A new investigative strategy to diagnose β-thalassemia syndrome in past human populations

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Abstract
The study of thalassaemia syndromes in archeological human remains is of growing interest in the field of paleopathology. However, a definitive diagnosis of the disease in skeletonized individuals remains difficult. Several non-specific bone lesions have been suggested as the most likely evidence of β-thalassaemia syndrome. In particular, skull lesions have been considered by several scholars as the most indicative of this hematopoietic disorder, while other authors have identified postcranial lesions as the best evidence of β-thalassemia. In this study, we reviewed the main features that have been identified in β-thalassaemia patients thanks to an extensive bibliographic research of clinical cases, radiological and microscopic analyses. Our aim was to discern between those skeletal lesions that can be considered “indicative/diagnostic” and those that are “indicative/non-diagnostic” of β-thalassaemia syndrome. With this knowledge, we developed a new evaluation form (Eva-BeTa) and tested it on previously published archeological cases. Based on our results, we believe that Eva-BeTa can be a valid diagnostic tool for the identification of ancient individuals potentially affected by β-thalassemia for further genetic confirmation.

Keywords Anthropology · Archaeology · Forensic · Paleopathology · β-Thalassemia syndrome

Introduction

Thalassemias are the most common hemoglobinopathies worldwide. The WHO estimates that almost 270 million people are nowadays carriers of the syndrome, of which 70 million are carriers of β-thalassemia (De Sanctis et al. 2017, 2018). This hereditary form of anemia is also called “Mediterranean anemia,” since it was first observed and reported in patients from the Mediterranean areas (Frassetto 1918; Chini et al. 1939; Gatto 1942; Silvestroni and Gentili 1946; Martuzzi Veronesi and Zanotti 1973; Zanotti et al. 1973; Silvestroni and Bianco 1975; Martuzzi Veronesi and Gualdi-Russo 1976; Dacie 1988). Yet, to date, thalassemic individuals are widespread not only in the Mediterranean basin, but also in Africa, India, south-eastern Asia, Melanesia, Pacific Islands (Kountouris et al. 2014), on the so-called thalassemia belt, and, currently, through population migration, in many other parts of the world.

Thalassemia is a genetic form of anemia characterized by reduced or absent synthesis of the α- or β-globin chains forming the hemoglobin (Hb) molecule in the HBB gene, which is placed on chromosome 11. Gene sequencing has identified more than 100 different mutations involved, which consist mostly of point mutations (Kumar et al. 2011; Thein 2013; Wong et al. 2016). Depending on the mutations involved, thalassemias may afflict patients with different degrees of severity (Weatherall 1997). The three main forms of β-thalassemia are briefly described in Table 1.

As a rule, the reduced synthesis of β-globin chains causes anemia by reducing the lifetime of the red blood cells (RBCs) and of their precursors: many RBCs precursors have a damaged membrane and die by apoptosis. In the most severe cases of β-thalassemia, it is estimated that 70–85% of the RBCs undergo apoptosis (Weatherall 1997; Rund and Rachmilewitz 2005; Galanello and Origa 2010). In addition, deficient Hb synthesis produces RBCs with insufficient hemoglobin (i.e., hypochromic, microcytic RBCs), thus with a lower oxygen transport...
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knowledge acquired in recent times on thalassemic syn-
tical analysis of their skeletal remains. Considering the
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it remains challenging to identify individuals who may
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Strong selective pressure for malaria resistance has led to
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the goal of this work was to develop a diagnostic
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scoptic analyses for selecting samples to submit to ancient
DNA (aDNA) analyses. The genetic assessment remains
indeed the only method for a certain diagnosis of β-
thalassemia.
Hitherto, few studies based on aDNA analyses have
allowed identifying β-thalassemia’s mutations in human skele-
tons from the thalassemic belt from up to 12,000 years ago
(e.g., Béraud-Colomb et al. 1995; Filon et al. 1995; Viganó et al. 2017). Yet, in an aDNA study carried out on 4000 years
old human remains from Crete (Hughes et al. 2012), no path-
ological variation was detected in the PCR-investigated individ-
als. All these studies, but in particular the latter molecular
investigation, which was carried out on sixty-nine specimens
from 49 individuals, were not preceded by any osteological
study that could have restricted the number of samples to be
submitted to genetic investigation. Hence, to carry out further
and wider molecular investigations, which can also help in
reconstructing the evolutionary history of the mutations in-
volved, it would be of importance to previously select skeletal
individuals on the basis of precise characters detectable
through osteological analyses.
With this study, we aim to propose a new evaluation form
and a flowchart to make a preliminary diagnosis of β-
thalassemia on osteological material. It should be noted that
the proposed procedure can be the only possible tool in the
analyses of ancient human remains when DNA is not pre-
served. This may be of primary importance since malaria
areas, where thalassemia is more widespread, may negatively
contribute to aDNA preservation due to peculiar environmen-
tal factors (temperature, humidity).
In brief, we can summarize this research project in two
steps: (i) after reviewing appropriate literature, we devel-
oped a work-flow and an evaluation form, which are based on the main skeletal and environmental features
relevant for the diagnosis of β-thalassemia; (ii) we applied this methodologic approach to published cases of
suspected thalassemia to verify the potential of this first
differential diagnosis.

### Table 1: Characteristics of β-thalassemia syndromes (from Kumar et al. 2011, modified)

| Syndrome               | Alleles  | Laboratory details                                      | Clinical features                                      |
|------------------------|----------|--------------------------------------------------------|-------------------------------------------------------|
| β-thalassemia major    | β⁺/β⁺    | Severe anemia, microcytosis. Fragmented RBCs and striking morphologic abnormalities (e.g., anisocytosis and poikilocytosis) | Patients need frequent transfusions. Iron overload results in endocrine abnormalities and chronic organ damage. |
| (Cooley’s Disease)     |          |                                                        |                                                       |
| β-thalassemia intermedia | β⁺/β⁻ or β⁻/β⁺ | Moderate anemia, with RBCs morphological abnormalities (e.g., microcytosis and low fragmentation) | Clinical phenotype intermediate between β-Tm and β-TM |
| β-thalassemia minor    | β⁻/β⁻ or β⁺/β⁻ | Mild anemia. Low RBCs morphological abnormalities (e.g., hypochromia, and microcytosis) | Asymptomatic in life |

β-Tm, β-thalassemia minor; β-TM, β-thalassemia major; RBCs, red blood cells
β⁺, defective HB; β⁻, absence of the β-globin chains in the HbA molecule
detectable on human skeletal remains. Unfortunately, most skeletal abnormalities associated with thalassemia are not specific and can also be found in other forms of anemia and metabolic disorders. Some of the features identifiable in individuals suffering from thalassemia are also found in rickets, scurvy, infections, and parasitosis, although other kinds of lesions are missing or completely different. As a general rule, the retrospective diagnosis of thalassemia is a complex task; hence, in this work, we have focused only on one form of thalassemia, β-thalassemia, which is more frequent in the Mediterranean basin. Given that it is any-
way hard to distinguish between β-thalassemia and other forms of severe anemia on the basis of morphological traits, the goal of this work was to develop a diagnostic
tool based on anthropological, radiographic, and micro-
scoptic analyses for selecting samples to submit to ancient
DNA (aDNA) analyses. The genetic assessment remains
indeed the only method for a certain diagnosis of β-
thalassemia.

The alteration of the globin chain synthesis in thalasse-
emic individuals provides resistance against malaria. Consequently, thalassaemia has a high prevalence in geo-
ographical areas where malaria is historically endemic
(Kuesap et al. 2015). Deaths from malaria would have in-
creased between 10,000 and 5000 years ago due to changes
in agricultural and settlement development (Hedrick 2011).
The consequence of the ineffective erythropoiesis in thalasse-
semic patients is a massive erythroid hyperplasia of the bone
marrow. This results in an expanded mass of the RBCs pre-
cursors, which erode the cortical bone, compromise bone
growth and cause skeletal abnormalities, including porotic hyperostosis of the skull (Myers et al. 1986; Kumar et al. 2011; Vuch et al. 2013).

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in agricultural and settlement development (Hedrick 2011).
Strong selective pressure for malaria resistance has led to
the high frequency of some harmful genetic diseases, such as β-thalassemia in Mediterranean populations. It is not
unexpected, therefore, to find cases of thalassemia in an-
cient populations that populated malarial areas. However,
it remains challenging to identify individuals who may
have been affected by the syndrome through anthropolog-
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Materials and methods

Work-flow structure

As a first measure, the study of thalassemia syndrome in a skeletal human population requires the sample to be divided into two age categories: children vs. adolescents and adults. This distinction is necessary because the life expectation of children with Cooley’s disease did usually not exceed the age of 8 years in the past (Ortner 2003). In other words, adolescent and adult skeletal individuals with pathognomic traits should be affected by the mild form of β-thalassemia (see Table 1).

For the successive steps, our work-flow involves the use of an evaluation form (Eva-BeTa, appositely developed and described later in the text). The form provides an assessment score for the presence of thalassaemic syndrome based on pathognomic traits identified in clinical as well as in bioarchaeological literature. This evaluation form considers different investigation methods (macroscopic, microscopic, and radiographic analyses), as reported in the following paragraphs. At the completion of the work-flow, depending on the assessment score obtained for an individual and considering chronological framework and environmental conditions during his/her lifetime, we obtain a differential diagnosis of β-thalassemia that can be verified with genetic analyses.

Literature review

The scientific peer reviewed literature for skeletal markers of the β-thalassemia syndrome published in the last decades (June 1996–December 2018) was screened by one author (F.S.) with the generic engine “Google”. Further, more specialized web search engines (“J-Stor”, “WorldCat”, “Firstsearch”, “Pub-Med”, “Google Scholar”, “ResearchGate”, “Elsevier Journal”, and “Wiley Online”) were consulted. The following keywords were used to identify studies on the topic: “β-thalassemia syndrome”, “thalassemia minor”, “thalassemia intermedia”, “thalassemia major”, “thalassemia”, “thalassemia & bone markers”, “thalassemia & skeletal evidences”, “thalassemia & paleopathology”, “Cooley’s anaemia”, “thalassemia & cribra”, “thalassemia & porotic hyperostosis”, “thalassemia & malaria”.

We carried out a first screening of the retrieved publications by excluding all those publications that strictly regarded pathological conditions of soft tissues; doing so, we selected 104 articles out of 409. After duplicates removal, we selected only those papers that considered macroscopic and microscopic skeletal markers in human bone. In total, 46 full-text papers were assessed for eligibility. All authors contributed to the decision to include the full-text studies. As further criteria for selection, we included only papers in English, which had undergone a peer review process. As a result, 8 publications (Hershkovitz and Edelson 1991; Tayles 1996; Lagia et al. 2007; Lewis 2010; Fornaciari 2015; Rohnbogner 2016; Thomas 2016; Tomczyk et al. 2016) were used to define the characters to be included in the evaluation form and for its subsequent applications. This stringent approach was essential to carefully identify the traits that are most frequently considered as skeletal markers of thalassemia.

Results

To identify probable β-thalassemic individuals in skeletal populations, we developed a strategy resumed in a work-flow (Fig. 1) based on the application of a new evaluation form. To develop the strategy, we used the standardized crossing of features already proposed for the probabilistic diagnosis of other biological features (like sex and age-at-death/Ascadi and Neméškeri 1970; WEA 1980), which increases the retrospective diagnostic potential of the work-flow.

We started selecting 10 skeletal indicators of thalassemia syndrome (Table 2), as suggested in the relevant literature (Hershkovitz and Edelson 1991; Tayles 1996; Lagia et al. 2007; Lewis 2010; Fornaciari 2015; Rohnbogner 2016; Thomas 2016; Tomczyk et al. 2016). We classified these indicators as “non-specific”, “indicative/non-diagnostic”, and “diagnostic”, also taking in account relevant clinical case studies (Aksoy et al. 1973; Moseley 1974; Martuzzi Veronesi and Guirdi-Russo 1976; Lehmann 1982; Lawson et al. 1983; Kalef-Ezra et al. 1995; Wonke 1998; Dresner et al. 2000; De Roeck et al. 2003; Voskaridou and Terpos 2004; Azam and Bhatti 2006; Tyler et al. 2006; Lewis 2010; Galanello and Origa 2010; Baggieri and Mallegni 2011; Haidar et al. 2010, 2012; Hattab 2012; Perisano et al. 2012; Jha and Jha 2014; Wong et al. 2014; Wong et al. 2016; Rivera and Mirazón Lahr 2017; Risoluti et al. 2018).

The term “non-specific” indicates that the trait is present in a wide range of diseases and cannot be considered diagnostic of a single pathology. Four indicators were proposed as “indicative/non-diagnostic”; these are complementary indicators, which are present in disorders related to anemia. We considered them as “complementary” because their presence significantly increases the chance of achieving a more accurate diagnosis of β-thalassemia. We also identified four indicators as “indicative/diagnostic” of β-thalassemia, since they are considered strictly connected to the syndrome. They are easy to detect because of their effect on the bones, but they are only present in an advanced stage of the disease.

The concomitant presence or absence of these indicators and their mutual association allow to calculate a final score from the evaluation form for the preliminary assessment of β-thalassemic syndrome on archeological remains.

Although we are aware that porotic hyperostosis and hair-on-end are two faces of the same phenomenon, we decided to
include both indicators in the evaluation form on the basis of two reasons: (i) because they are detected with two different methods (hair-on-end can be identified only with radiological analysis, whereas porotic hyperostosis can be detected by macroscopic observation of the skull), and (ii) because hair-on-end represent a later stage of porotic hyperostosis and can be found only in extreme severe cases of anemia. Since of the two markers only hair-on-end can be related to cases of thalassemia, we defined it as indicative/diagnostic, whereas the porosity of the vault was considered as a non-specific indicator.

**Development of an evaluation form for β-thalassemic diagnosis (Eva-BeTa)**

The main objective of this research was to develop a new evaluation form to be implemented in the work-flow (Fig. 1). In the evaluation form (Evaluation form for Beta-Thalassemia, thus Eva-BeTa, Fig. 2), we reported the most relevant bone skeletal characteristics associated with β-thalassemia which have been previously identified through the literature review. The form is subdivided in two sections for cranial and postcranial indicators. This subdivision enables the analyses to be carried out also on partial human skeletons. For each indicator, we assigned a different weight based on its pathognomonic significance. We defined arbitrarily the pathognomonic significance (“degree of importance”) for each indicator, yet taking into account multiple parameters retrieved from the scientific literature, like the frequency of the character in patients or carriers, its severity, and its occurrence within the pathology. The highest degree of importance (3) was assigned to those relevant characters that are mostly associated to β-thalassemia syndrome and other severe anemias; an intermediate value (2) to those that are associated with other indicators and could be supportive of the presence of β-thalassemia syndrome; the lowest value (1) to those characters that are present in a wide spectrum of diseases, but are associated with anemia and could also be found complementarily in β-thalassemic syndrome. Missing data is indicated with a neutral value (empty field) in the form.

To ensure that the newly developed evaluation form is functional, we established, again conventionally, that a minimum number of 4 evaluable macroscopic markers or, at least, 2 evaluable macroscopic markers plus a marker detected through another specific analysis (X-ray investigation; Microscopy or thin-ground-section microscopy).
(Schultz 2001) should be delivered. The use of these specific analyses would enhance the diagnostic potential, given that many bone changes which cannot be observed or which can be mistaken by macroscopic analysis are revealed by CT and microscopic techniques (Schultz 1988; Rühle et al. 2000). Since diagnostic criteria may be relatively limited in paleopathology, it is necessary to combine and use more methods and techniques, especially at the microscopic level, to make such diagnoses as reliable as possible (Schultz 2001).

Filling out Eva-BeTa, a score will be obtained with a cut off of 0 (Table 3). Scores > 0 indicate that a putative β-thalassemic individual has been identified, while scores < 0 suggest that (severe, moderate, as well as mild) β-thalassemia is unlikely. Samples positive to Eva-BeTa could be submitted to aDNA analyses with a higher probability to identify thalassemic mutations.

**Application of the evaluation form Eva-BeTa to data from the literature**

We applied the new evaluation form on a selection of relevant historical and pre-historical archeological putative β-thalassemia cases taken from the scientific literature (Table 4; see also SI for the forms generated for any single case). The authors had mainly formulated their diagnoses using morphological analyses, and only occasionally they have used radiological methods, while none of the examined studies resorted to microscopic techniques.

From the 17 archeological cases considered (Table 4), we found 3 individuals with evidences suggestive of β-thalassemia major and 4 who may potentially be cases of intermedia or minor β-thalassemia. For further 4 individuals, we can confirm a generic diagnosis of β-thalassemia.
Thus, we obtained a total of 11 individuals (64.7% of the sample), who may have been affected by thalassemia, while 3 of the remaining six were not compatible/not suggestive of β-thalassemia and 3 were not evaluable/diagnosable due to the lack of a minimum number of detectable indicators.

### Discussion and conclusions

The identification of pathognomonic features of β-thalassemia major in ancient populations is always a challenge, because many of the observable skeletal lesions may also have resulted from other forms of anemia. With the application of Eva-BeTa, we expect to identify all bone...
alterations, which are affected by the severity of the syndrome, thus to narrow the field to at least severe forms of anemia so that suitable samples can be selected for further aDNA analyses.

As a rule, pathologic bone changes due to severe anemias, including Cooley’s disease, occur prevalently in the cranium (e.g., hair-on-end) and in the splanchnocranium (e.g., hypertrophy of zygomatic bones, pars basilaris, and alae maioris), due to local enlargement of the bone marrow. This evidence might be sufficient for a preliminary diagnosis of β-thalassemia major, when the individual is less than 8 years old.

More problematic in archeological populations is the diagnosis of heterozygous individuals for thalassemia; in fact, several lesions are non-specific for β-thalassemia but are the expression of a wide range of hematological disorders, including hypovitaminoses, such as scurvy and rickets (Klaus 2018), as well as chronic hemorrhagic diseases caused by fragile vessels and hypervascularization (Ortner 2003; Schultz 2003; Zuckerman et al. 2014). Starting from the morphological analysis of human dry bones, a reliable diagnosis of heterozygotes is difficult to obtain, although some facial features of thalassemia major might be present attenuated even in the mild form of the disorder, as observed in living patients (Martuzzi Veronesi and Gualdi-Russo 1976; Galanello and Cao 1998; Galanello and Origa 2010; Pollak et al. 2008). The strategy developed here should also help to identify moderate forms of anemia caused by genetic mutations to be verified with aDNA procedures.

Among the other causes leading to the development of anemia is the lack or inadequate supply of iron. Since iron is a key component of hemoglobin, iron deficiency in the diet or the binding of iron with dietary substances that make it inaccessible for hemoglobin synthesis can lead to severe anemia (Ortner 2003). Further, abnormal blood loss through “bleeding,” which is related to a variety of causes (e.g., gastrointestinal tract infection and abundant menstrual cycle) (Buikstra and Roberts 2012) can be another cause of anemia. However, this type of causes is transient and leaves no visible mark on the bones. Thus, individuals suffering from anemia due to iron deficiency will not be revealed by Eva-BeTa as possible cases of thalassemia.

The alterations that can be detected on the bones by Eva-BeTa are the result of prolonged stress as in the case of marrow hyperplasia induced by chronic anemia. They consist in an expansion of the medulla, thinning of the cortical bone, and resorption of the cancellous bone. Increased bone resorption can also result from marrow hyperplasia with the release of cytokines, which stimulate the osteoclast activity along with increased oxidative stress (Dede et al. 2016). The outcome of this process is a generalized loss of bone density (Tunaci et al. 1999; Ortner 2003) and the expression of the classical porotic features, such as those observed in Porotic Hyperostosis and Cribra Orbitalia (Walker et al. 2009). The precise understanding of the interaction between iron/magnesium (Polo et al. 1999; Castiglioni et al. 2013; Rude and Gruber 2004; Rude et al. 2009) and thalassemia could be a crucial challenge in the study of bone lesions in the β-thalassemia syndrome. One of the best indicators for the diagnosis of β-thalassemia syndrome is the hair-on-end appearance. This rare condition is referred to as the result of bone marrow proliferation with periosteum detachment and expansion of the diploë (Tomeczyk et al. 2016; Weatherall and Clegg 2001). Hair-on-end is well detectable by radiological inspection and appears as vertical striations extending behind the outer table. This indicator is also present in a wide spectrum of disorders, including congenital anemia, iron deficiency anemia, and tumors (Steinbock 1976; Lagia et al. 2007). Its presence depends on the severity and duration of the disorder, making it an excellent indicator in the evaluation of β-thalassemia major and intermedia if concomitant with the other traits considered in Eva BeTa.

The skeletal indicators that were selected for the evaluation form were mostly observed in patients with Cooley’s disease. Thus, the appearance of these markers in skeletons of subadults under 8 years of age-at-death can be attributed with confidence to β-thalassemia major. On the other hand, adolescents and adult individuals with positive scores of Eva-BeTa would be representative of thalassemia intermedia phenotypes. Among the published individuals re-analyzed with

| Range | Diagnosis                          | Range | Diagnosis                          |
|-------|------------------------------------|-------|------------------------------------|
| −0.51 to −1.0 | Non compatible with β-thalassemia syndrome | +0.51 to +1.0 | Compatible with β-thalassemia syndrome |
| −1.1 to −2.0 | Low chance of β-thalassemia syndrome | +1.1 to +2.0 | Fair chance of β-thalassemia syndrome |
| < −2 | Absence of β-thalassemia syndrome | > +2 | Possible presence of β-thalassemia syndrome |

The value 0 indicates that the case is not evaluable.
Table 4 Application of Eva-BeTa to specimens from published studies. The results obtained (score and diagnosis) are reported in the last column.

| Archeological site / reference | Country | Period | Ind no / burial no | Individual age-at-death/age class | Skeletal status | Radiological analysis | Score and diagnosis |
|-------------------------------|---------|--------|--------------------|----------------------------------|----------------|-----------------------|----------------------|
| Atilt-Yam/ Hershkovitz and Edelson (1991) | Israel | 6th mill. BCE | Homo 25 | 16–17 years (Adolescent) | Skull fragment, postcranium complete | Humerus | 0 not evaluable (min number of indicators not available) |
| Khuk Phanon Di / Tayles (1996) | Thailand | 2nd cent. CE | 21 | 8 years (Child) | Skull, upper and lower limbs and extremities | Metatarsal | +0.2 suggestive of β-thalassemia syndrome |
| | | 2nd cent. CE | 24 | 25 years (Young adult) | Skull and upper limb | Humerus | -1.0 not compatible with β-thalassemia syndrome |
| | | 2nd cent. CE | 56 | 45 years (Middle adult) | Skull (fragmented) | – | 0 not evaluable (min number of indicators not available) |
| | | 2nd cent. CE | 88 | 9 months (Infant) | Skull, lower limbs (fragmented) | Tibia and fibula | -1.0 not compatible with β-thalassemia syndrome |
| | | 2nd cent. CE | 101 | 15 months (Infant) | Skull (fragmented) | – | 0 not evaluable (min number of indicators not available) |
| | | 2nd cent. CE | 121 | 15 months (Infant) | Skull (fragmented), humerus | Humerus | +0.66 compatible with β-thalassemia syndrome |
| | | 2nd cent. CE | 150 | 30 months (Infant) | Skull (fragmented), femurs | Femur | +0.6 compatible with β-thalassemia syndrome |
| Greece/ Lagia et al. (2007) | Greece | 20th cent. CE | ABH-76 | 14 years (Adolescent) | Skull, vertebrae, ribs, scapulae, coxae, long bones | Skull, ribs, coxae, long bones | +1.6 fair chance of β-thalassemia syndrome |
| Poundbury Camp / Lewis (2010) | UK | 1st-5th cent. CE | PC525 | 1 year (Infant) | Parietal bones, thoracic vertebrae, ribs, left humerus, radius and ulna and left femoral shaft. | Parietal bones, ribs | +2.0 fair chance of β-thalassemia syndrome |
| | | | PC1083 | 6 months (Infant) | Skull (fragmented), ribs (fragmented), vertebral column, femurs (fragmented), left ileum, phalanges (undet) | Ribs | -1.0 not compatible with β-thalassemia syndrome |
| | | | PC920b | 9 months (Infant) | Skull and ribs (fragmented) | – | +1.0 compatible with β-thalassemia syndrome |
| San Giovenale / Fornaciari (2015) | Italy | 3rd cent. BCE | Tomb III | 4–5 years (Child) | Skull, long bones (fragmented) | Skull, left femur | +0.8 compatible with β-thalassemia syndrome |
| | | | Tomb V | 4–5 years (Child) | Skull, long bones and vertebral column (fragmented) | Skull, vertebrae, humerus | +1.57 fair chance of β-thalassemia syndrome |
Eva-BeTa, we identified two possible cases of thalassemia intermedia, individual ABH-76 (Lagia et al. 2007) and MK11G107 (Tomczyk et al. 2016), which represent ideal candidates for a genetic diagnosis of β-thalassemia.

Considering the results obtained with the evaluation form on the cases indicated in literature, most of the information used for a preliminary identification of putative β-thalassemic individuals comes from the analysis of the skull. Among all the analyzed specimens, 47.1% showed porotic hyperostosis, 41.2% cribra orbitalia, 29.4% hair-on-end, and 35.3% maxillary hypertrophy. Further indicators compatible with the diagnosis of thalassemia can also be found in the postcranium. Radiological and microscopic investigations carried out in previous studies confirmed that the cortical porosity of long bones is present mainly in sub-adults as a result of marrow expansion. These changes were more commonly observed in the humerus and femur, while short tubular bones were more commonly affected in children than in adults (Aufderheide and Rodriguez-Martin 2000; Tyler et al. 2006; Buikstra and Roberts 2012). We observed these changes in 35.3% of the reviewed individuals and in particular on the individual No. 21 (Khuk Phanon Di) along with enlarged foramina of the hand’s phalanges (Tayles 1996).

X-ray and CT examinations detected another important feature which suggests the presence of β-thalassemia syndrome, since it was often observed in living patients. The rib-within-a-rib appearance displays a subperiosteal extension of haemopoietic tissue through the rib cortex and is noted particularly in its middle and anterior portions. This is the most striking rib change present in patients with thalassemia who were never transfused (Currarino and Erlandson 1964; Aksoy et al. 1973; Lawson et al. 1981; Tunaci et al. 1999; Bedair et al. 2008). This indicator has been observed in five of the reconsidered studies (see also SI for detailed information). These subjects were sub-adults in 90% of cases.

The spine is also a relevant skeletal part for the diagnosis, in particular the vertebral bodies (Lagia et al. 2007), which show vertical striation due to thickened vertical trabeculae in individuals affected. In severely affected patients, biconcavity of the superior and inferior margins of the vertebral bodies or fractures may occur by compression (Wonke 1998; Aufderheide and Rodriguez-Martin 2000; Ortner 2003; Haidar et al. 2012). This characteristic was observed only in three cases from literature on archeological human remains. Yet, we should consider that in the other fourteen cases, the spine was not present.

In conclusion, it is hard to distinguish between skeletal anomalies due to thalassemic syndrome and chronic anaemias such as dietary iron deficiency (Steinbock 1976; Ascenzi et al. 1991; Ortner 2003). Therefore, we think that our work-flow, which is based on Eva-BeTa, and takes into account biological, as well as historical and environmental information, provides important insights for the differential diagnosis of β-thalassemia syndrome in archeological remains, thus can be a valuable tool to select samples for aDNA analysis. In the absence of detectable genomic material, the evaluation form Eva-BeTa will be the quickest and most effective means of determining the likelihood of a case of β-thalassemia.

An alternative cause of severe anemia in Mediterranean environments could be malaria itself (Carter and Mendis 2002). Severe anemia affecting children is a prominent feature of all forms of chronic malaria but only in areas of high transmission (endemic malaria), where the disease is circumscribed to the few years of life (Crawley 2004). If children survive malaria, in their adulthood, most malaria infections will be asymptomatic, but they will have been already developed the bone traits of chronic anemia (White 2018). Taking this information into account, samples from individuals identified as positive with Eva BeTa and resulting negative to the genetic test for β-thalassemia could be excellent candidates for aDNA detection of the Plasmodium spp. causing malaria.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s12520-020-01261-5.
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Code availability Not applicable.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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