Medical diagnostic methods applied to a medieval female with vitamin D deficiency from the North of Spain.

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Abstract

Vitamin D deficiency is a pathological condition that affects bone metabolism by preventing proper mineralization, which eventually leads to bone deformities and other pathological conditions such as osteoporosis, increased bone fragility and fractures. The aim of this study is to present a case of vitamin D deficiency, but also to note how the application of several complementary techniques is a fundamental step in the establishing an accurate diagnosis. These techniques range from classical palaeopathological analysis to modern clinical practice. After the macroscopic examination of a medieval female skeleton from Palencia (Spain), where various bone deformations were observed, a differential diagnosis could not establish a definitive cause. Radiological, bone density, and histological studies were carried out, finally allowing to confirm a vitamin D deficiency suffered in both childhood and adulthood. This is a clear example, with practical applications, of the importance of interdisciplinarity to reveal insights about the life history and physical health of ancient individuals.

Key words: palaeopathology; metabolic bone disease; radiograph; bone mineral density; histological study.

1. Introduction

The identification of archaeological cases of vitamin D deficiency is not always simple. Interdisciplinary studies enable new approaches to solve diagnostic difficulties, and thus avoid overlooking interesting cases. In addition, combining several techniques allows to establish the moment of the life cycle in which the deficit occurred.

The aim of this paper is to present the advantages of applying procedures typically used in the modern clinic, along with traditional methods of palaeopathology, for diagnosing disease in past populations. This can help establishing not only the pathological condition present in ancient remains, but also the point of life in which this deficit occurred.

We describe the determination of a case of medieval vitamin D deficiency, explaining and evaluating the techniques used for its diagnosis. For this we performed a macroscopic assessment combined with a radiological analysis, bone density estimation and histological studies in bone and teeth. Through an interdisciplinary approach, we evaluate the diagnostic ability of these techniques when dealing with archaeological remains.

2. Background

2.1 Vitamin D: acquisition and metabolism

Humans typically obtain the necessary vitamin D from sunlight (Ginde et al. 2009; Holick 2008) but also from their diet and from dietary supplements (Berry et al. 2002; DeLuca 2004; Holick 2006; Joiner et al. 2000). There are two sources of vitamin D, cholecalciferol or vitamin D_3 and ergocalciferol or vitamin D_2. Vitamin D_3 is produced endogenously in the skin following exposure to ultraviolet radiation whilst vitamin D_2 is produced by some mushrooms and yeasts (Holick 2007). Throughout this article, to refer to vitamin D_3 or D_2 indistinctly, we use the term "vitamin D".

Vitamin D has no biological activity by itself, and its activation requires two hydroxylation steps. Skin cells contain pro-vitamin D_3 (7-dehydrocholesterol) which during exposure to sunlight is transformed by ultraviolet radiation into pre-vitamin D_3 (Wacker and Holick 2013). This pre-vitamin D_3 is rapidly converted to vitamin D_3 by a temperature-dependent process and is transferred to the extracellular space (Wacker and Holick 2013). Vitamin D_3 formed in the skin and orally-ingested vitamin D_3 and D_2 are transported to the liver, where the first hydroxylation occurs and vitamin D is converted to 25-hydroxyvitamin D (25(OH)D) (Zittermann 2003). The 25(OH)D is not stored in the liver but is released into the bloodstream where it has a half-life of 12-19 days (Zittermann 2003). In the kidney, it suffers a second hydroxylation by the action of the 25-hydroxyvitamin D-1-α-hydroxylase enzyme (1-α-

hydroxylase), and is converted into 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), which is the biologically active form of vitamin D (Berry et al. 2002; Holick 2006; Zittermann 2003).

2.2 Vitamin D deficiency

Vitamin D deficiency can be caused by various reasons, often interconnected by homeostatic processes. These can be grouped by the life moment when the change happens and the mechanisms that trigger it: intake deficiency, lack of sunlight, secondary hyperparathyroidism, genetic defects and other various causes (Figure 1). The immediate effect of a vitamin D deficiency is the inability to mineralize the osteoid correctly. Since a non-mineralized matrix cannot provide structural support, deficiency can cause growth retardation and bone deformities in infancy and childhood. In adults, can cause or exacerbate osteopenia, osteoporosis, musculoskeletal pain, weakness, risk of fractures, and osteomalacia (Holick 2007). Vitamin D is also involved in non-calcaemic roles, such as immune function, cardiovascular health, and suppression of tumour growth (Heaney 2008; Wacker and Holick 2013; Zittermann 2003).

2.3 Rickets and osteomalacia

Rickets occurs when the vitamin D deficiency happens during growth and the newly formed bone is not mineralized properly. Clinical manifestations depend on the age of onset and the severity of the deficiency (Berry et al. 2002); they are more marked if the deficiency coincides with a period of rapid growth. Bone deformities, such as bowing or twisting, are typical of rickets, and occur as a consequence of the weight endured by a weakened skeleton. In addition to the deformities of the bone shaft, dramatic changes in the metaphyses are common (Ortner and Mays 1998; Pinhasi et al. 2006). Given that the mineralization process of the teeth is comparable to that of the skeleton, the dentition is also affected by vitamin D deficiency (D'Ortenzio et al. 2016). This causes, as a primary manifestation, the presence of spaces known as interglobular dentin (Brickley et al. 2017; D'Ortenzio et al. 2016; Vital et al. 2012).

When impaired mineralization affects bone remodelling, it leads to osteomalacia, or softening of bone, which is typically associated with generalized pain and muscle weakness (Holick 2003). If deficiency begins in adulthood, metaphyseal changes are not observed, since growth has already ceased. In advanced cases, weakening of the bone makes it elastic and susceptible of being deformed due to normal weight-bearing, movement and muscle function (Brickley et al. 2005; Francis and Selby 1997). Also, pseudofractures can be seen macroscopically. These are minimal fractures where non-mineralized or poorly made osteoid is accumulated and are considered the most characteristic expressions of bone fragility in osteomalacia (Brickley et al. 2007; Pinhasi and Mays 2008).

Radiographic changes can be seen in osteomalacia. For example, demineralization may appear as osteoporosis (Waldron 2009) or pseudofractures. According to this author, they can be seen as radiolucent zones that correspond to accumulations of osteoid.

Histologically, both rickets and osteomalacia result from a failure of the osteoid matrix to calcify normally; thus the primary feature in both children and adults is the excess of uncalcified matrix. The Haversian system is also poorly developed, with few osteons. Mineralization deficits of the bone can be observed as increased trabecular thinning and increased resorption. The cortical bone usually presents incomplete mineralization of layers of bone and enlarged osteocyte lacunae, which can be adjacent to cement lines In general, large spaces are seen where the unmineralized osteoid would be accumulated in a fresh bone biopsy (Brickley et al. 2007; Pinhasi and Mays 2008).

In adults, “healed rickets” can also be found, which are remnant signs of a past episode of the illness during childhood. Mineralization works correctly, so there is no excess of osteoid and pseudofractures tend to heal, but typically maintain deformities and can be seen as thinning and thickening of the cortices (Brickley et al. 2007; Brickley et al. 2010; Waldron 2009). Unlike the bones, the dental tissue is not remodelling, so the presence of interglobular dentin makes it possible to establish past episodes of rickets in adults (D’Ortenzio et al. 2016).

Previous studies have described vitamin D deficiency in ancient populations (Brickley et al. 2005; Brickley and Buckberry 2015; González et al. 1999; Haduch et al. 2009; Malgosa et al. 1996; Ortner and Mays 1998; Schamall et al. 2003, among others). Although its incidence increased with industrialization in urban areas, it was likely prevalent a long time before (González et al. 1999; Littleton 1998). In Spain, the studies from González et al. (1999) and Malgosa et al. (1996) are examples of vitamin D deficiency in children from the Bronze Age to the modern age. Presumably, Spanish adults would also have had vitamin D deficiency, though no confirmed case can be found in the literature. The study from Haduch et al. (2009) is particularly interesting as it shows a case of osteomalacia or residual rickets, and the moment in life in which the deficiency occurred could not be assessed. The methods shown in the present article can help establishing not only the pathological condition present in ancient remains, but also the point of life in which it occurred.

3. Material and methods

3.1 Archaeological Context

The skeletal remains of this study were recovered in an archaeological recovery carried out between November 2007 and March 2008 in the monastery of San Andrés de Arroyo, located in the northern area of the province of Palencia (Spain) (Figure 2a and 2b). The remains were recovered from a burial in the northeast of the exploration area (Lobby of the faithful), 80 cm from the northern wall and below the foundation of east wall, under which the feet of the individual were located (Figure 2c). Inside there was a nearly complete adult skeleton in a supine position with arms crossed over the chest, and articulated.

3.2 *Biological Profile*

Biological sex estimation was based on methods that relied on classical pelvic and cranial morphological characteristics of sexual dimorphism (Buikstra and Ubelaker 1994; Klepinger 2006). Age-at-death was estimated from the main macroscopic changes of the pelvis,

following the criteria for the metamorphosis of the pubic symphysis (Buikstra and Ubelaker 1994). Height estimation was performed using the regression equations proposed by Nunes de Mendoça (1998), which were developed using individuals from the Iberian Peninsula as reference. Equations for long bones were computerized in MATLAB (Moler 2009) and WinBUGS (Spiegelhalter et al. 2007) making use of functions adapted from Konigsberg et al. (2006). Results were generated using the MATBUGS framework (Murphy and Mahdaviani 2005).

An analysis of pathologies and entheseal changes throughout the skeleton was undertaken, following Buikstra and Ubelaker (1994), Genant et al. (1993), Capasso et al. (1998), and Estévez Gonzalez (2002). Observable characteristics of a metabolic disease made the skeleton a putative case of vitamin D deficiency, so an exhaustive study was carried out using different techniques that allow discarding or confirming this diagnosis.

3.3 Macroscopic analysis of pathology

For the analysis of the macroscopic features (and also radiological and histological features), after an extensive bibliographical review, we decided to follow the manifestations associated with metabolic bone diseases reviewed by Brickley and Ives (2008b), due the fact that this study provides a differential diagnosis for each feature associated with metabolic disorders in all bones of the skeleton. The adequacy of these has also been shown in more recent publications (Brickley and Buckberry 2015; D'Ortenzio et al. 2016; Ives and Brickley 2014).

3.4 Radiologic analysis of pathology

A radiographic study of the anatomical regions that usually show traits indicative of metabolic diseases was carried out in the radiodiagnosis service of the Central University Hospital of Asturias (HUCA by its Spanish acronym). A complete radiological series of the lower limbs, *ossa coxae* and *claviculae* were performed in anteroposterior position, and also in a medial position for the *femora*, using a 10 mA beam at 90 kV for 10 ms on a film for X-rays Kodak Industrex AX Ready Pack Film.

3.5 Bone mineral density (BMD) analysis

To obtain data on the individual's bone mineral density and to establish comparisons with healthy individuals, a dual-energy X-ray absorptiometry (DXA) was performed on this subject along with other individuals from the same necropolis, as part of a comparative study of the BMD in historical and modern populations conducted by one of the authors (García-Manrique 2012). The bones chosen, given the good results they provide and their frequent use in studies of this type, were the femur, more specifically the femoral neck region in all individuals, as well as the lumbar region of the spine (Gómez Alonso 1992). A commercial DXA scan Hologic QDR-1000, coupled to an NCR 80386SX-PC computer was used to produce the digitization and the image analysis. The study employed a two-beam pulsatile emission of 70 and 140 keV of energy.

3.6 Histological analysis

A histological biopsy of the upper iliac crest obtained images for differential diagnosis. Also, a histological study of the teeth assessed the presence of interglobular dentin. The teeth chosen for the study were the left upper first molar and the lower left canine. To establish the moment when interglobular dentin could have been formed, we follow the stages of development of the teeth described by AlQahtani et al. (2010).

Due to the fragility of the remains, a 1.5 cm. wedge shaped sample was obtained using a surgical saw and cutting perpendicular to the crest of the ilium. From this piece a longitudinal section was made where both cortices and the trabecular tissue between them could be observed. The sample was prepared following the protocol described by Baron et al. (1983). Sections were stained by Haematoxylin-Eosin and Goldner's Trichrome in order to differentiate healthy mineralized bone from pathological. The histological study was performed using a Carl Zeiss Axioplan 2 light microscope, with a Canon EOS 300D digital image capture system. As in the case of densitometry, a control was used to establish comparisons, taking samples from an apparently healthy individual from the same population.

Tooth samples were prepared with a method adapted from D'Ortenzio et al. (2016). The samples were embedded in epoxy resin EPO-TEK and mounted on glass microscopes slides. The samples were sectioned in a buccolingual direction with a Buehler PetroThin sectioning system and polished with a Logitech LP30 and 800 grit carburundum paste, followed by a diamond grinding disc and polishing cloth with diamond paste. Additionally, the samples were ultrasonicated in water to eliminate possible incrustations between each step. The histological study was performed with a 5M 300x USB Digital Microscope from Mustech Electronics Co., Ltd., with MicroCapture Pro image system, and a Carl Zeiss Axiolab polarized light microscope, with an AmScope digital image capture system. The scoring system for interglobular dentin used was described by D'Ortenzio et al. (2016).

4. Results

4.1 Macroscopic analysis

After grossly examining the skeletal features, we estimated that it was a female according to the shape and breathing of the greater sciatic notch and the expression of the subpubic concavity of the pelvis, and the smooth nuchal crest and supraorbital ridges of the skull, among other features. Changes on the pubis symphysis classify it between phases 6-7 of Todd's method (in Buikstra and Ubelaker 1994), which provided an age range within 30 and 39 years. The woman's height was approximately 136.12 ± 2.41 cm. This estimated stature is potentially diminished due to the deformities present in the leg bones. An estimation from the undeformed bones of the upper extremities suggested a stature of 142.11 ± 8.44 cm.

Eleven teeth had been lost antemortem; moderate dental wear appeared in the conserved pieces along with a moderate amount of dental calculus. There was also periodontal disease, as well as caries in the cement-enamel junction on the buccal surfaces in both lower first molars. There were no examples of linear enamel hypoplasia or dental abscesses. The most striking

feature of the dental study was the shape of the second right upper molar, which is much smaller than expected and displaced from its normal position to a more buccal position (ectopic tooth).

The macroscopic examination of the bones highlighted the location of the pathological features. Both femora presented marked anteroposterior bending in the upper third of the shaft, without showing signs of neck or diaphysis fracture. There were no external signs of pseudofractures. With a similar degree of curvature, the difference in size between the two *femora* is noteworthy, with the left being 10 mm longer than the right. The lateral curvature of tibiae and fibulae is even more striking, with the right extremity showing more extreme changes. In addition, both fibulae were flattened anteroposteriorly. Therefore, as with the femora, the left tibia is longer than the right. It was not possible to measure the length of the fibulae, since they were incomplete. This difference in size is due to the different degree of bending, which also caused their markedly reduced maximum length. Aside from the deformations present in the lower limbs, the left clavicle also showed an altered morphology, with a curvature greater than the normal range of variability perpendicular to the major axis of the bone, curved towards the top of the same (Figure 3).

After reconstructing the fragmented vertebrae and assembling the spine in its anatomical position, absence of kyphosis was observed. However, the apparent normality of the spine disappeared when the sacrum was added and positioned with the hip bones, which showed a slight scoliosis to the left within the lumbar region associated with a slight abnormal curvature of the body of the sacrum. This deformity is due to a loss of height of the third segment of the sacrum, which causes skewing to the left (Figure 4a), affecting the spine similarly. A wedge-shaped vertebra, T11, was also observed (Figure 4b). The result was a reduction of the anterior height of 22.7% with respect to the posterior height, which classifies it as grade 1 (mild deformity).

4.2 Radiologic analysis

Anteroposterior radiographs of the femora show normal trabeculae in the neck region and greater trochanter for a person of this estimated age. Absence of pseudofractures can be observed on the femoral neck region and the medial subtrochanteric region (Figure 5). In lateral view, the x-rays of the femora indicate thinning in the curvature zone, showing a reduced cortex in the anterior region of the bone, presumably as structural reinforcement due to bending (Figure 5). On the other hand, radiologically both tibiae and fibulae showed radiodense lines transverse to the diaphysis through cortical, identified as Harris lines (Figure 6). Finally, the radiograph of both claviculae allowed to rule out a fracture as a cause of the left clavicle deformation due to the absence of the typical traces (fracture calluses) left by the remodelling process that occurs during healing (Figure 3).

4.3 Bone mineral density analysis

The BMD values are shown in Table 1. The average BMD of the female population of San Andrés de Arroyo was 0.913 ± 0.125 g/cm^2 for the femoral neck region, whilst the BMD of our individual is 0.931 g/cm^2, which falls within the first standard deviation, being the mean age of the female population of 45.1 ± 11.1 years. From the BMD values, T-scores were calculated (Table 1), which are the basis of the diagnostic criteria for osteoporosis established by the WHO. The femoral neck T-value is the most used clinically, which was -1,064 for our individual, which classifies it as a case with mild osteopenia or low bone density.

Table 1. BMD and T-score values of the lumbar region of the spine (L2-L4) and the femoral neck obtained in DXA test [BMC: bone mineral content; BMD: bone mineral density].

Region	Area (cm^2)	BMC (g)	BMD (g/ cm$^{2)}$	T-score
Lumbar region				
L2-L4	33.59	31.31	0.932	-0.953
Femoral neck region				
Neck	3.11	2.25	0.724	-1.064
Trochanteric	9.74	6.22	0.639	-0.576
Intertrochanteric	15.31	17.75	1.159	0.836
Ward triangle	1.13	0.69	0.609	-1.168
TOTAL	28.16	26.22	0.931	0.124

4.4 Histological analysis

Due to the inconclusive results obtained so far, we carried out a histological study to show the state of the bone at the microstructure level and its state of metabolic activity. Bone structure alterations can be seen microscopically. With Haematoxylin-Eosin stain, poor mineralization could be observed in both cortical and trabecular bone (Figures 7a and 7b). The cortical bone showed enlarged empty spaces, which are areas in which the organic content has been lost by decay, common in archaeological samples. The trabecular bone was seen as small and fine fragments, the product of osteoclastic resorption and subsequent formation of osteoid that did not mineralize. In the same way, sections stained with Goldner's Trichrome showed mineralized areas surrounded by damage corresponding to degraded unmineralized osteoid (Figures 7c and 7d). The samples also show cracks and taphonomic alterations, which can be observed in the controls as well, but while the latter are distributed in a random scatter, mineralization defects follow the distribution of the bone tissue. Hence, we could confirm that the mineralization process was not occurring correctly at the time of death, which is indicative of an active vitamin D deficiency.

On the other hand, histological samples from teeth show an area with presence of interglobular dentin, both in the first molar and the canine, classified as grade 3 (Figure 8). This corresponds to a phase of vitamin D deficiency during childhood, approximately between the age of 3 and 5 years in accordance with the stages of development of the teeth.

Therefore, we propose that the individual under study suffered during her life at least two successive episodes of vitamin D deficiency.

5. Discussion

Palaeopathological studies are one of the fundamental tools used in human ecology studies to assess the quality of life of ancient populations, or to reinforce conclusions from palaeo-demographic studies. Precise diagnoses of diseases that occur in a population under

study lead to greater information is obtained about its way of life. Therefore, the combination of different diagnostic techniques is important in palaeopathological studies, since each method provides different information in a complementary way which leads to better diagnostic accuracy.

The skeletal remains presented here show a putative metabolic bone disorder that, at first, fits the diagnosis of a vitamin D deficiency, either osteomalacia or healed rickets. Not all the pathological features typical of vitamin D deficiency are shown. Therefore, it is necessary to describe each pathological sign and take into account all possible causes for it, trying to infer a diagnosis that encompasses all the signs.

5.1 Differential diagnosis

Discarding other pathologies that can deform long bones is a necessary step to be able to assume a metabolic disorder. Brickley and Ives (2008b) report different situations, in addition to vitamin D deficiency, which result in curved long bones. Which bones are deformed and in what direction bending occurs can be useful to distinguish the cause of the deformations (Brickley et al. 2010). Note that all changes in bone shape, assuming normal bone metabolism, are not necessarily associated with pathological histological results. In the same way, bending due to mobility patterns and musculoskeletal stress results in normal histological results, though paired with marked entheseal changes in the lower limbs.

If deformations were due to trauma, the marks of a healed fracture would appear on the radiographs. Also, both in trauma and in pre-natal bowing or birth defects, the correct mineralization would show a non-pathological histological study.

Osteogenesis imperfecta is caused by a mutation that induces type I collagen to form defectively. Onset can manifest even before birth. In cases where the affected individual reaches adulthood, significant deformities could be seen on long bones. Also, this disease is characterized by an increased brittleness of bones which leads to multiple fractures, as well as a

low BMD and osteoporosis (Ortner 2003; Waldron 2009). This disorder therefore does not fit our observations from these skeletal remains.

Blount's disease causes an alteration of the growth in the proximal epiphysis of the tibia. It is mainly related to childhood obesity and can result in knee osteoarthritis (Brickley and Ives 2008b; Natoli et al. 2016). Although this can lead to modifications of the condylar region of the femur, these changes do not correspond with the lesions observed.

Metaphyseal chondrodysplasia is a congenital disorder that also begins in childhood. Affected individuals present short stature with short legs, long bones bowing, and a limp. Also, due to the age of onset, the epiphyseal changes are severe, with evident cupping and metaphyseal widening (Brickley and Ives 2008b; Higuchi et al. 2016). These features have allowed us to dismiss it as a cause.

Infantile cortical hyperostosis or Caffey's disease is a pathological condition of unknown cause that begins to develop within a few months of life. It usually disappears before the age of two years and its effects tend to be corrected. If fusion of adjacent bones has occurred, deformities can be maintained throughout life (Guerin et al. 2012). In any case, mineralization patterns would be normal in the adult, so a histological study would be non-pathological.

Treponemal infection may be associated with deformities, especially in the tibia. However, it is known to affect the skull and other bones, with well-described destructive and reactive lesions, so it can be ruled out as a diagnosis in this case (Lopez et al. 2017; Ortner 2003; Pinhasi and Mays 2008; Waldron 2009).

Excess weight can cause deformities in the bones due to bone overload, especially at the level of the joints. By itself, obesity does not fit with what is observed in this case, since mineralization would also be normal and the histological study non-pathological. Although it does not seem to be the main cause of the defect described here, an excess of body fat is usually

related to other pathologies, including a deficiency of vitamin D, since body fat has a high affinity for it, capturing it and preventing the body from processing it (Vanlint 2013).

In addition to the deformations of the lower limbs, the left clavicle showed an exaggerated curvature. Since the radiological study rules out a fracture as a cause, we attribute this deformity to the same process that caused the bending of the long bones. At this point, accepting that the deformations and many of the pathological traits had been caused by a metabolic bone disorder involving the deficiency of vitamin D, the next objective was to look for distinctive signs that allowed to identify what moment of life the disease was active.

Rickets and osteomalacia share a number of common characteristics, since both are the response to a deficit of vitamin D where essential processes are affected, such as osteoid mineralization. In addition, bone tissue has a limited number of ways for reacting to a metabolic disorder. However, despite these common features, both disorders can be differentiated because these affected processes occur in two very different moments of the life of the individual. Therefore, we think that a detailed analysis of the pathological traits present in an individual with presumed osteomalacia or healed rickets, akin to modern medical practice, is crucial to differentiate the moment when the vitamin D deficiency occurred.

Several cases of rickets and osteomalacia have been described in ancient populations (Brickley et al. 2005; Brickley et al. 2007; Brickley and Buckberry 2015; Haduch et al. 2009; Ortner and Mays 1998; Schamall et al. 2003; van der Merwe et al. 2018, among others). In their review of indicators of vitamin D deficiency in adults, Brickley et al. (2005) point out that the appearance of long bones deformities in an adult skeleton may be a sign reminiscent of a past phase of rickets. In the same article they affirm that in light of this fact, the search of pseudofractures in the femoral neck and the subtrochanteric region becomes necessary to perform accurate differential diagnoses: Osteomalacia becomes likely if pseudofractures are found, while their absence indicates putative healed rickets.

The skeleton described here shows a curvature of the long bones of the lower extremities and absence of pseudofractures, consistent with infantile rickets. However, it should be noted that clinical cases in which vitamin D deficiency is present in adults do not always show pseudofractures (Azad et al. 2012; El-Sagheer et al. 2016; Rai and Rai 2016; Shin et al. 2015), and skeletal deformations have also been noted (Cañas and Iglesias 2005; Chalmers et al. 1967; Watanabe et al. 2015). This suggests that, although pseudofractures are considered to be pathognomonic of osteomalacia, they are not essential in all cases, and vitamin D deficiency may occur without them. In fact, clinical investigations show that radiological changes are not observed before two years after the onset of the deficit and should be intense and prolonged for such manifestations to be observed (Brickley et al. 2007). Pseudofractures are usually described in advanced cases, since sufficient time is needed for enough unmineralized osteoid to weaken the bone, given a certain amount of osteoid is always present. In addition, a prolonged pressure must be produced for the formation of stress micro-fractures. It is possible that day-to-day movements, as well as the simple fact of supporting the body weight, can cause the bones to deform without producing enough stress to fracture them.

On the other hand, in rickets the presence of nutritional stress indicators are frequent, such as enamel hypoplasias (Brickley and Ives 2008b). Other signs can also appear, such as the delay in the sequence of eruption of the dentition or porosity of skull bones or epiphysis of long bones, and even symptoms of vitamin C deficiency. Unlike the latter, since the dental enamel is not renewed, enamel hypoplasias remain throughout life, as long as the teeth are conserved or wear is not excessive, and are a sign of deficiencies during development. In the case presented by Haduch et al. (2009), the skeleton presented enamel hypoplasias, which made possible the inference of past episodes of nutritional insufficiencies. On the contrary, our skeleton had no hypoplasia in any of its preserved teeth. However, the right upper second molar exhibits a smaller size than the rest of the teeth as well as, presumably, its antimere, although this cannot be confirmed since that antimere was lost postmortem and not recovered in the excavation. This malformation appears to be restricted to the upper second molars, but the relationship with

vitamin D deficiency is uncertain. Since the histological study of the teeth showed interglobular dentin, and this has been linked to vitamin D deficiency in the paper by D'Ortenzio et al. (2016), it is likely that the individual suffered rickets in her childhood, between the age of 3 and 5 years. The individual also presented Harris lines that were seen in radiographs of *tibiae* and *fibulae*. In turn, both characteristics may be related to the short stature of the individual, significantly lower than the female average of this population (López et al. 2012).

Bone density loss is another feature that can be related to vitamin D deficiency (Wacker and Holick 2013). However, this is not the case given that the BMD values shown in our individual are normal. Nevertheless, histological characteristics of osteomalacia are observable in both cortical and trabecular bone tissue, and are particularly noteworthy after comparing their appearance and distribution with control individuals. This process of osteomalacia must have been active close to the moment of death, since the acquisition of vitamin D would have led to the mineralization of osteoid. Previous histological studies which have identified episodes of past deficiency during growth (D'Ortenzio et al. 2016), or active episodes (van der Merwe et al. 2018), have compared the use of optical with scanning electron microscopy (SEM). Both studies agree that any method can provide essentially the same diagnostic information, which validates our choice of optical microscopy as the cost and difficulty of this is much lower than SEM.

In summary, we can distinguish two phases of deficiency suffered by this individual. A first one during childhood, that could explain the short stature, and which is supported by the presence of interglobular dentin. The second phase would have occurred in adult life, beginning no more than two years before the death of the individual, since no pseudofractures were found.

As for the deformations of the bones, it is difficult to estimate the moment when they occurred. However, the femora present a structural reinforcement due to a thicker cortical on the dorsal region that required an active mineralization process. This suggests that the bones were deformed during the first episode of deficiency, although it is possible that the second episode aggravated the curvature.

Microscopic analysis can provide information that is not evident in a macroscopic evaluation. This is why histological studies are considered gold standards for the diagnosis of osteomalacia. There may be obvious cases in which they are not necessary, and the analysis does not have to be performed, e.g. if pseudofractures were present. However, in many others more ambiguous or doubtful, the deficient mineralization resulting from a disorder of vitamin D metabolism can only be appreciated in a histological analysis, thus confirming or refuting the presence of osteomalacia (Brickley et al. 2007; Ives and Brickley 2014).

Regarding the possible cause of the inferred vitamin D deficiency, one of the first things to consider is that it is the only individual that presents this condition in the recovered population. That rules out global events that would affect the entire population. Genetic disorders usually occur during childhood, although they might appear later in some cases (Bergwitz et al. 2006; López et al. 2008). Some of the hereditary disorders mentioned in Figure 1 could be the cause, such as X-linked hypophosphatemia (XLH), which is the most common hypophosphatemic syndrome accounting 80% of the cases of genetic hypophosphatemia (HYP Consortium 1995; López et al. 2008). However, a defining feature of genetic disorders is that, once they manifest, result in a constant deficiency without proper treatment. This allows us to discard them in this case, since we have shown the signatures of different episodes with recovery between them. The deficiency suffered here is likely to have been caused by a limited exposure to sunlight, maybe caused by seclusion, for which the diet did not compensate, which is the most common cause of deficiency for archaeological cases (Brickley and Ives 2008b; D'Ortenzio et al. 2016).

While is known that cases of osteomalacia and rickets became common in Europe after the Industrial Revolution, cases from earlier periods have been found (Littleton 1998; Ortner and Mays 1998). The case presented here belongs to a rural population of the Spanish Middle Ages, long before the Industrial Revolution. Agriculture-related tasks, which would have been very common activities in all individuals in this population, would have provided sufficient exposure to UVB radiation. Therefore, some specific cause had to confine this woman away

from her daily activities so that the deficiency developed as it did. The deformations of the long bones that had appeared during childhood would have been a likely impediment to proper movement. It is possible that at some point, a lifestyle change led the individual to indoor duties, which could have led to the deficiency.

6. Conclusion

Vitamin D deficiency is a condition that over time can lead to osteomalacia or rickets. This affects bone differently when abnormalities in the mineralization process impair bone remodelling or bone growth. When studying ancient populations, it is important to be able to accurately establish when these conditions occurred in order to correctly make inferences about quality of life.

The macroscopic study of the remains showed deformations that could be due to several causes since different diseases can lead to the same features. With a radiological and BMD analysis, several possibilities were dismissed. The histological study finally allowed to discard the remaining disorders allowing to establish a diagnosis of vitamin D deficiency, confirming episodes during both growth and adulthood. Therefore, the diagnosis can be illustrated with a glimpse into two different periods of the individual's life.

Histological studies are a complement to the palaeopathological analysis that should not be relegated only to suspicious cases. Despite the destructive nature of the test, its usefulness is undeniable. It should be used on a regular basis, since when a large number of pathologies can be overlooked if studies are limited to macroscopic assessments. We therefore consider that, as accurate diagnoses are needed to provide useful information of the evolutionary history of health and disease, palaeopathological studies should widely adopt interdisciplinary approaches based on the combination of anthropological and clinical methodologies.

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Figures

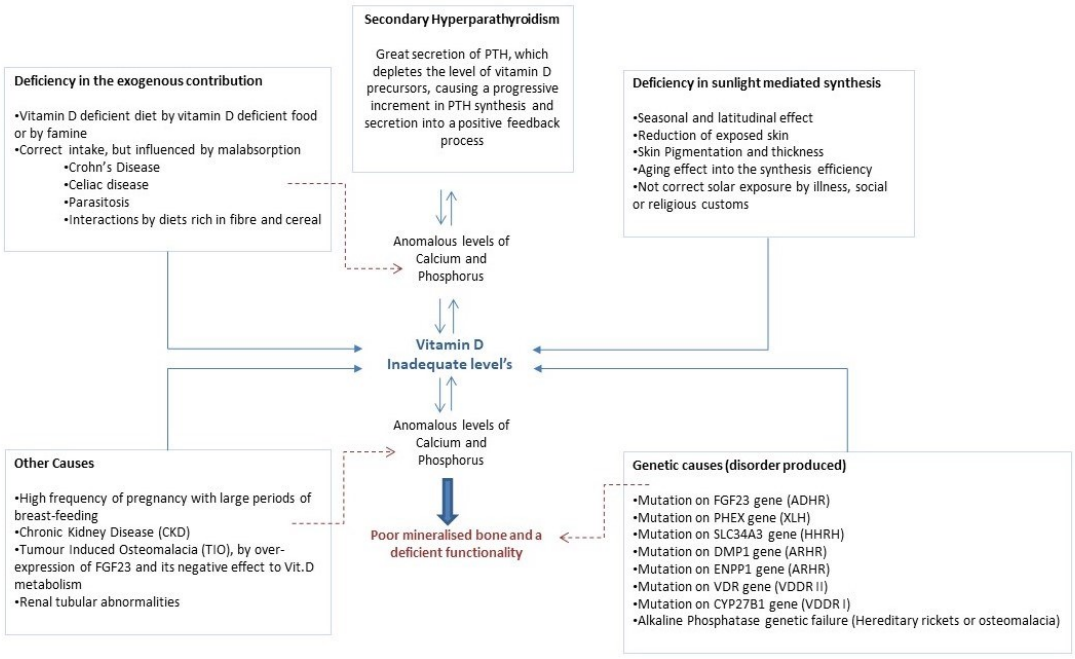

Figure 1. Causes of vitamin D deficiency (arrows with continuous lines), their inter-relationships due to homeostasis (bidirectional arrows) and their effects on calcium and phosphorus levels (arrows with dotted lines) [PTH: parathyroid hormone; FGF23: Fibroblast growth factor 23; ADHR: Autosomal dominant hypophosphatemic rickets; XLH: X-linked hypophosphatemia; HHRH: Hereditary hypophosphatemic rickets with Hypercalciuria; ARHR: Autosomal recessive hypophosphatemic rickets; VDDR: Vitamin D dependent rickets] (Brickley and Ives 2008b; Holick 2007; Lorenz-Depiereux et al. 2006; Lorenz-Depiereux et al. 2010).

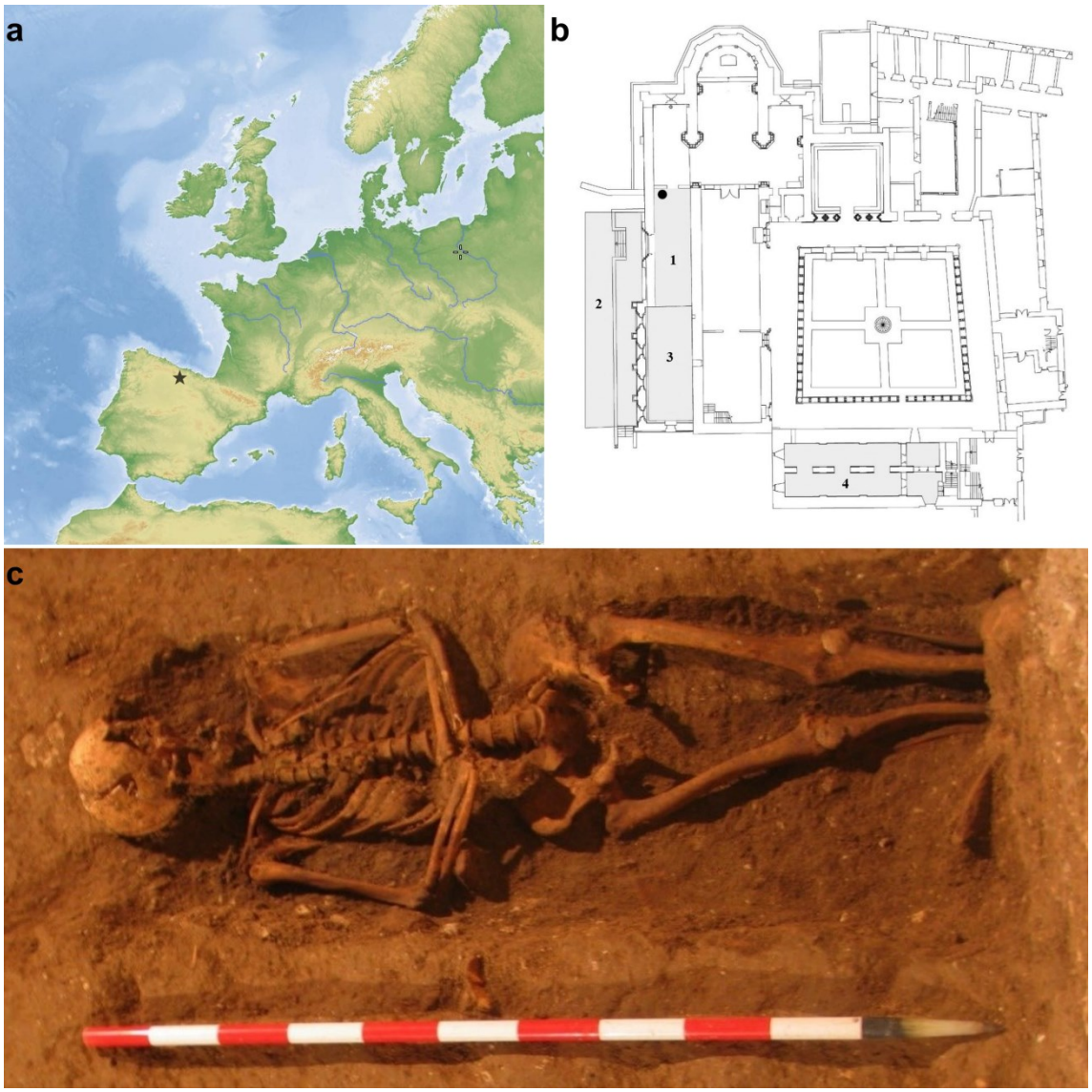

Figure 2. a) Approximate location of San Andrés de Arroyo (Palencia, Spain) marked with a black star. b) View of the monastery where the excavated areas are seen in gray (1: Lobby of the faithful; 2: Courtyard; 3: North Hall; 4: Granary). The place where the individual under study was found is marked with a black dot. c) Top view of the grave where the individual was recovered. Note the feet are not visible due the east wall of the construction.

Figure 3. Photograph and radiograph of the *claviculae* (right clavicle al the left; left clavicle at the right). A greater curvature can be seen in the left clavicle. No signs of fracture are observed on the radiograph as that could have caused the deformity.

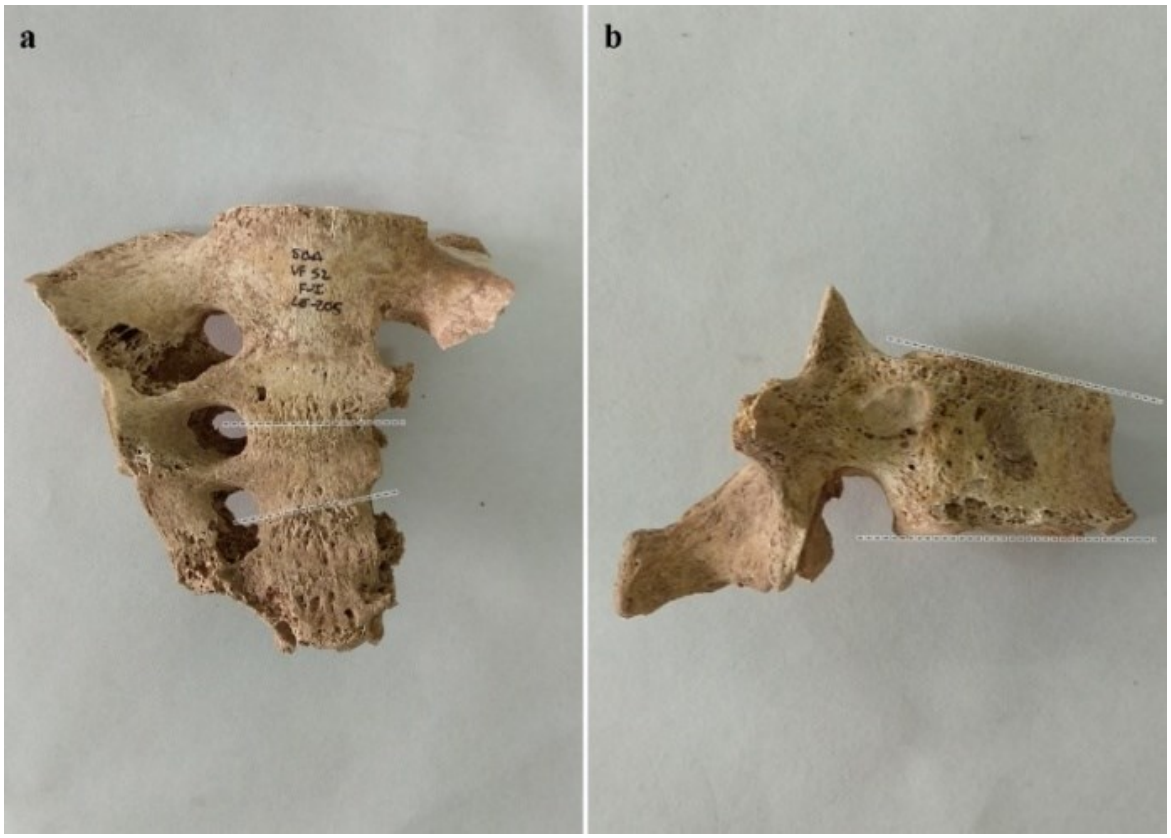

Figure 4. a) Photograph of the sacrum where a deformity of the third segment can be observed (between dotted lines). b) Photograph of vertebra T11, a reduction of the anterior height can be seen between the dotted lines.

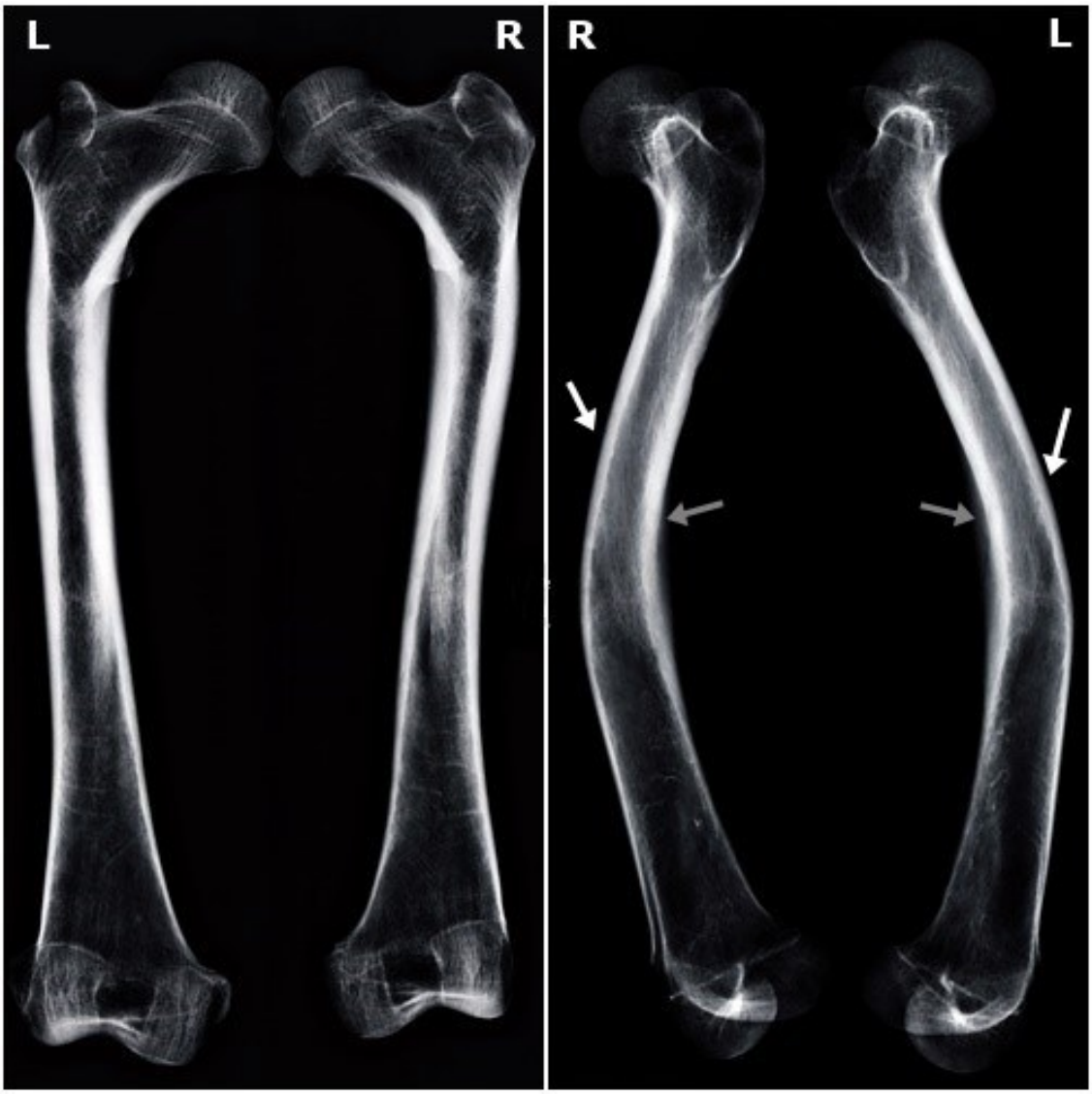

Figure 5. *Femora* radiographs of the individual with suspected vitamin D deficiency, posterior view on the left and medial view on the right. Note the different thickness between the anterior (white arrows) and the posterior (grey arrows) cortical bone.

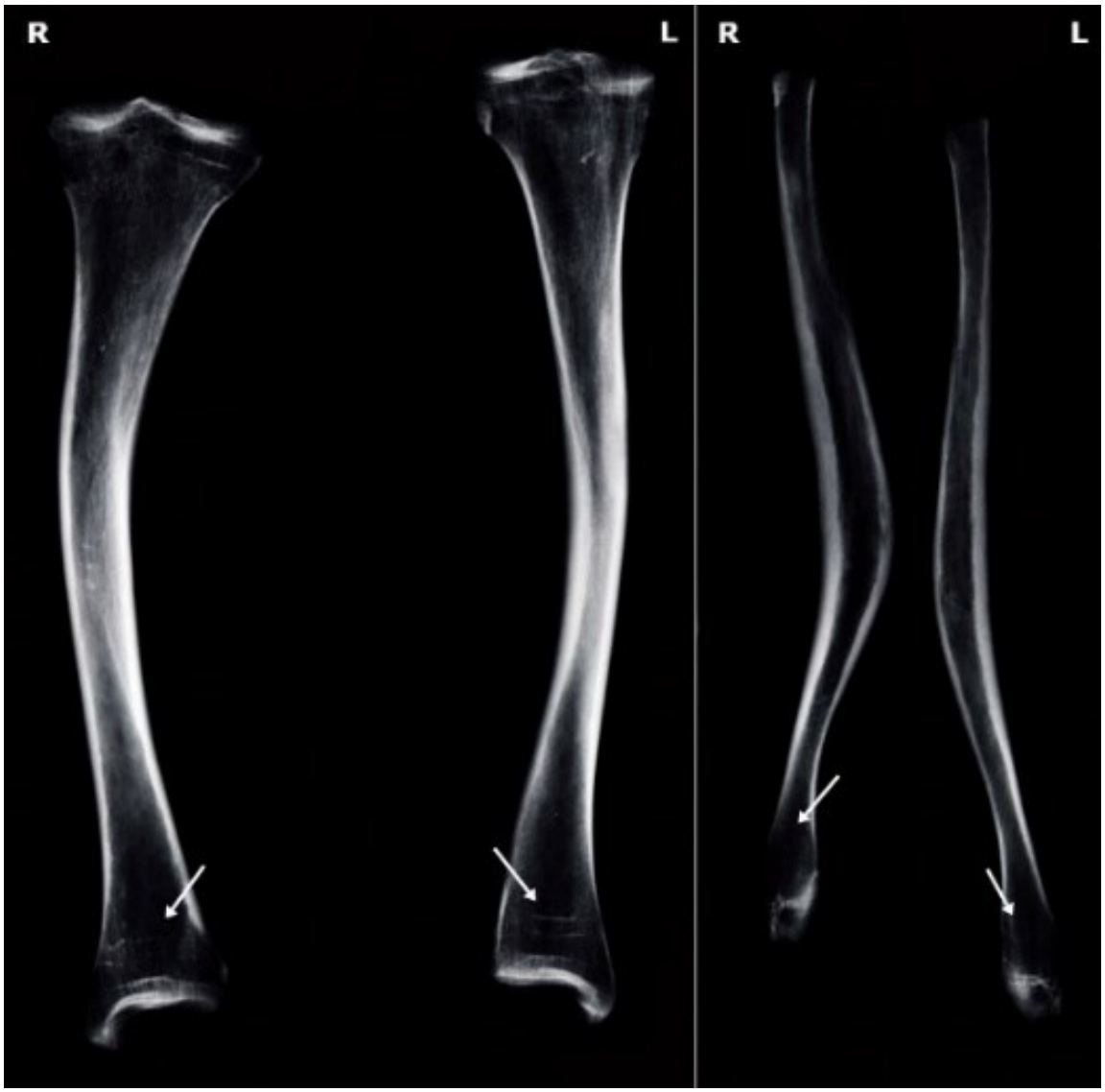

Figure 6. *Tibiae* and *fibulae* radiographs of the individual with suspected vitamin D deficiency. Note the presence of Harris lines at the distal end in all bones (arrows).

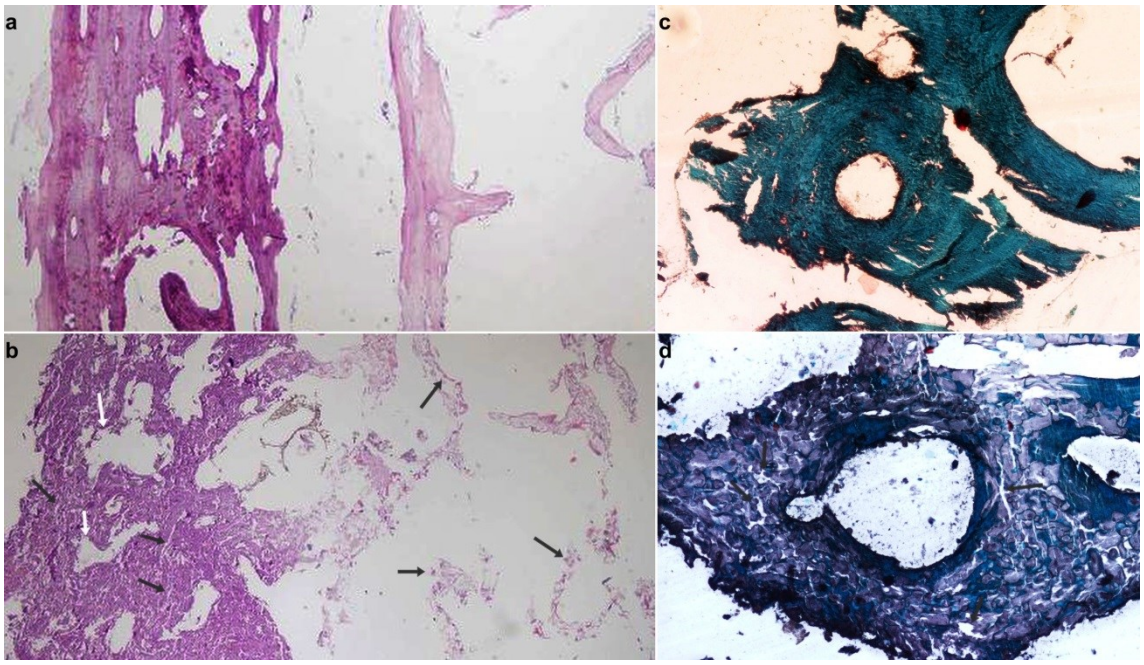

Figure 7. Histology comparison of the suspected vitamin D deficiency (bottom) versus the control healthy individual (top) in iliac crest samples. Note the difference between the microstructure in both images, the suspected deficiency case shows bone mineralization defects attributable to active osteomalacia, with enlarged space in cortical bone (white arrow) and defective mineralization in both cortical and trabecular bone corresponding to unmineralized osteoid whilst the control shows normal mineralization (grey arrows) (a and b: Haematoxylin-Eosin stain, 4x; c and d: Goldner's Trichrome, 10x).

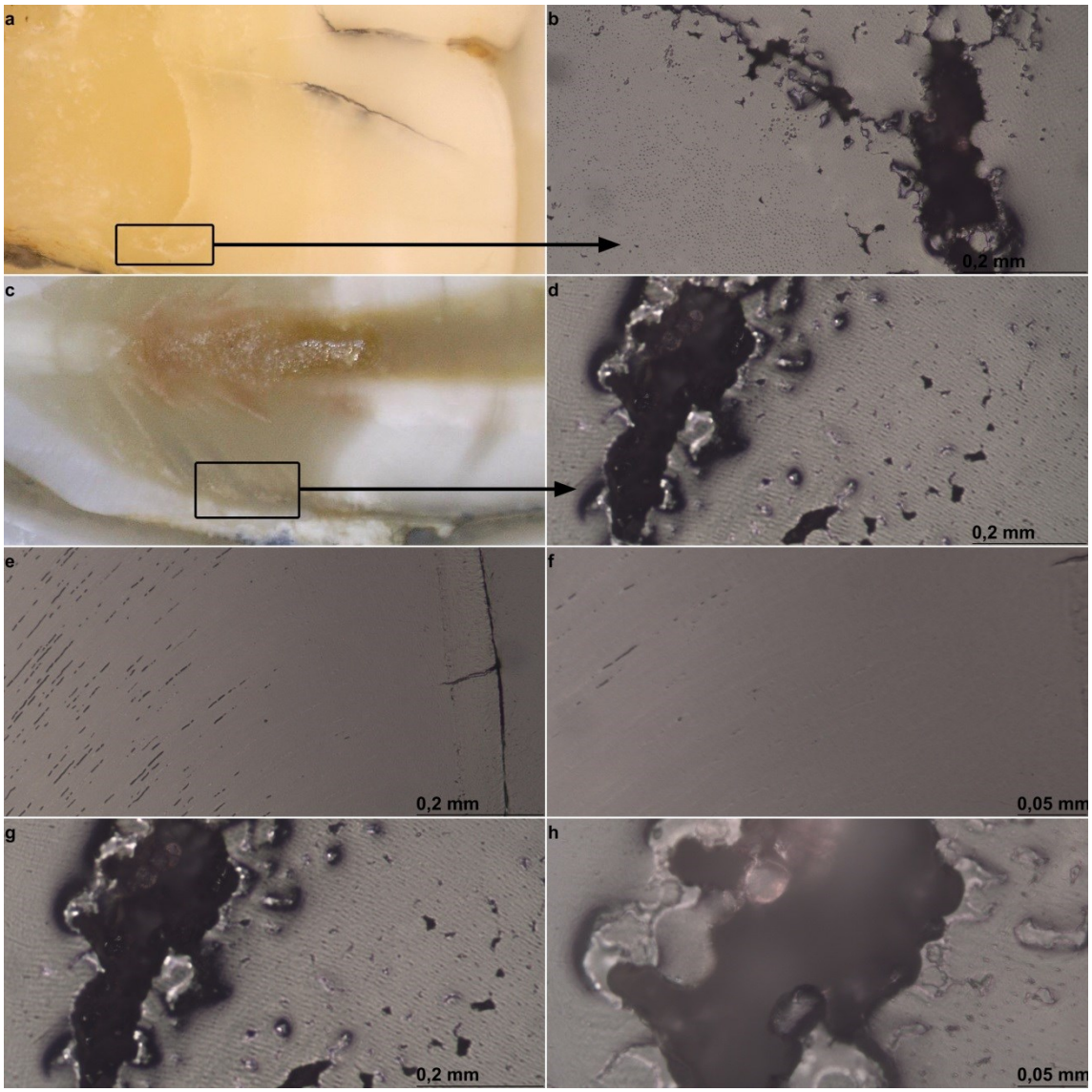

Figure 8. Histology of the tooth samples. a) Image of the first molar taken with the USB digital microscope where one of the areas with presence of interglobular dentin is indicated. b) Detail of interglobular dentin present in the marked area from the previous image of the first molar (10x). c) Image of the canine taken with the USB digital microscope where one of the areas with presence of interglobular dentin is indicated. d) Detail of interglobular dentin present in the marked area from the previous image of the canine (10x). e) Image of the tooth sample from the control healthy individual (10x). f) Image of the tooth sample from the control healthy individual (20x). g) Image of the tooth sample from the suspected vitamin D deficiency case (10x). h) Image of the tooth sample from the suspected vitamin D deficiency case (20x). Note the spaces in the g and h images, corresponding to interglobular dentin.

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