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Virtual histology based on 3D X-ray microscopy imaging for non-destructive age-at-death estimation of incinerated teeth from the Tophet of Motya (Sicily, 6th century BC)

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ABSTRACT
The reconstruction of infant biological history and mortality profiles provides biocultural insights into adulthood morbidity, maternal health, parental caregiving practices, and social dynamics. However, interpreting biological data from cremated infant remains in archaeological contexts can be challenging due to their often poor preservation and potential biases. Tophets, sanctuaries for distinct burial of cremated infant remains present in several Phoenician-Punic colonies in the Mediterranean area, offer an ideal case study. This study presents the first virtual histology performed in X-ray Microscopy-based imaging (XRM) on a sample of crowns of cremated deciduous teeth from the Tophet of Motya (Sicily, 6th century BC). The observation of the Neonatal Line (NL) in enamel tissue offers important informations on the community demographic profile, age-at-death classes and life-histories of the buried individuals. Results are consistent with previous studies on other human remains found in the main Tophets in Mediterranean basin and highlight the importance of using non-invasive techniques to collect and analyse data that are useful for the interpretation of newborns and infants burial practices.

Section: RESEARCH PAPER

Keywords: age-at-death estimation; XRM imaging; virtual histology, neonatal line; Motya Tophet; measurement

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1. INTRODUCTION

In bioarchaeological research, the study of the odontoskeletal remains of immature individuals assumes a pivotal role in understanding past human societies, as the infant and juvenile segment of the population in a skeletal sample can be considered a dependent variable for measuring living conditions and resilience in ancient communities [1]-[3]. In particular, the reconstruction of infant biological histories and mortality profiles provides important biocultural information about adulthood morbidity patterns, maternal health, parental caregiving practices and social dynamics between adults and children in different populations [2]-[4]. Indeed, in case of infant remains found in archaeological contexts, a clear and unbiased interpretation of the bioarchaeological record can be a particularly difficult task due to their near absence in the anthropological records [2], [5]-[7]. In this context, the Tophet,

Figure 1. View of the island of Motya from the North – West; in the foreground the Tophet. Copyright: Archaeological Expedition to Motya (MAM), Sapienza University of Rome (https://www.lasapienzamozia.it/).

sanctuary typical of Phoenician culture in the Mediterranean, represents an ideal case study.

1.1. Tophet burials: a still unsolved dilemma

In a few colonies of the Phoenician-Punic western world, particularly in North Africa, Sicily and Sardinia, there is documented evidence of burial areas which represent an identifying and distinctive element of the communities, known as Tophets. These particular sepulchral sites, located in marginal areas of the settlement, were dedicated to housing in cinerary urns the cremated remains of children who died in perinatal or infant age and, as well as, not infrequently, those of small animals. Shrines and other structures related to the funerary cult were also placed there, including numerous steles depicting deities or sacred symbols [8]-[10].

Given the peculiarity of these burials, the nature and exact function of the Tophets have been and continue to be subjects of debate [5], [11], [12]. According to the reconstruction of the remains mortality profiles [13]-[15] and the historiographical records [16]-[18], some scholars suggest that the incinerated were offered in ritual sacrifices; others, conversely, consider the differentiation of these burial areas and the modalities of deposition to be linked to the socio-cultural significance attributed to the very young age of the buried, regardless of their cause of death [19]-[21], highlighting also a strong presence of premature foetuses and perinatal individuals among the individuals preserved in the urns [22]-[24]. Nevertheless, new investigations into the age of the cremated individuals placed in Tophets could help gather new data useful for interpreting what, at the current state of research, remains an incineration necropolis exclusively dedicated to infants.

1.2. Age-at-death estimation in immature individuals

Methods for estimating age at death in immature individuals typically rely on comparative measurements in different skeletal regions – primary ossification centres, length of diaphysis of long bones – and the state of tooth formation and eruption [25]-[29]. However, the anthropological analysis of cremated human remains can be challenging, especially when it involves perinatal and neonatal remains. The destructive nature of the combustion process increases the likelihood that cremated remains will be fragmented. This, in turn, may increase the potential for error and poor results in the application of examination techniques [22], [30], [31].

In this context, the most accurate method of estimating chronological age is through the observation of growth marks that form during the daily deposition of the enamel matrix of deciduous teeth and the first permanent molar [32]-[35]. The Neonatal Line (NL) is the most significant marking concerning perinatal age, as it unequivocally indicates the birth of an individual and its actual survival, separating tissues formed in the intrauterine environment from those post-partum [22], [36]-[38]. Histology, whether conventional [33], [34], [39] or virtual [40]-[44], is currently the only method available for high-resolution observation of dental enamel microstructure. However, this type of analysis entails certain constraints, such as sample destruction in traditional histology and limited access to Synchrotron facilities in the case of the virtual technique.

This study presents the first analysis in virtual histology using X-ray Microscopy-based imaging to determine the age-at-death range in a sample of deciduous incisors from the Tophet of Motya (Sicily, 6th century BC).

X-ray Microscopy (XRM) is a non-destructive characterization technique capable of visualizing the internal architecture of an object, providing microstructural and compositional information about the sample with submicrometric spatial resolution. Recently applied to a wide range of materials and fields, such cultural heritage [45], [46] and biological samples [47], XRM is particularly suitable for multi-scale and multi-modal approaches. Similar to microtomographic techniques, XRM can extract a three-dimensional model of the sample from its two-dimensional scans, which can be virtually handled without physical alteration [48]-[50].

1.3. The Phoenician colony of Motya (Sicily, Italy)

Motya, in the centre of the Marsala Lagoon (Trapani), on the western cusp of Sicily, was a Phoenician port city founded around 800 BC, for four centuries one of the most flourishing centres in the central Mediterranean [51]-[53]. The explorations carried out since the mid-1960s by the Archaeological Mission (MAM) of the Sapienza University of Rome, in collaboration with the BB.CC.AA Superintendency of Trapani and the “G. Whitaker” Foundation, led to the discovery and excavation of the entire area of the Motya Tophet (Figure 1), located on the north-western coast of the island, near the main necropolis [8], [54]. The sanctuary, excavated continuously for ten years between 1964 to 1973 and subsequently re-explored by ongoing missions, has yielded over two thousand deposits and an equal number of steles distributed across seven layers, covering a chronological span ranging from the second half of the 8th century to the end of the 4th century BC [52]-[56].

2. MATERIALS AND METHODS

2.1. Sample recovery and first macroscopic analysis

A sample of 22 crowns of deciduous incisors, upper and lower regardless of the side, from Motya Tophet was selected for the present study (Table 1). The remains were preserved in five different cinerary urns (MT93.139/1579, MT93.29, MT93.143, MT93.153, MT93.49) dated the second half of the 6th century BC.

None of the examined urns were sealed, except for MT93.139/1579, which was filled with compact sediment, the central parts of which yielded the best-preserved remains, while the lower and superficial layers contained only fragments. Micro-excavations by arbitrary units were carried out on the remaining urns due to the inconsistency of the sediment. Following the current guidelines [57], all material was passed through a stack of sieves with mesh sizes from 3 to 0.5 mm. The most important specimens were retrieved with precision cross-action tweezers

Table 1. Study sample (*N* = 22). Ldi1 = lower central deciduous incisor, Ldi2 = lower lateral deciduous incisor, Udi1 = upper central deciduous incisor, Udi2 = upper lateral deciduous incisor. Cr ½ = crown half completed with dentine formation; Cr ¾ = crown three quarters completed; Crc = crown completed with defined pulp roof; (B) = broken cervical plane.

Sample ID	Tooth type	Urn ID	Crown formation stage [27]
M01	Ldi2	MT93.139/1579	Crc
M03	Ldi2	MT93.139/1579	Crc (B)
M04	Udi2	MT93.139/1579	Cr ¾
M05	Udi2	MT93.139/1579	Cr ¾
M06	Udi1	MT93.139/1579	Crc
M11	Udi2	MT93.29	Crc
M12	Udi1	MT93.29	Cr ¾
M13	Udi1	MT93.29	Crc
M21	Udi2	MT93.143	Cr ¾
M31	Udi2	MT93.153	Cr ½
M32	Udi1	MT93.153	Cr ½
M33	Udi2	MT93.153	Cr ½
M34	Ldi2	MT93.153	Cr ½
M35	Ldi2	MT93.153	Cr ¾
M41	Udi1	MT93.49	Crc
M42	Udi1	MT93.49	Cr ¾ (B)
M43	Udi2	MT93.49	Crc ¾
M44	Udi2	MT93.49	Crc
M45	Ldi2	MT93.49	Crc
M46	Ldi1	MT93.49	Crc
M47	Ldi2	MT93.49	Crc
M50	Ldi2	MT93.49	Cr ¾ (B)

characterized by low elastic force to avoid further damage the fragile remains. Finally, a photographic documentation of the contents of each urn is taken, subdividing the remains by skeletal districts.

All the urns contained burnt human bones and teeth and in one case (MT93.49) also faunal remains. Among the few bone fragments, in a poor state of preservation, portions of long bones, skull, ribs, hearing bones, vertebral arches and clavicles were recovered in each sampled urn. At gross examination, no cut marks were detected on the vertebrae and/or long bones.

MT93.143 urn only yielded pelvic girdle bones (two right ischia and a left ischium) and some dental crowns; however, it was not possible to assign these remains to a particular individual or pair them with the teeth found. Only the dental remains were sufficiently intact, but due to their fragility it was in any case impractical to carry out accurate caliper measurements.

2.2. X-ray Microscopy scans acquiring procedures

A special sample holder with a small pocket in the centre was designed and 3D printed at the Fab Lab SAPeri&Co Research Centre of the Sapienza University of Rome to acquire the scans in the least traumatic way possible. The tooth was inserted into a cavity carved in a block of beeswax that filled the housing. This method ensures a secure seal without the need for excessive force. Beeswax does not interfere with scans and can be easily removed through gentle heating or the use of organic solvents, without affecting the integrity of the sample.

Each sample was examined using a ZEISS Xradia Versa 610 3D X-ray Microscope [58] available at the Research Center on Nanotechnology Applied to Engineering (CNIS, Sapienza University of Rome) and part of the ATOM (open infrastructure for Advanced Tomography and Microscopies) project. Scans were acquired operating in absorption contrast, with a pixel size range between 10.71 μm and 13.18 μm, an exposure time of 1.5 seconds, a voltage of 70 kV, and 4 x optical magnification.

2.3. Images post-processing

XRM datasets for each sampled tooth were reconstructed using ZEISS Reconstructor Scout-and-Scan (Version 16.1.14271, Carl Zeiss, Oberkochen, Germany) [59]. The three-dimensional reconstructions were imported into the image analysis software Dragonfly Pro (Version 2020.1 Build 797, Object Research System ORS, Montréal, Canada) [59]. The reconstructed crown's volume (Figure 2) was rotated to position the cutting plane along the longitudinal direction, which passes through the widest buccolingual diameter and the tip of the dentine horn [61] (Figure 3). This process identifies a group of slices of interest, forming an image stack.

The volume was then imported into ImageJ software (Version 1.54i 03, National Institutes of Health NIH, Bethesda, USA) [62]. A set of reformatted slices, parallel to the cutting plane, was extracted. The final image was obtained as the average of the stack, resulting in a single slice from the original pixel-sized slices.

Microstructural detail as the Neonatal Line, if present, were enhanced on the coronal enamel portion by increasing the contrast between pixels using a series of *sharpen* and *smooth* filters in succession.

Extra-uterine life-time was calculated by dividing the length of the enamel formed after birth, i.e. the section identified from the Neonatal Line to the edge of the tooth at its thickest point, by the post-natal Daily Secretion Rate (DSR), i.e. the daily rate at which enamel matrix is secreted, according to the values obtained by Peripoli *et al.* [34] on a sample of individuals from the Motya archaic necropolis (7^{th} - 6^{th} century BC). Considering that the cervical and outermost portions of enamel in forming teeth, representing approximately one week and half of growth, may be lost in post-depositional processes [33], [63], it was deemed reasonable to add two weeks to the chronological age count, while also taking into account the cremation process to which the remains were subjected.

3. RESULTS

Table 2 shows the presence of the Neonatal Line in the sample, which was successfully identified in 10 crowns, one central lower incisor, two lateral lower incisors, two central upper incisors and five lateral upper incisors, respectively. In four samples, the presence of the NL remains undetermined due to buccolingual surfaces in a fragmentary state (M42, M50) or the cervical margin deformed by the cremation process (M13, M45). Therefore, for the latter it was not possible to estimate an age-at-death, so they are excluded from further analysis. For the remaining sample, in which the Neonatal Line was not detected (N = 8), it was assumed that individuals died before its formation, either in the prenatal period or immediately after birth.

Figure 4 and Table 3 show the demographic profile of the sample (N = 18) for which it was possible to estimate a chronological age at death from the presence of NL (N = 10). Those individuals who did not show this distinct line (N = 8) were classified as preterm-perinatal. The morphological age is also estimated based on the observation of the average stages of crown formation [25], [27]. It is noted that in the three individuals (M41, M44, M47) the two ages do not agree, especially in the case of M47, with a morphological age of approximately one and a half months, where the virtual histological analysis had not detected the presence of the NL.

Figure 2. 3D rendering of XRM volumes of the total sample (N = 22). (a) M01, Ldi2; (b) M03, Ldi2; (c) M04, Udi2; (d) M05, Udi2; (e) M06, Udi1; (f) M11, Udi2; (g) M12, Udi1; (h) M13, Udi1; (i) M21, Udi2; (j) M31, Udi2; (k) M32, Udi1; (l) M33, Udi2; (m) M34, Ldi2; (n) M35, Ldi2; (o) M41, Udi1; (p) M42, Udi1; (q) M43, Udi2; (r) M44, Udi2; (s) M45, Ldi2; (t) M46, Ldi1; (u) M47, Ldi1; (v) M50, Ldi2. Note the fractured buccolingual portion in M42 (p) and M50 (v) and the heat-deformed cervical plane in M13 (h) and M45 (s). Scale bar = 1 mm.

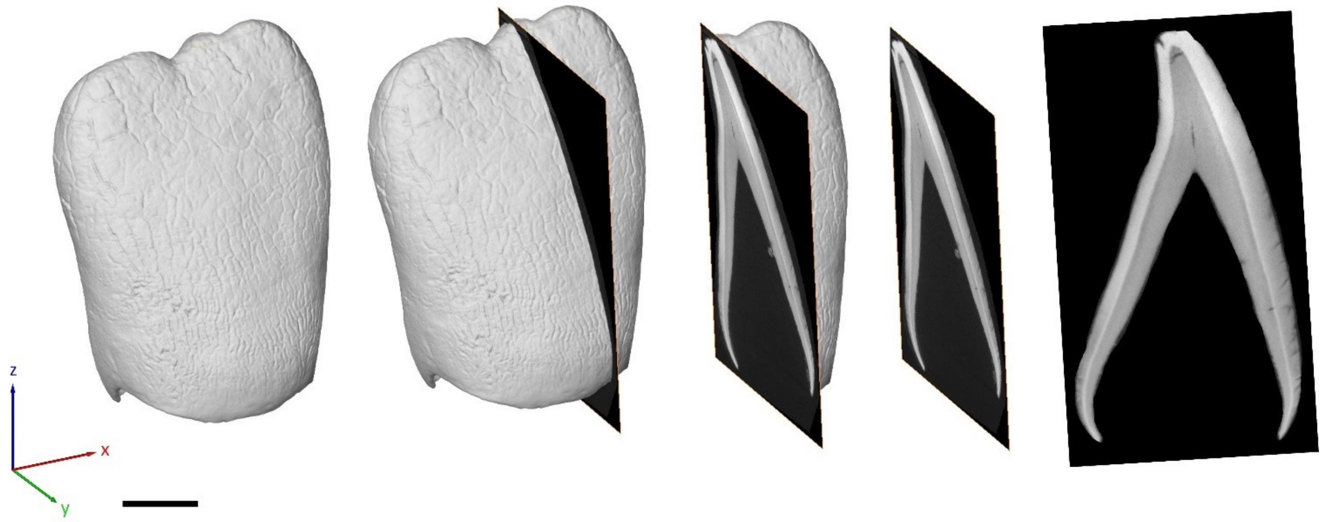

Figure 3. Virtual sectioning procedure. Red arrow marked the enamel-dentin junction (EDJ). Scale bar = 1mm.

The appearance of the Neonatal Line in an XRM-based imaging virtual section filtered for contrast enhancement is shown in Figure 5. Virtual histology analysis also revealed the presence of additional Accentuated Lines (AL) on the prenatal enamel of four incisor crowns (M04, M05, M21, M43). ALs formed before birth indicate that physiological stresses, such as nutritional deficiencies or infectious diseases, have affected the mother or child during pregnancy [40], [43].

4. DISCUSSIONS

4.1. Bioarchaeological perspective on Tophets function

Over the span of more than a century, archaeological expeditions in Motya necropolises have uncovered a broad spectrum of rituals, bearing witness to the ideological complexity of Phoenician-Punic communities across the Mediterranean basin [51], [64], [65]. Within this multifaceted context, the ongoing so-called *querelle* surrounding the purpose of the Tophet, burial areas designated for the interment of cremated infant remains in urns, continues to remain unresolved.

Table 2. Presence of the neonatal line in the virtual sections. Y = yes, N = no, ND = undetermined.

Sample ID	Tooth type	Urn ID	Neonatal Line
M01	Ldi2	MT93.139/1579	Y
M03	Ldi2	MT93.139/1579	Y
M04	Udi2	MT93.139/1579	Y
M05	Udi2	MT93.139/1579	Y
M06	Udi1	MT93.139/1579	Y
M11	Udi2	MT93.29	Y
M12	Udi1	MT93.29	N
M13	Udi1	MT93.29	ND
M21	Udi2	MT93.143	N
M31	Udi2	MT93.153	N
M32	Udi1	MT93.153	N
M33	Udi2	MT93.153	N
M34	Ldi2	MT93.153	N
M35	Ldi2	MT93.153	N
M41	Udi1	MT93.49	Y
M42	Udi1	MT93.49	ND
M43	Udi2	MT93.49	Y
M44	Udi2	MT93.49	Y
M45	Ldi2	MT93.49	ND
M46	Ldi1	MT93.49	Y
M47	Ldi2	MT93.49	N
M50	Ldi2	MT93.49	ND

In response to hypotheses suggesting Tophets as sacrificial sanctuaries, Schwartz *et al.* [22] performed conventional histological analysis on a sample of 50 deciduous incisors from the Tophet of Carthage (Tunisia), identifying the Neonatal Line in 24 dental crowns, confirming the survival of these individuals for at least two weeks post-partum. In the remaining 52% of the sample ($N = 26$), the absence of the Neonatal Line suggested that most individuals were stillborn, spontaneously aborted, or died during the first week after birth, and thus could not have been offered in sacrifice. Further study by the same authors [24] reviewed the ages at death of human remains preserved in more

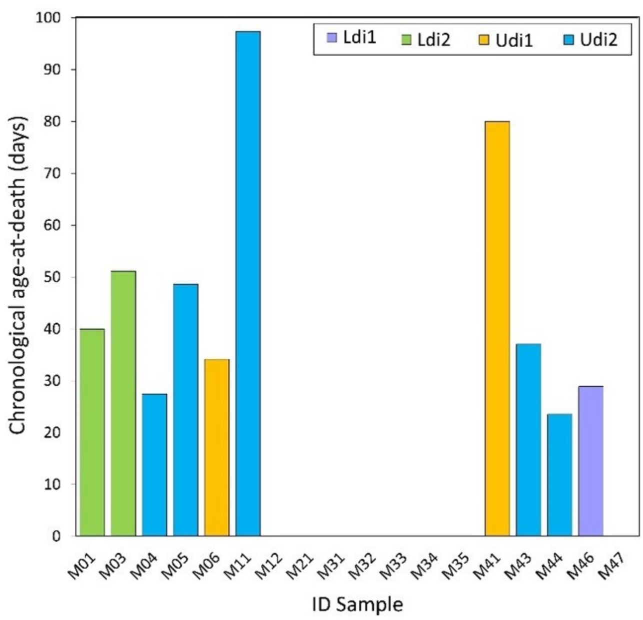

Figure 4. Demographic profile by age-at-death distribution, defined as days of post-natal life.

than 1,000 urns found in the Phoenician colonies Tophets of Hadrumentum, Sousse, and Carthage in Tunisia, Sulci in Sardinia, and Motya. According to various standard methodologies, such as the long bones length, odontoskeletal development, and Haversian canal configuration, most of the samples analysed were found to be preterm, peri-, and neonatal-aged individuals (see [24] for the complete references). In the present study, the Neonatal Line was observed in 55% of the sample, with a mean survival of 45 days from birth, excluding teeth that were fragmented or deformed at the cervical plane and therefore not observable (Table 2) (Figure 4). This survival rate is slightly higher than that previously estimated [22]. Prenatal and perinatal individuals constitute a smaller proportion (44%) of the total sample.

Considering the small number of specimens analysed compared to these previous studies, the results obtained here are consistent with previous observations, both in terms of demographic estimates of Tophet cremations and archaeological debate. Furthermore, the discrepancy observed between chronological and morphological ages for three individuals (Table 3) supports the assumption that reference standards for age-at-death estimation based on modern dental series [25], [27] may be potentially inappropriate when applied to archaeological remains, as pointed out by Nava *et al.* [33], [43]. Considering this specific burial context, it is also reasonable to assume that the estimated morphological ages may have been influenced by the condition of these remains. In contrast, the chronological ages derived from the histological approach, whether virtual or traditional, are not affected by these factors.

4.2. Brief notes on tooth/crown shrinkage

One of the main topics of discussion regarding the validity of an anthropological study on Tophet cremated remains concerns the possible alteration of crown morphology, and consequently tooth microstructure, caused by the cremation process [13], [14], [23], [24]. Bone and teeth react differently to heat, and because tooth enamel contains significantly less organic matter than bone, it is less susceptible to deformations that would not visibly affect the overall morphology or state of crown formation [23]. In the specific case of crowns on developing and unerupted teeth, the bone around the crypts in the maxilla and mandible can significantly reduce thermal exposure by protecting the tissue from the direct effects of heat [57]. In fact, although the state of

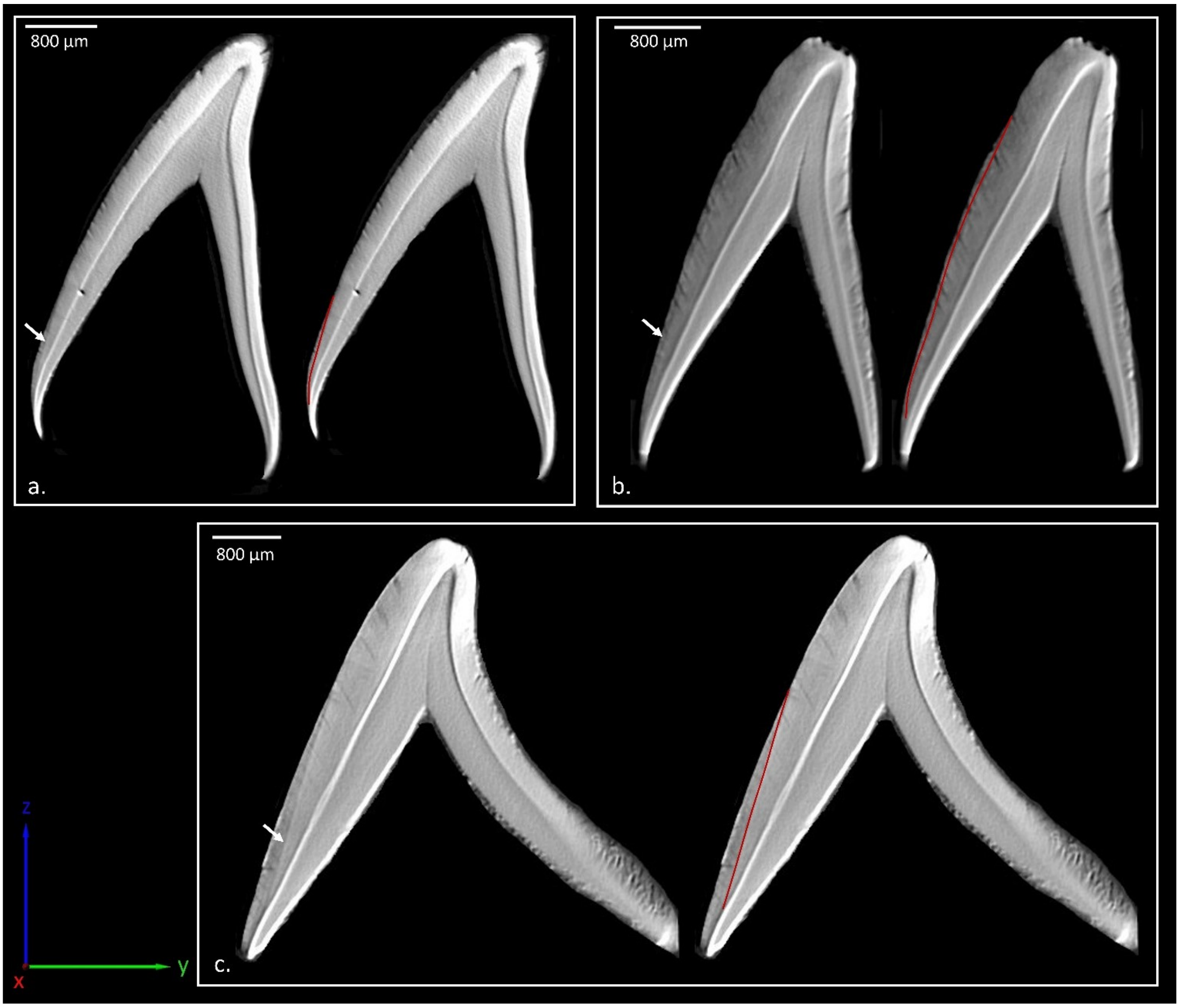

Figure 5. Virtual histology of M01 (a), M04 (b) and M41 (c). The white arrows indicated the Neonatal Line, marked in red.

Table 3. Comparison of the calculated chronological age with the morphological one estimated based on crown completion rate.

Sample ID	Tooth type	Mean Post-natal DSR (μm/day) [34]	Chronological age	Morphological age [25]
M01	Ldi2	4.6	41 days	1.5 months
M03	Ldi2	4.6	51 days	1.5 months
M04	Udi2	4.6	27 days	Perinatal – 1.5 months
M05	Udi2	4.6	49 days	Perinatal – 1.5 months
M06	Udi1	4.34	34 days	1.5 months
M11	Udi2	4.6	97 days	4.5 months
M12	Udi1	4.34	Preterm – perinatal	34 weeks *in utero* – perinatal
M21	Udi2	4.6	Preterm – perinatal	Perinatal – 1.5 months
M31	Udi2	4.6	Preterm – perinatal	34-38 weeks *in utero*
M32	Udi1	4.34	Preterm – perinatal	30 weeks *in utero*
M33	Udi2	4.6	Preterm – perinatal	34-38 weeks *in utero*
M34	Ldi2	4.6	Preterm – perinatal	30-34 weeks *in utero*
M35	Ldi2	4.6	Preterm – perinatal	34 weeks *in utero* – perinatal
M41	Udi1	4.34	80 days	1.5 months
M43	Udi2	4.6	37 days	Perinatal – 1.5 months
M44	Udi2	4.6	24 days	4.5 months
M46	Ldi1	4.34	29 days	Perinatal – 1.5 months
M47	Ldi2	4.6	Preterm – perinatal	1.5 months

preservation of the study specimen was precarious, with fractures and deformations along the cervical line in some elements, the Neonatal Line remained intact (Figure 5).

4.3. Motherhood in Phoenician Motya

The Neonatal Line is considered the first of the Accentuated Lines, a mark left on the enamel of primary teeth and the first permanent molar due to stress during the transition from the intrauterine to the extrauterine environment, appearing at least one to two weeks after birth, following the daily periodicity of enamel deposition [22], [33], [36]. The peak in infant mortality is attested in these very early days of life. Moreover, it is reasonable to assume that in pre-antibiotic societies, mothers and infants were exposed to numerous episodes of physiological stress due to different sanitation conditions compared with current times.

In the present study, Accentuated Lines, indicative of intrauterine stress, were found in three individuals who died within the first two months of life, respectively at 27 days (M04), 49 days (M05), and 37 days (M43), and in one individual (M21) who died pre- or perinatally, correlating with morphological age. These observations align with those made by Peripoli *et al.* [34] on the prenatal health status of infants buried in the archaic necropolis of Motya, where prenatal Accentuated Lines were identified in four individuals These data, along with the ALs observed on the prenatal enamel of Ostuni (Upper Palaeolithic, Italy) [43] and Arma Veirana (early Mesolithic, Italy) [40] foetuses further support recent hypotheses suggesting that the quality of intrauterine and early months life can strongly influence the likelihood of early mortality [66], [67].

5. CONCLUSIONS

Over the last decade, technologies originally developed for biomedical and engineering purposes have been increasingly applied to the analysis of fossil and archaeological human remains [68], [69]. This approach, known as Virtual Anthropology [70], has become a fundamental analytical method for studying the external and internal morphology of bone and dental tissues, allowing the non-invasive acquisition of morphostructural information acquisition with a same resolution equal to standard techniques.

This study presents the first virtual histology based on X-ray microscopy imaging on a sample of deciduous teeth cremated crowns. Despite the compromised preservation conditions and extreme fragility of the remains, the Neonatal Line was identified in ten crowns of the total sample analysed (N = 22), demonstrating the potential of XRM as an alternative, reasonable, and non-destructive method. The application of advanced, non- invasive, and analytical techniques demonstrates to be of primary importance not only for preserving remains, whatever the reasons, but also in cases where it is difficult to use measuring instruments.

In agreement with previous studies regarding these burial sites, the presence of remains belonging to individuals deceased in prenatal and perinatal ages agrees with the interpretation that considers Tophets as dedicated necropolis for premature and newborn infants (who, regardless of the cause of death, always represent the most fragile segment of the population), contributing to the understanding of funerary practices dedicated to the infant and juvenile portion of the Phoenician-Punic populations of the Mediterranean.

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