A new approach for the diagnosis of b-thalassemia in archaeological contexts: the relationship between congenital anaemia and dentinogenesis defects through micro-CT

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Abstract

The application of radiology as an aid for paleoanthropological analyses is seeing a widespread increase. The aim of this study is to examine a number of radiological dental aspects related to hereditary haemoglobinopathies, such as b-thalassemia, on the human remains of a past community by way of micro-CT.

A sample from the early medieval (10th-11th century) archaeological site of Vetricella (Scarlino, Grosseto), located in an area historically affected by malaria and part of the nEU-Med Advanced ERC project, was selected. Macroscopic paleopathological analysis revealed features typical of b-thalassemia. As a result, the skeletal remains underwent CT and micro-CT examination. CT analysis of the ribs, as demonstrated in previous studies, confirmed the initial hypothesis of the presence of b-thalassemia in the group. The dental micro-CT carried out on a sample of 7 deciduous incisors belonging to 7 sub-adult individuals allowed observing for the first time a direct relationship between the degrees of b-thalassemia recorded on the skeletons and dentinogenesis defects, identifying also a new radiological evidence which will be termed as «iris-like» appearance. A sample of three individuals from a coeval, non-thalassemic community was used for comparative purposes. These observations constitute a new approach to the diagnosis of b-thalassemia in archaeological contexts, providing an additional tool for differential diagnoses while also furthering our knowledge of the natural history of this disease.

1. Introduction

Hereditary haemoglobinopathies are a group of anaemias linked to a qualitative defect in haemoglobin, an historically widespread malady in the Mediterranean area. The most recurrent is thalassemia, a genetic anaemia resulting from defects in haemoglobin production (Higgs et al. 2012), the presence of which testifies to a long history of genetic adaptation to the parasitic disease of malaria. Malaria was endemic in many lagoon and coastal areas of the Italian peninsula at least until the end of the 19th century (Corti 1987; Frassine 2007). Its connection to hereditary haemolytic anaemias, in particular b-thalassemia, from the most (*major*) to the least (*minor*) severe forms, is a natural evolutionary response of human communities living in marshland environments infested by *Anopheles* mosquitoes, vectors of *Plasmodium falciparum*, the cause of malaria (Weatherall 2018; Vlok et al. 2021). While malaria per se does not cause changes in the skeleton, chronic anaemia associated with malaria, such as b-thalassemia, can leave non-specific traces detectable in the bioarcheological record (Buikstra 2019). B-thalassemia, caused by a decrease in b-globin production, affects multiple organs and is associated with significant morbidities and ultimately loss of life (Cunningham et al. 2004). Bone lesions are entirely due to compensatory hyperplasia of the bone marrow, leading to expansion of the medullary cavity (Helmi et al., 2017).

The study of thalassemia syndromes in archaeological human remains is of growing interest in the field of paleopathology, however, a definitive diagnosis of the disease in a skeletal sample remains difficult. To facilitate diagnosis in archaeological skeletal samples, a protocol (Eva-BeTa) has recently been

developed to assess which skeletal lesions can actually be considered as diagnostic of b-thalassemia syndrome (Scianò et al. 2021). A number of these can be obtained from the radiological record, highlighting the importance of radiology in the diagnosis of b-thalassemia (Tyler et al. 2006). For this reason, radiological diagnosis was attempted in the first phase of this study .

Although difficult, it is nevertheless possible to distinguish, in skeletal samples from archaeological contexts, individuals with thalassemia *intermedia* or *minor* who had time to develop pathognomonic features of the disease on their bones (Lagia et al. 2007; Balikar et al. 2013). On the contrary, the detection of b-thalassemia in the skeletal remains of children is far more complex, as individuals affected by the homozygous form of thalassemia would not have survived long enough to develop typical features such as «hair-on-end» or marrow hyperplasia of the facial bones, except in rare cases (Lewis 2012). Consequently, the identification of individuals with thalassemia *major*, i.e. Cooley's disease, in archaeological contexts, remains problematic (Ascenzi, Balistreri 1977; Hershkovitz et al. 1998; Salvadei et al. 2001; Keenleyside e Panayotova 2006).

The discovery of an early medieval community with several characteristics attributable to b-thalassemia, including high childhood and youth mortality, allowed us to focus on these issues, while also seeking further evidence of the presence of thalassemia in the age group up to 6 years.

In order to exclude that the traces of anaemia depended on nutritional factors, stable isotope analyses were carried out on the entire sample, including anaemic and non-anaemic individuals. The results showed no significant changes in $\delta^{15}N$ and $\delta^{15}C$ bone collagen values, even in the presence of anaemia. This confirmed that the disease was not acquired, as access to food appeared as homogeneous for both anaemic and non-anaemic individuals. Excluding malnutrition, the hypothesis of the presence of a congenital haemolytic anaemia appeared more likely (Viva et al. 2021).

Aim of this work was therefore to provide an additional tool for the diagnosis of the homozygous form of b-thalassemia in immature individuals from archaeological contexts, thanks to the study of dentinogenesis defects observed in micro-CT. For this purpose, a sample of individuals affected by congenital anaemia was analysed and compared with a sample from a contemporary community not affected by this pathology.

2. Archaeological And Environmental Context

The archaeological site of Vetricella (Scarlino, Grosseto) in south-western Tuscany is located in the centre of the coastal plain of Scarlino, crossed by the River Pecora and lying at the foot of the homonymous castle (Fig. 1) (DMS 42° 56' 59.294" N, 10° 49' 1.174" E).

Over the past decades the area underwent extensive archaeological research (Marasco 2018) and was recently included among the contexts investigated within the European ERC Advanced project “nEU-Med: Origins of a new Economic Union (7th to 12th centuries): resources, landscapes and political strategies in a Mediterranean region”, funded by the European Research Council (ID: 670792). Aim of the project was

to study the means and timing of economic growth in this part of the Mediterranean before the major developments of the 12th century (Bianchi and Hodges 2018, 2020 www.neumed.unisi.it). The site occupied a natural ridge in what was once a wetland landscape, not far from the edges of a now reclaimed lagoon. The nEU-Med project adopted a multidisciplinary approach based on various 'biodiverse' proxies, such as archaeological, anthropological, paleoenvironmental and geomorphological data, centred on a classic Mediterranean riverine corridor. Here, fluvial and alluvial dynamics influenced a wide range of settlement, land management and resilience strategies to survive the hazardous habitat, including diseases such as the historically attested presence of malaria. This environment was probably the direct cause of hereditary haemolytic anaemia, clearly visible in the anthropological analysis of the skeletal sample of a local indigenous community (Viva 2020; Viva et al. 2021), radiocarbon dated between the 9th and 11th centuries AD. For comparative purposes, the archaeological site of San Genesio (San Miniato, Pisa; DMS 43° 41' 30.59" N, 10° 52' 58.3" E), was selected, located along the Via Francigena between Pisa and Florence – geographically not far from Vetricella (about 85 km) – and historically not affected by the presence of malaria (Viva 2017). Fieldwork activities recorded the development phases of this site, ranging from the 6th to the 13th century AD, and constituting one of most important medieval religious centres in the region (Cantini et al. 2017; Cantini and Viva 2022; Viva et al. 2020; Viva et al. 2022).

3. Materials And Methods

The anthropological sample from the medieval cemetery of Vetricella consists of 51 individuals and is characterized by a high prevalence of infant mortality (0–1 year; 29.4%) and youth mortality in general (0–18 years; 70.6%), evidence that has already been partly discussed in previous work (see, Viva 2020). Consequently, the paleopathological study focused on the condition of immature individuals in order to understand the causes of their decease. Evidence of anaemia in the form of porotic hyperostosis (PH: 48.6%; Fig. 2) and *cribra orbitalia* (CO: 72.7%) was recorded on various skulls from the total sample (Viva 2020).

In order to understand its origin, a differential diagnosis was made between acquired anaemia and congenital haemolytic anaemia. Of the 27 individuals affected by PH and/or CO, 17 (63.0%) presented osteopenia on the post-cranial skeleton (PC), i.e. cortical erosions and pathological porosity on different anatomical regions, particularly on the distal metaphysis and epiphyses of femurs, tibiae and fibulae and proximal of the humeri (Fig. 3) (congenital haemolytic anaemias are characterized by low bone density: Jensen et al. 1998; Vogiatzi et al. 2005; Vlok et al. 2021). Of these, ten were children, five of whom died within two years of age; four were sub-adults between 13 and 17 years of age, and three were adult women. Two individuals characterized by abnormal diploe thickness, the so-called «hair-on-end» appearance (Fig. 4), would lend support to the b-thalassemia hypothesis (Ascenzi, Balistreri 1977; Buikstra 2019; Chaichun et al. 2021; Scianò et al. 2021). A third skull showed such severe PH that it appeared as «hair-on-end», however the feature did not develop due to death at 9–18 months of age (Fig. 2); unfortunately this individual had no teeth to analyse in this work.

To further verify the presence of b-thalassemia within the sample, 15 ribs belonging to 9 individuals, ranging in age from early childhood to adulthood, were subjected to a CT scan, performed with GE 128-layer equipment and a layer thickness of less than 1 mm. Observations focused on measuring rib density and changes in the trabecular component. Due to the impossibility of carrying out a detailed morphological study of the complete dentures, an analysis of dental tissue, enamel and dentine was considered in order to ascertain any alterations directly related to the anaemic condition caused by the genetic disorder. Likewise, for conservation purposes, micro-CT image analysis was preferred to histological procedures, which necessarily entail the destruction of the osteological sample.

Images of seven deciduous upper first incisors from individuals aged between 0–6 years were analysed. Deciduous teeth was chosen because it is assumed that individuals with more severe forms of b-thalassemia died no later than two years of age (Lewis 2012). The crown of the first deciduous upper incisor begins to form around the 30th week of gestation, the root finishing its development at around two years of age (AlQahtani et al. 2010).

A Xalt micro-CT scanner was used to obtain high-resolution micro-CT images (Panetta et al. 2012); teeth were scanned at 50 kVp, 2 mm Al filtration, 960 views over 3608, 1.6 mAs/view. Images were reconstructed using a modified Feldkamp algorithm (Feldkamp et al. 1984) with an isotropic voxel size of 18.4 μm on a 512×512×1200 volume dataset and cropped to the nearest tooth bounding box. Micro-CT results underwent visual examination, each individual reading carried out in both longitudinal and cross-section, as well as quantitatively through segmentation.

Upon autoptic examination, dentinogenesis defects (DD) were assigned a gradient, from absent (degree 1) to most severe (degree 3). To assess these defects in relation to the evidence of anaemia in the skeletal sample taken into consideration, the degrees of the observed features were classified in order of severity: *cribra orbitalia* (CO), porotic hyperostosis (PH), for which reference was made to degree 0 to 3 proposed by Steckel (2006), and osteopenia of the post-cranial skeleton (PC), for which, similarly, three degrees were adopted, from 0 to 3, where 0 indicates the absence of the skeletal part that is therefore not observable. The mean value (M) was calculated taking into account only observable features. Formation characteristics of dentinogenesis defects were also assessed according to radiographic appearance.

Micro-CT images were segmented virtually using Seg3D v2.2 (CIBC, University of Utah, USA, www.seg3d.org). Micro-CTs were obtained by image filtration (median and anisotropic diffusion filters); segmentation was followed by region-growing-assisted voxel thresholding with manual corrections and integrations. Segmentation allowed to analyse the quantitative dentinogenesis defects in each tooth, indicated in mm^3.

No congenital anaemic pathologies were detected in the comparative anthropological sample from San Genesio and the individuals from which three deciduous incisors were selected presented no skeletal traces of anaemia (Viva 2017).

4. Results

4.1 CT rib test scans

Rib observation was carried out on a random sample in order to confirm the widespread diagnosis of b-thalassemia throughout the community from Vetricella. Of the 15 ribs belonging to 9 individuals ranging from early childhood to adulthood, CT analysis recognized all cases as compatible with the presence of b-thalassemia, with ribs showing signs of hyperostosis (Fig. 5A) and the «rib-within-a-rib» appearance, a condition resulting from extensive marrow expansion within the rib shafts, appearing radiographically as sclerotic bands within the ribs due to extramedullary hematopoiesis (Fig. 5B).

4.2 Micro-CT dental tests

Micro-CT observations were performed on longitudinal and cross dental sections. Defects observed in cross-sections consist, in the most severe cases, of elongated lacunae arranged radially around the pulp chamber or, in less severe cases, in micro lacunae formations, again set in a radial pattern around the pulp chamber. Longitudinally, it can be observed that in more severe cases the defects affect the entire tooth, from the crown to the root, while in less severe cases they are concentrated either in the crown or in the root. Larger fractures through dentin and enamel should not be misleading, as these are tooth micro-fractures of the tooth that have occurred as a result of diagenetic processes.

This radial arrangement of the hypoplastic dentine is typical of cases diagnosed as thalassemic with skeletal features and rib X-rays, a pattern we have termed as «iris-like» appearance because, especially in severe cases, it closely resembles the characteristic arrangement of iris pigments around the pupil in the human eye.

Table 1 shows the results of the degrees of *cribra orbitalia* (CO), porotic hyperostosis (PH), manifestations of anaemia on the post-cranial skeleton (PC); the mean value of these (M)[1] and the degree of dentinogenesis defects (DD) assigned to each individual (Table 1). Spearman's correlation coefficient between M and DD ($r = 0.7159$) indicates statistical significance (P-Value is .0198).

In Table 2, the degrees assigned were compared with the images of the tooth sections most representative of the condition of each specimen analysed. Two cross-sections are shown, the one closest to the root apex and the one nearest to the crown, along with a longitudinal section (Table 2). Cases with the most clearly visible defects are specimens VT26 and VT45 (Fig. 6), both 2 years old and both with severe evidence of anaemia on the cranial and post-cranial skeleton. Two similar individuals with slight dentine defects are VT13 (4 years old) and VT19 (6 years old); the former shows minor traces of skeletal anaemia while in the latter these appear quite acute. VT40 (9 months old) presented severe evidence and degree 2 dentine defects. Lastly, two individuals with an age at death of about 1.5 years, VT33 and VT46, were characterized by slight dentine defects (similar to those of VT13 and VT19) and no apparent defects, respectively. Both had no skeletal signs of anaemia.

With regard to the sample from San Genesio: SG174 (3 years old) show a dentine defect, albeit different from that observed in individuals from Vetricella. The lacunae are not arranged radially as, for example, in VT45 (Fig. 6), but appear as spots near the enamel-dentin junction. SG197 (3 years old) and SG207 (6 years old) do not show any dentine defects (Table 2).

4.3 Micro-CT segmentation

Segmentation allowed to quantify dentinogenesis defects, i.e. the volume occupied by hypoplastic dentine in each tooth. Table 3 shows the complete results of the segmentation of enamel, pulp chamber, total dentine, normal and hypoplastic dentine. As the present sample consists of archaeological material, we must consider that segmentation results may be affected by diagenetic alterations. However, the tooth with the highest number of defects, corresponding to individual VT45, is the one least affected by post-depositional processes such as fractures and inclusions in the pulp chamber, showing the highest values of hypoplastic dentine (HD = 18.4%). The other teeth also present values that correspond to the visual observations of the micro-CT sections. SG174 shows dentinogenesis defects already visible in the micro-CT sections; hypoplastic lesions, although extensive (HD = 4.9%), have different characteristics from those of thalassaemic teeth: they do not show the typical «iris-like» appearance. Individual SG217 was excluded from segmentation because it presented extensive cavities that altered the tooth.

The Spearman correlation coefficient between DD and %HD (r = 0.7422) indicates statistical significance (P-Value is .0220).

[1]The mean value is calculated only on observable aspects; where the field is 0 it is not averaged.

5. Discussion And Conclusion

This research stemmed from the joint analysis of the demographic, paleopathological and isotopic observations of the sample, keeping in mind the surrounding natural environment, the coastal plain where the archaeological site of Vetricella is located, an area historically affected by the presence of malaria until its eradication in the 20th century (Majori 2012) (Fig. 7). The correlation between the presence of malaria and the evolution of congenital anaemia in human communities has been amply demonstrated (Weatherall 2018; Vlok et al. 2021). Recent aDNA studies of thalassaemia-related genetic mutations show that malaria in Italy was endemic even before the Middle Ages (Viganò et al. 2017).

The demographic study showed a high infant and youth mortality rate; paleopathological analyses revealed high percentages of anaemia, with the presence of some individuals with pathognomonic features of b-thalassemia (Viva 2020). Isotopic analyses of the paleo-diet ruled out the possibility that this could be due to poor nutrition (Viva et al. 2021).

Radiology was used to achieve two objectives: confirm the diagnosis by means of one of the radiological features of b-thalassemia, namely the «rib-within-a-rib» appearance, already documented in previous

studies (Lawson et al. 1981; Scianò et al. 2021; Vlok et al. 2021) and observed on several individuals of all ages in the present archaeological sample.

The high infant and youth mortality in this context suggests that many of the sub-adults, especially the younger ones, may have died from a severe form of this disease, i.e. Cooley's anaemia. However, for the reasons described above (Lewis 2012), the diagnosis of b-thalassemia is very difficult for these age groups. We are also aware that not all children would have died from Cooley's disease; causes of death could include malaria or other infectious diseases typical of childhood. In order to distinguish the possible causes of death in this age group and identify those who actually died of thalassemia, an innovative study was conducted on the micro-CT of deciduous teeth.

In scientific literature, several studies correlate congenital haemolytic anaemia with dental defects, ranging from orthodontic defects to dental tissue formation disorders and biochemical flaws (Novak 1944, Kaplan et al. 1964, Poyton, Davey 1968, Mourshed, Tuckson 1974, Cox, Soni 1984, De Mattia et al. 1996, Hazza'a, Al-Jamal 2006, Chaudhary et al. 2012, Costa et al. 2013, Kashid et al. 2013, Singh et al. 2013, Lopes et al. 2018). Some works on histological changes in the enamel, dentin and cementum of sickle cell anaemia patients relate dental tissue alterations, such as dentin hypomineralisation, hypercementosis and calcified areas in the pulp, to the congenital disease (Chaudhary et al. 2012). As early as 1966, a micro-radiographic study of individuals with Cooley's anaemia revealed a normal appearance of the dental enamel but pronounced pathological changes in the dentine and cementum, showing, in particular, that dentine formation was disturbed and poorly mineralized, resulting in the formation of lacunae (Soni et al. 1966). The microradiographic tooth characteristics observed by Soni et al. (1966) appear as very similar to those observed in the Vetricella sample through micro-CT.

At this point, it was possible to carry out a correlation study between degrees of skeletal anaemia and dentinogenesis defects. VT26 and VT45 were most likely affected by a severe form of thalassemia (b-thalassemia *major* or Cooley's disease) and their age at death, around two years, is in line with past life expectancy if affected by this pathology. VT13 (4 years) and VT19 (6 years), having passed the critical threshold of two years, may have suffered from *intermedia* or *minor* b-thalassemia, despite VT19 having the highest degree of skeletal anaemia evidence (2,3) and an intermediate degree of dentinogenesis defects. In this case, it is possible to observe how even the minor degree of anaemia, which usually leads to a higher survival rate, manifests itself more markedly on the skeleton as the years go by. The presence of similar traces of dentinogenesis defects made it possible to diagnose a form of intermediate b-thalassemia in younger individuals who may have died of other concomitant causes (VT33); in one case, one of the five individuals who died under the age of two (VT46) appears not to have been affected by Cooley's anaemia, as he presented no dentinogenesis defects. The micro-CT cross-section analysis made it possible to observe for the first time the typical radiological appearance of deciduous teeth in thalassemic subjects, defined in the present study as the «iris-like» appearance, as a result of its similarity to the human eye.

Lastly, the segmentation provided results corresponding to the visual observations of the micro-CT sections and the degree of anemia observed. It can therefore be said that volumetric quantification is also possible on materials from archaeological contexts as it provides reliable results, although visual examination remains essential in order not to confuse possible dentinogenesis defects.

Thus, for the first time, it was possible to identify a statistical significant correlation between degrees of skeletal anaemia and dentinogenesis defects in the remains of children under the age of 6 from an archaeological context. In many cases, the skeletons of sub-adults were either poorly preserved or did not have the time to develop the typical features of b-thalassemia; in contrast, the teeth, which are usually better preserved in the archaeological record, can provide a significant amount of information on this disease.

The results of the present study emphasize the great potential offered by micro-CT analysis, the comparison between skeletal anaemia and dentinogenesis defects providing a new method of diagnosing b-thalassemia in immature subjects, helping to distinguish the causes of death in individuals under two years of age from a community affected by b-thalassemia.

All these observations are useful in supplementing our knowledge of the natural history of this hereditary disease which is currently on the rise across Europe, continuing to appear in present-day human communities where it proves fatal in contexts lacking the necessary pharmacological treatments.

Declarations

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Author Contributions

SV and PFF conceived the study. SV and GB assembled archaeological materials and provided information on archaeological context. SV performed analysed data. GV viewed the micro-CT and carried out the segmentation. AP provided ribs CT. DP provided dental micro-CT. SV wrote the manuscript with input from all co-authors.

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Tables

Table 1 Skeletal sample from Vetricella (VT) and San Genesio (SG); the degrees of *cribra orbitalia* (CO), porotic hyperostosis (PH), and traces of anaemia on the post-cranial skeleton (PC); mean degree values of anaemia (M); degree values related to dentinogenesis defects (DD)

SK	CO	PH	PC	M	(CO, PH, PC)	DD
VT46	0	1	1	1		1
VT13	0	1.5	1	1.3		2
VT19	3	2	2	2.3		2
VT33	2	1	1	1.3		2
VT40	0	0	2	2		2
VT26	2	2	2	2		3
VT45	2	2	2	2		3
SG174	1	1	1	1		2
SG197	1	1	1	1		1
SG207	0	1	1	1		1

Table 2 is available in the Supplementary Files section.

Table 3 Results of micro-CT segmentation. Values are in mm^3

SK	Enamel	Pulp	Total dentine	Normal dentine (ND)	Hypoplastic dentine (HD)	% HD
VT46	30.28	43.71	108.72	108.72	0.00	0.0
VT13	19.22	19.47	140.33	138.93	1.40	1.0
VT19	22.07	13.28	114.53	114.43	0.10	0.1
VT33	25.34	44.51	79.99	79.62	0.37	0.5
VT40	26.66	32.02	50.03	49.10	0.93	1.9
VT26	20.94	20.71	112.78	105.06	7.72	6.8
VT45	21.97	38.97	108.19	88.23	19.96	18.4
SG174	20.31	26.72	113.26	107.74	5.52	4.9
SG197	20.42	23.74	120.52	120.52	0.00	0.0

Figures

Figure 1

Location of the two archaeological sites object of the present study

Figure 2

One of the most severe cases of porotic hyperostosis with characteristic «hair-on-end» appearance on a skull of a 9-18 month old subject

Figure 3

Post-cranial osteopenia in sub-adults. Left: distal end of femurs. Right: above, scapula; below, proximal end of femurs.

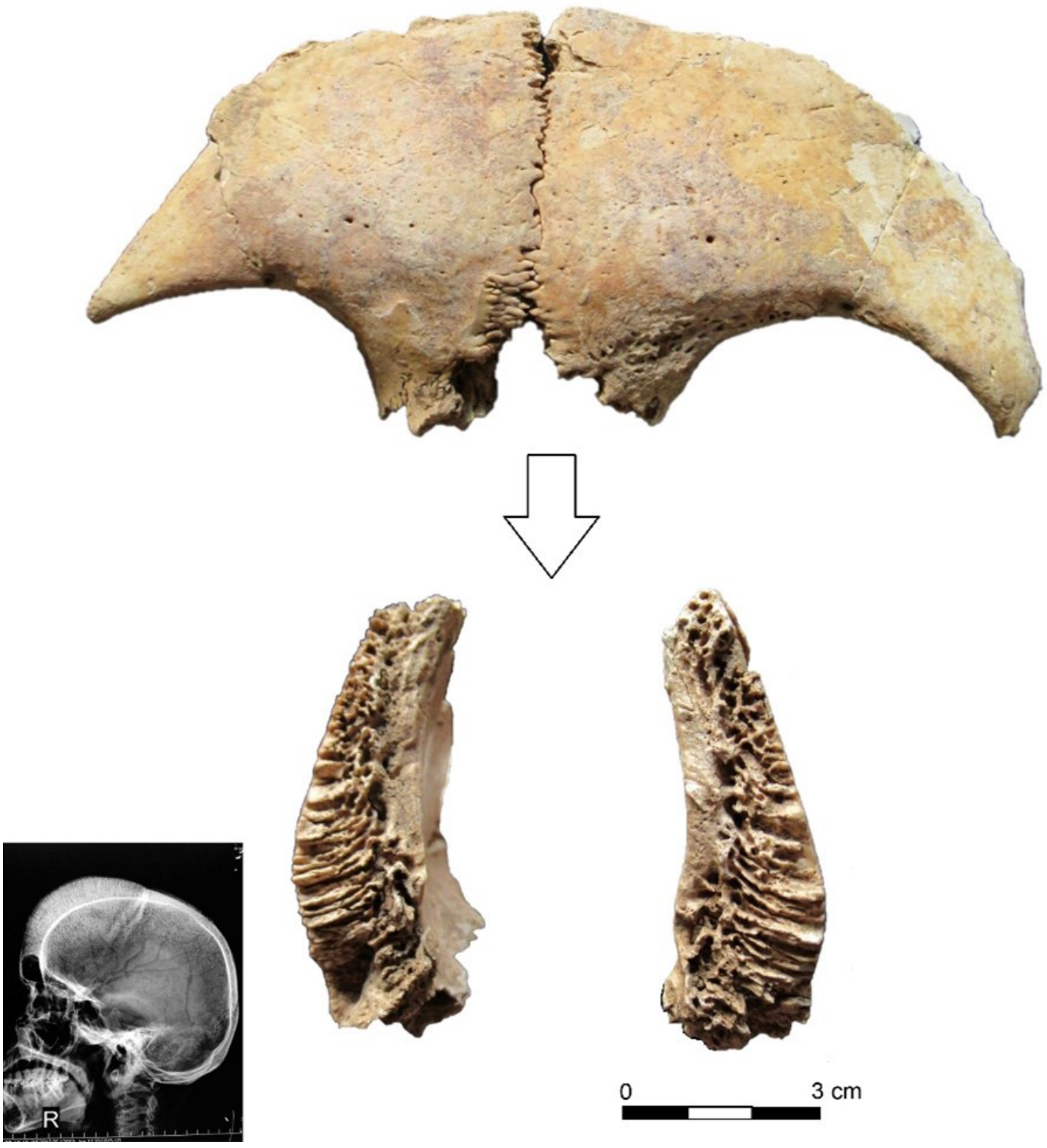

Figure 4

«Hair-on-end» appearance on frontal bones (VT37). In the box below, radiological example from Balikar et al. 2013

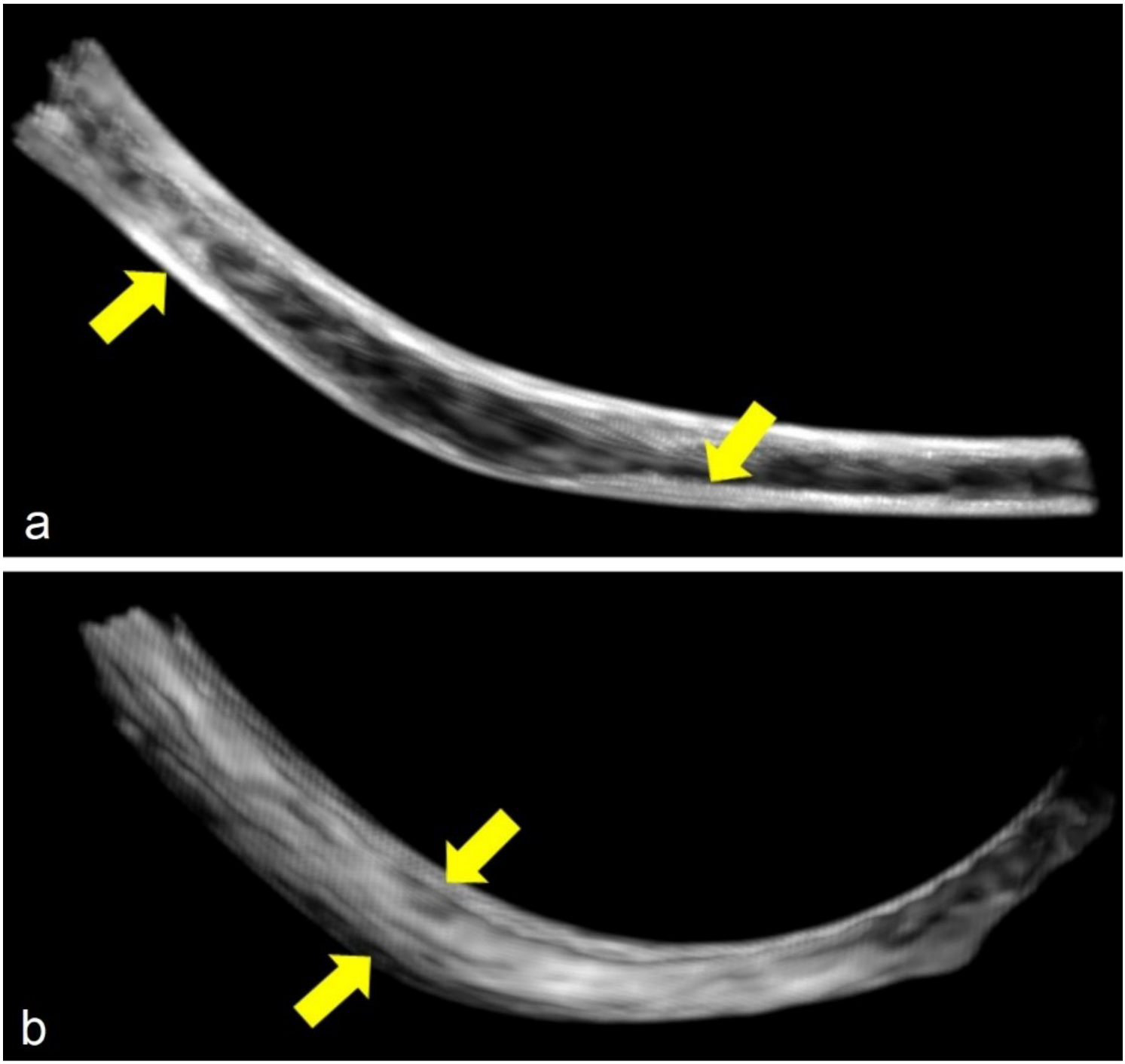

Figure 5

a 3D MIP reconstruction of a rib showing signs of hyperostosis (VT37). **b** 3D MIP reconstruction of a rib showing the «rib-within-a-rib» appearance in the medial portion (VT42)

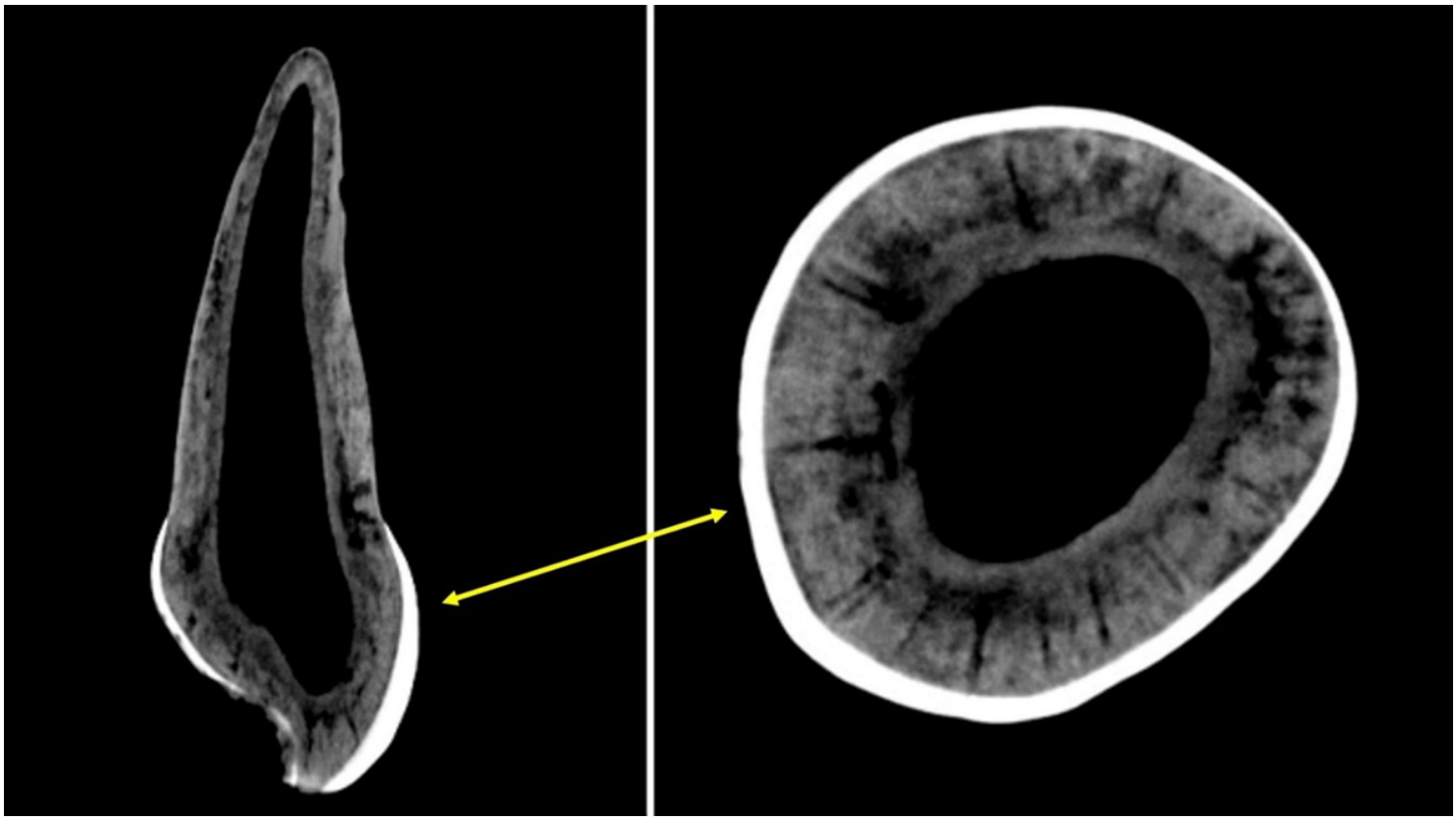

Figure 6

Deciduous incisor with evident dentinogenesis defects recorded in the skeletal remains of the individual most affected by anaemia within the sample (VT45). Left: a longitudinal micro-CT section; right: a micro-CT cross-section with «iris-like» appearance

Figure 7

Map of malaria distribution in Italy. Torelli, Firenze, Pellas, 1882. (Modified from Majori 2012). Areas in dark grey indicate very high malaria presence in 19th century; in red the archaeological site of Vetricella

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table2.docx