not from Perdigões site and spent their life's in some other settlement. Further research on this extensive and important site is necessary and it will be continued in the context of the Global Research Program of Perdigões.

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APPENDIX A: PERDIGÕES SITE LOCATION

Figure 30 *Location of Perdigões site location, using Google Earth program*

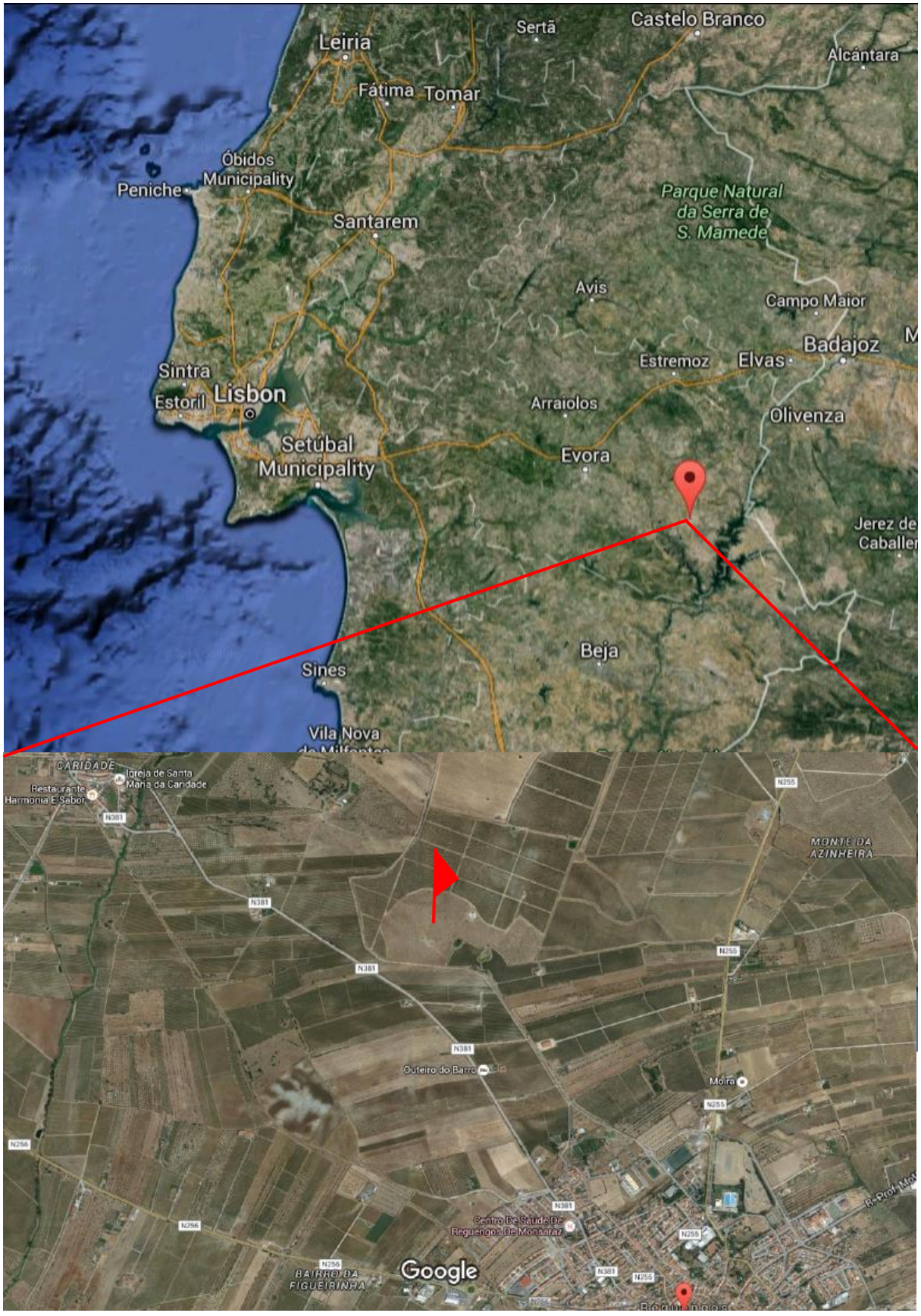
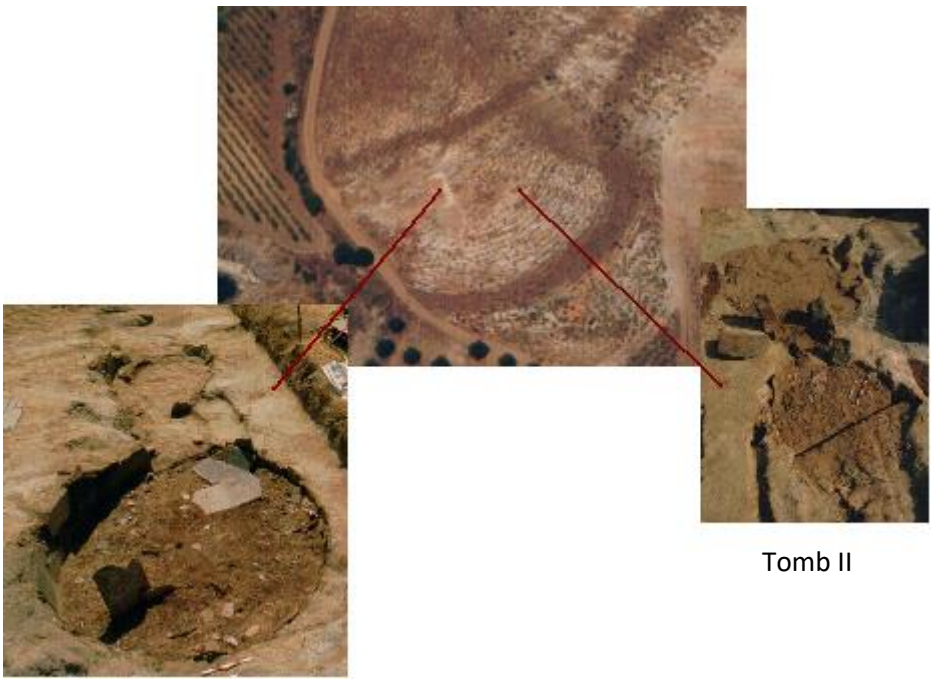


Figure 31 *Perdigões archaeological comples and tomb I and tomb II location (images taken from http://www.nia-era.org/perdigoes-project/281-os-perdiges-apresentao-presentation.)*



Tomb II

Tomb I

APPENDIX B: LIST OF SAMPLES

Table 4 *List of faunal samples from Perdigões Chalcolithic layers*

Archaeological Nº	Sector	Structure	U.E.	Assigned Nº in the lab	Species	Element
2	P	Fosso 7	91	FPerd-1	Canis familiaris	Mandible
3	P	Fosso 7	72	FPerd-2	Canis familiaris	Mandible
4	P	Fosso 7	91	FPerd-3	Sus sp.	Mandible
5	P	Fosso 7	85	FPerd-4	Sus sp.	Maxilla
6	P	Fosso 7	93	FPerd-5	Sus sp.	Maxilla
7	P	Fosso 7	93	FPerd-6	Ovis/Capra	Mandible
8	P	Fosso 7	94	FPerd-7	Ovis/Capra	Maxilla
9	P	Fosso 7	96	FPerd-8	Ovis/Capra	Maxilla
10	P	Fosso 7	77	FPerd-9	Cervus elaphus	Mandible
11	P	Fosso 7	74	FPerd-10	Cervus elaphus	Metapodial bone
12	Q		333	FPerd-11	Cervus elaphus	Mandible
13	Q		340	FPerd-12	Bos Taurus	Mandible
14	P	Fosso 7	71	FPerd-13	Bos Taurus	Maxilla
16	P	Fosso 7	91	FPerd-15	O. cuniculus	Mandible
17	P	Fossa 47	60	FPerd-16	O. cuniculus	Iliac
18	P	Fosso 7	86	FPerd-17	O. cuniculus	Radius

Table 5 *List of human samples from Perdigões tomb I (Chalcolithic period)*

Archaeological Nº	U.E.	Phase	Assigned Nº in the lab	Element
1422	304	2a	HPerd-1	Mandible
1468	310	2a	HPerd-2	Mandible
1331	304	2a	HPerd-3	Mandible
364	175	2b	HPerd-4	Mandible
253	172	2c	HPerd-5	Mandible
181	172	2c	HPerd-6	Mandible
133	172	2c	HPerd-7	Mandible
3339	174	2c	HPerd-8	Mandible
D4#85	173	2c	HPerd-9	Mandible
D6#13	96	2d	HPerd-10	Mandible
D4#38	97	2d	HPerd-11	Mandible
A6#40	97	2d	HPerd-12	Mandible
F6#13	90	3b	HPerd-13	Mandible
D3#2a	91	3b	HPerd-14	Mandible
D3#2b	91	3b	HPerd-15	Mandible
1380	63	3c	HPerd-16	Mandible
B2#16	63	-	HPerd-17	Humerus
ND	63	-	HPerd-18	Humerus
Plano L	63	-	HPerd-20	Humerus
E7#79	97	-	HPerd-21	Humerus
C6#119	97	-	HPerd-23	Humerus
C2#43	97	-	HPerd-24	Humerus
B4#18	97	-	HPerd-26	Humerus
E5#12	97	-	HPerd-27	Humerus
E7#92	169	-	HPerd-28	Humerus
#243	ND	-	HPerd-29	Humerus
#700	-	-	HPerd-30	Humerus
C2#60	178	-	HPerd-32	Humerus
#1787	ND	-	HPerd-33	Humerus
#1829	ND	-	HPerd-34	Humerus

Table 6 *List of human samples from Perdigões tomb I and tomb II (Chalcolithic period) from Emslie et al. (2015) study*

Sample name	$\delta^{13}C$	$\delta^{15}N$	C:N
Perdigões Tomb I, 458, 169 E7, 105	-25.3	9.9	3.2
Perdigões Tomb I, 1759, 997, 802	-24.7	9.2	3
Perdigões Tomb II Chamber S5 (E)	-19.6	9.4	3.16
Perdigões Tomb II Chamber S10 (J)	-20.1	9.5	3.39
Perdigões Tomb II Chamber S11 (K)	-19.6	10.2	3.2
Perdigões Tomb II Atrium S13 (M)	-19.9	9.8	3.11
Perdigões Tomb II bag 63, 231 #227	-20.4	9.5	3.5
Perdigões Tomb II Atrium #108, 3955, 261	-20.9	9.2	3.6
Average	*-21.3*	*9.6*	
std.dev.	*2.3*	*0.4*	

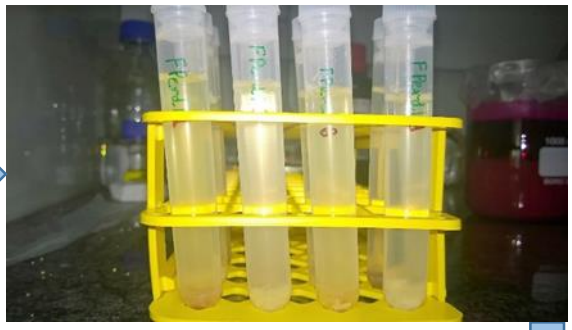
APPENDIX C: ISOTOPE ABUNDANCE

Isotope	Abundance (%)*
^{12}C	98.93
^{13}C	1.07
^{14}N	99.632
^{15}N	0.368
^{84}Sr	0.56
^{86}Sr	9.86
^{87}Sr	7.00
^{88}Sr	82.58

* The natural abundance (%) data is obtained from the 1997 report of the IUPAC Subcommittee for Isotopic Abundance Measurements by K.J.R. Rosman, P.D.P. Taylor. In Pure and Applied Chemistry, 1999, Vol. 71, p. 1593-1607.

APPENDIX D: SIMPLIFIED PROCESS OF COLLAGEN EXTRACTION

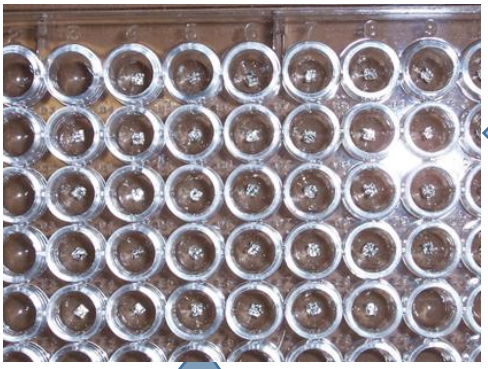
1st step: to clean ~500 mg of compact bone and break it into small pieces



2nd step: demineralization and removal of contaminants. Followed by gelatinization

3rd step: filtration and freeze-drying

4th step: put 'pure' collagen into Sn capsules



Last step: analysis by IRMS

APPENDIX E: SUPPLEMENTARY TABLES AND GRAPHS

__Table 7__ ATR-FTIR analysis results of faunal samples (numbers in bold showing max. and min. values)

Assigned Nº	IRSF	Mean crystal length (nm	C/P	Am/P
FPerd-1	**3.7**	**58.9**	0.16	0.035
FPerd-2	3.4	53.7	**0.52**	**0.028**
FPerd-3	3.5	55.8	0.20	0.063
FPerd-4	3.3	51.6	0.21	0.044
FPerd-5	3.5	54.8	0.19	0.035
FPerd-7	3.7	60.0	**0.14**	0.036
FPerd-8	**3.7**	**60.0**	**0.14**	0.044
FPerd-9	3.4	52.7	0.20	0.062
FPerd-10	**3.2**	**49.5**	0.25	**0.109**
FPerd-11	3.3	51.6	0.22	0.071
FPerd-12	3.5	55.8	0.21	0.063
FPerd-15	3.3	51.6	0.37	0.063
FPerd-16	3.3	51.6	0.23	0.068
Mean value	3.4	54.4	0.23	0.055

__Table 8__ ATR-FTIR analysis results of human samples (numbers in bold shows highest and lowest values)

Assigned Nº	IRSF	Mean crystal length (nm)	C/P	Am/P
HPerd-2	4.2	70.4	0.21	0.027
HPerd-3	4.1	68.3	0.16	0.018
HPerd-4	3.9	64.1	0.14	0.018
HPerd-5	4.1	68.3	0.12	0.027
HPerd-6	4.3	72.5	0.12	0.018
HPerd-7	4.2	70.4	0.12	0.027
HPerd-8	4.1	68.3	0.26	0.027
HPerd-10	**3.6**	**57.9**	0.17	**0.055**
HPerd-11	4.0	66.2	0.13	0.030
HPerd-12	4.1	68.3	0.14	0.026
HPerd-13	4.2	70.4	0.12	0.018
HPerd-14	4.2	70.4	0.19	0.018
HPerd-15	4.3	72.5	0.12	0.022
HPerd-16	4.0	66.2	**0.32**	0.018
HPerd-17	4.0	66.2	0.15	0.018
HPerd-18	3.9	64.1	0.17	0.036
HPerd-20	4.0	66.2	0.14	0.026
HPerd-21	4.0	66.2	0.12	0.018
HPerd-23	**4.7**	**80.8**	**0.11**	0.017
HPerd-24	3.9	64.1	0.21	0.036
Hperd-26	4.4	74.5	**0.11**	**0.016**
HPerd-27	4.0	66.2	0.14	0.026
HPerd-28	4.3	72.5	**0.11**	0.026
HPerd-29	4.1	68.3	**0.11**	0.026
HPerd-30	4.4	74.5	**0.11**	0.026
HPerd-32	4.2	70.4	0.22	0.018
HPerd-33	3.9	64.1	0.15	0.035
HPerd-34	3.9	64.1	0.14	0.027
Mean	4.1	68.4	0.15	0.025

Figure 32 *Correlation between IRSF values and mean crystal length by species.*

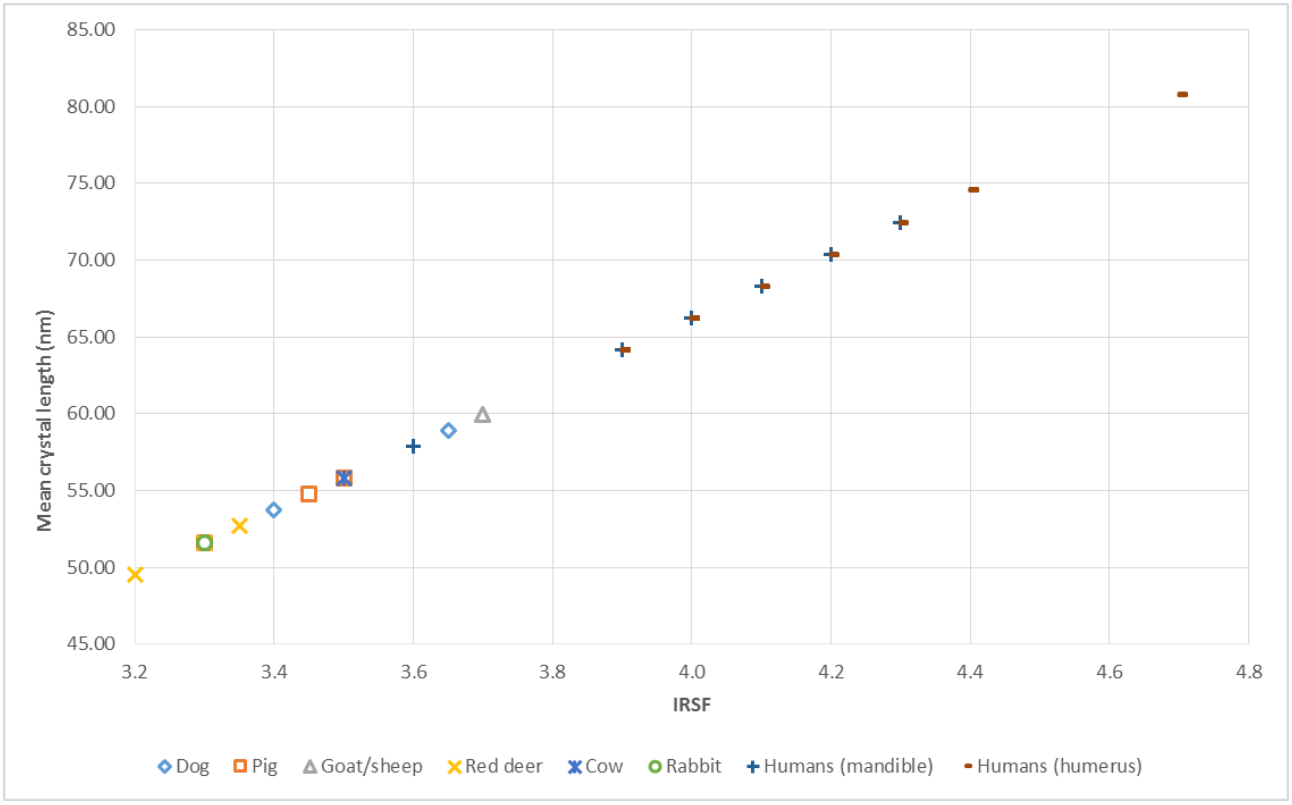


Figure 33 *Relationship between Am/P and IRSF ratios by species*

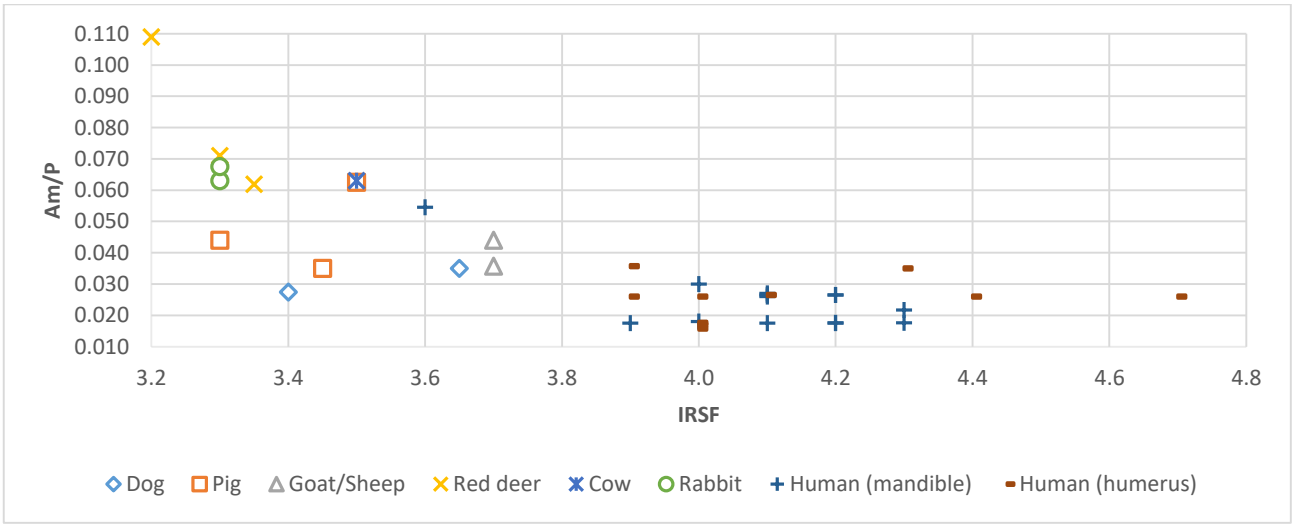


Figure 34 *Relationship between C/P and Am/P ratios.*

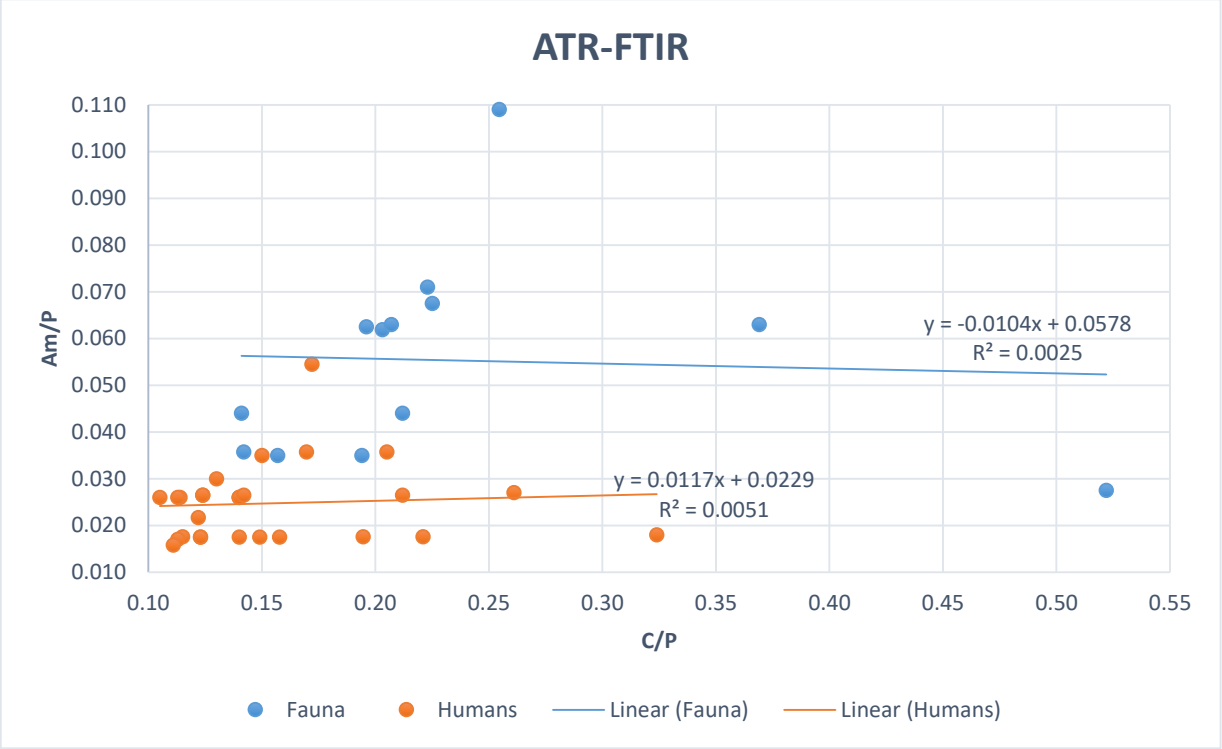


Table 9 *Mean stable isotope results of Late Neolithic and Chalcolithic period human (adults) samples from the Iberian Peninsula*

Site name	Country	Mean $\delta^{13}C$ (‰)	±	Mean $\delta^{15}N$ (‰)	±	N	Source of data
Montelirio, Valencina-Castilleja	Spain	-19.4	0.6	9.1	0.4	11	(Fontanals-Coll, et al., 2015)
Avenc dels Dos Forats	Spain	-19.1	0	10.2	0.3	2	(Fontanals-Coll, et al., 2015)
Cova La Pastora	Spain	-19.3	0.2	9.5	0.8	7	(McClure, et al., 2011)
Bolores	Portugal	-20.2	0.5	8.8	0.8	10	(Lillios, et al., 2014)
Tholos Borracheira	Portugal	-19.8	0.1	8.8	0.2	6	(Fontanals-Coll, et al., 2015)
Lapa Rainha II	Portugal	-19.6	0.2	8.9	0.2	3	(Waterman, et al., 2016)
Zambujal	Portugal	-20.4	0.4	9.3	0.8	2	(Waterman, et al., 2016)
Feteira II	Portugal	-20.3	0.3	8.3	1.2	13	(Waterman, et al., 2016)
Paimogo I	Portugal	-20.3	0.5	8.7	0.5	11	(Waterman, et al., 2016)
Cova da Moura	Portugal	-19.9	0.7	9.4	0.9	10	(Waterman, et al., 2016)
Cabeço da Arruda I	Portugal	-19.8	0.3	8.6	0.9	8	(Waterman, et al., 2016)
Perdigões (this study+Emslie, excluding two outliers)	Portugal	-19.9	0.5	9.4	0.5	9	(Emslie, et al., 2015)
Perdigões (this study)	Portugal	-19.6	0.1	9.1	0.4	3	-

APPENDIX F: MAP OF THE SITES USED FOR COMPARISON

Figure 35 *Map of the sites mentioned in this study (adapted from < https://www.dreamstime.com/royalty-free-stock-photography-spain-map-national-borders-most-important-cities-rivers-lakes-image31930827>).*

Map of sites from Estremadura (taken from (Waterman, et al., 2016)).