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Federico Lugli[a,*], Carla Figus[a,*], Sara Silvestrini[a], Valentina Costa[a], Eugenio Bortolini[a], Sara Conti[a], Beatrice Peripoli[b], Alessia Nava[c,d,e], Alessandra Sperduti[c,f], Leonardo Lamanna[g], Luca Bondioli[b,c], Stefano Benazzi[a,h]

[a] Department of Cultural Heritage, University of Bologna, Ravenna, Italy

[b] Department of Cultural Heritage, University of Padua, Padua, Italy

[c] Bioarchaeology Service, Museum of Civilization, Rome, Italy

[d] DANTE - Diet and ANcient TEchnology Laboratory, Department of Maxillo-Facial Sciences, Sapienza University of Rome, Rome, Italy

[e] Skeletal Biology Research Centre, School of Anthropology and Conservation, University of Kent, Canterbury, UK

[f] Dipartimento Asia Africa e Mediterraneo, Università di Napoli L'Orientale, Napoli, Italy

[g] Soprintendenza Archeologia Belle Arti e Paesaggio di Cremona Lodi Mantova, Mantova, Italy

[h] Department of Human Evolution, Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany

* Correspondence to: federico.lugli6@unibo.it; carla.figus3@unibo.it

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**Abstract**

In this work, osteological and paleopathological analyses are combined with liquid-chromatography mass spectrometry to study life and death of 30 non-adult individuals from an Early Medieval Italian funerary context (Valdaro, 7$^{th}$-8$^{th}$ cent AD). We estimated individual sex by exploiting sexual differences in enamel-bounded peptides. Enamel proteins were extracted through an acid etching of the whole tooth crowns for 4 samples and through a partial digestion of small enamel chunks for the remaining 26 samples. Both protocols were informative on the sex of the individuals through the identification of amelogenin isoforms (AMELX and

AMELY). In addition, low-mineralized tooth germs were analysed and they provided reliable information on the infants' sex. We observed the presence of 13 males and 17 females among the non-adults of Valdaro, not significantly different from a random sample with an equal frequency of males and females. *Cribra cranii* and endocranial lesion occurrence showed an association with sex, with higher frequencies in male individuals.

## 1. Introduction

In the last two decades, we witnessed a growing interest in children in bioarchaeological theories and research, as shown by the large number of works focused on the non-adult component of ancient societies (Beauchesne and Agarwal, 2018; Figus et al., 2017; Halcrow et al., 2017; Halcrow and Tayles, 2011, 2008; Lewis, 2017, 2011; Murphy and Le Roy, 2017; Thompson et al., 2014).

After having been often neglected in anthropological research, non-adults are now considered a key evidence for understanding demography, social structure and biocultural dynamics of past populations, both as single individuals and as a socio-demographic class. The osteobiographic perspective explores the articulated relationships between multiple factors and events experienced during the individual's life course (Agarwal, 2016; Zvelebil and Weber, 2013). At the same time, life histories allow researchers to address the complex issue of identity and social role, as also emphasized by funerary rites. Indeed, while it is true that social relationships influence the biology of individuals (Sofaer, 2006), the opposite is equally true: sex, age, and health conditions are individual biological parameters that contribute in the assessment of the social status and social relationships of individuals within a community (Clark et al., 2020).

In a broader perspective, survival and living conditions of infants and children offer an assessment of fitness in past populations and of their responsiveness to environmental change (Goodman and Armelagos, 1989; Lewis and Gowland, 2007). Moreover, as highlighted by numerous modern epidemiological studies, living conditions in the course of intrauterine life and during growth influence frailty and mortality during adulthood (Almond and Currie, 2011; Barker, 2004, 1990). Recently, the study of non-adults relied on advanced techniques for the analysis of physiological stress indicators and dietary profiles combined with increasingly precise methods for age at death assessment (Nava et al., 2017). On the other hand, these inferences lacked information concerning sex of infants and children. The latter variable can shed light on issues such as possible gender inequality in childcare and feeding practices in past populations. While some insights may originate from retrospective studies on adults (Miller et al., 2019), the younger non-survivor individuals (DeWitte and Stojanowski, 2015) may yield a more comprehensive picture of the actual effects of such inequalities.

Along with age-at-death, the other pillar of biological profiling is sex assessment (Acsádi et al., 1970; Schutkowski, 1993). The robust assessment of sex in human skeletal remains is an essential part of any skeletal assemblage study, both in archaeology and forensic anthropology. Nonetheless, it is well-known that sexual dimorphism is not present – or not enough marked – in the human skeleton until the development of secondary sexual characters after puberty (Cardoso, 2008; Loth and Henneberg, 2001). This prevents anthropologists from accurately determining sex of immature skeletal remains (Loth and Henneberg, 2001). However, there is strong evidence suggesting that sex in humans is already established at the moment of conception (Loth and Henneberg, 2001; Stull et al., 2017) and, especially during the first year of life, the production of sex hormones is relatively high (Burger et al., 1991; Reinisch et al., 1991). Differences have been found also in body proportions and in biomechanical adaptations during growth (Stull et al., 2017), with males showing e.g. larger diaphyseal breadth than females, and longer bone length after adolescence (Humphrey, 1998). Also, the timing and duration of growth and maturation are different not only between males and females, but also between individuals (Cunningham et al., 2016), making the study of sexual dimorphism even more challenging. Hence, the inaccuracy or the absence of sex information can bias the results of investigation on skeletal growth, health, cultural behaviors, and, more in general, the understanding of the health and well-being of a past population (Mays, 2013). Considering the importance of sex information for the construction of the biological profile and in paleodemographic studies, numerous attempts to design reliable methods were made, with different degrees of success. Several studies showed high accuracy associated with non-adult sex assessment, but the same methods did not achieve robust results in other populations, due to inter-population differences (Stull et al., 2017). Various methods were developed for different parts of the skeleton (Vlak et al., 2008), with a preference for the districts that are less prone to be damaged by taphonomic events, and/or districts with a considerable dimorphism in adults (i.e., mandible, pelvis and teeth) (Cardoso, 2008; Monge Calleja et al., 2020). Geometric morphometrics methods (GMM) were also employed with varying degree of success, being possibly less dependent on the observer's experience and thus more unbiased (Miller et al., 2019; Wilson et al., 2008, 2017).

Despite the considerable amount of research directed towards these questions, sex assessment methods are still highly imprecise and biased, so that crucial details on past juvenile demography are lost. To date, only molecular investigations, namely ancient DNA, may help to untangle sexing of juvenile individuals with a high degree of confidence. The high costs and limitations of the method (e.g. DNA degradation), however, make them unsuitable for a large number of samples (Tierney and Bird, 2015). Recent advancements in liquid-chromatography mass spectrometry (LC-MS/MS) allow for effectively estimating individual sex from tooth enamel (Froment et al., 2020; Lugli et al., 2019; Parker et al., 2019; Stewart et al., 2017, 2016; Wasinger et al., 2019). In fact, even if almost entirely mineralized (95-99% wt.), mature enamel averagely contains between 1% and 3% (wt.) organic material, mostly proteinaceous (Castiblanco et al., 2015). Such enamel proteome consists in amelogenins (AMELX and AMELY), enamelin (ENAM), ameloblastin (AMBN), amelotin (AMTN), tuftelin (TUFT1), matrix

metalloproteinase 20 (MMP20) and kallikrein 4 (KLK4) (Bansal et al., 2012; Cappellini et al., 2019). These proteins and proteinases are mostly synthesized and secreted by ameloblasts during enamel deposition. Then, throughout enamel secretion and maturation phases, structural proteins are hydrolyzed by proteases (Castiblanco et al., 2015). As enamel maturation proceeds, the organic matrix is in fact resorbed and substituted by minerals.

In particular, amelogenins have recently demonstrated their pivotal role in sex estimation and taxonomical classification of humans and animals by LC-MS/MS (Cappellini et al., 2019; Lugli et al., 2019; Parker et al., 2019; Stewart et al., 2017; Welker et al., 2019). AMELY is expressed from the Y-chromosome and thus strictly linked to male sex. Previous works demonstrated how AMELY can be easily identified and discriminated from AMELX within high-resolution ion chromatograms of LC-MS/MS analyses, by checking the presence of peptide $SM_{(ox)}$IRPPY (monoisotopic $[M+2H]^{+2}$ = 440.2233 *m/z*) and possibly other AMELY-related peptides (Lugli et al., 2019; Parker et al., 2019). Subsequent $MS^2$ database searches (e.g. UniProt and NCBI) can be employed to further confirm the sex classification.

The high-efficiency of such analytical protocol originates from four main factors: 1) at the end of the maturation process, enamel proteins are physiologically digested by proteases into peptides, obviating laboratory enzymatic digestion (Stewart et al., 2016); 2) amelogenins are relatively abundant within the enamel proteome (Bansal et al., 2012); 3) teeth are generally well represented in the archaeological record (Ogden, 2007); 4) enamel and mineral-bounded peptides showed a high resistance to post-depositional diagenetic alterations (Cappellini et al., 2019; Demarchi et al., 2016; Lugli et al., 2019; Welker et al., 2019).

The goal of this study is twofold: first of all, we aim to test whether the analysis of peptides entrapped in tooth enamel can be employed to rapidly and inexpensively determine the sex of a relatively large group of non-adults (*n* = 30; age range 4.5 months to ~16 years), recovered from the Early Medieval site ($7^{th}$-$8^{th}$ cent) of Valdaro, Italy. Second, we aim to identify possible differences in juvenile mortality between males and females, as well as to identify the onset of stressful events, through correlations between sex, age, and specific proxies of health status (i.e. skeletal indicators of non-specific metabolic stress).

## 2. Materials and Methods

The skeletal record of the Early Medieval cemetery of Valdaro includes 40 skeletons of sub-adults. Overall, the state of preservation and the representation of the anatomical districts were poor. In most cases, the cortical bone was not well preserved, and the skeletal elements were often fragmented or even absent. A subset of the best preserved sub-adult individuals (n = 30) was studied at the Laboratory of Osteoarchaeology and

Palaeoanthropology – BONES Lab, of the Department of Cultural Heritage, University of Bologna (Ravenna Campus, Italy).

### 2.1. The archaeological context

The Early Medieval necropolis of Valdaro (Province of Mantua, Lombardy, northern Italy; Figure 1) pertains to a larger archaeological context. The necropolis was excavated in 2008 and 2009 by the *Soprintendenza Archeologia, Belle Arti e Paesaggio per le Province di Cremona, Lodi e Mantova* in Lombardy. The archaeological record yielded evidence of human occupation across the site from the Neolithic to the Early Medieval period. Seven Neolithic burials and a probable incineration – the latter dated to the Iron Age – indicates that the site was inhabited throughout prehistory. Additionally, the presence of a Roman villa, agricultural divisions and moats suggests an intensive use during the Imperial Roman Age. The Early Medieval necropolis yielded 82 inhumation burials grouped in small clusters. The majority of the graves were rectangular in shape, directly dug into the ground, with a west-east orientation. The skeletons were lying supine with the arms extended by their side. A few graves showed the presence of small funerary structures as a brick cover. Just one case, a juvenile grave (Tomb 43), yielded a pair of bronze circular shaped earrings with three smaller rings on the border, typical of the Early Medieval period (Figure 1). This item was probably part of the outfit worn by the deceased at the moment of inhumation.

### 2.2. Protein extraction and LC-MS/MS

The enamel of 26 specimens was manually pre-cleaned and sampled (chunks of ~5 mg) using a drill (Lugli et al., 2019). Four additional specimens were treated following the protocol of Stewart (Stewart et al., 2017), employing an acid etch cleaning and sampling. Thus, a total of thirty teeth pertaining to the same number of individuals were investigated through LC-MS/MS (Table 1).

Enamel specimens were demineralized for 45 min - 1 h with 200 µl of 5% HCl at room temperature. A first batch of acid was discarded before the actual extraction. The supernatant containing both minerals and peptides was transferred in a new Eppendorf tube. Samples were thus desalted and purified by HyperSep SpinTips (Thermo Scientific) with $C_{18}$ functionalized silica. Resin-bounded peptides were eluted using 20 µL of 60% acetonitrile in 0.1% formic acid. Samples were finally dried down at room temperature under a laminar flow hood (class 100). All the previously described protocols were carried out at the BONES Lab. In total, this extraction protocol required ~10 hours of work of one person.

Dry extracted peptides were resuspended in 35 µL of a mixture of water:acetonitrile:formic acid 95:3:2, before the LC-MS/MS measures. Analyses were conducted using a Dionex Ultimate 3000 UHPLC coupled to a high-resolution Q Exactive mass spectrometer (Thermo Scientific, Bremen, Germany). A total run time between 60 and 90 minutes was employed for each sample and blank. Centroided MS and $MS^2$ spectra were recorded from 200 to 2000 m/z in Full MS/dd-$MS^2$ (TOP2) mode, at a resolution of 35000 and 17500 respectively. The two most intense multi-charged ions (TOP2) were selected for $MS^2$ nitrogen-promoted collision-induced dissociation. An inclusion list comprising six entries with the m/z and possible charge states of the peptides of interest was included in the method ($[M+2H]^{+2}$ 523.7748; 440.2233; 540.2796; 525.2975; 575.7533; 656.3528 m/z). More details on the analytical protocol are reported in Lugli (Lugli et al., 2019). Raw MS data were deposited in Zenodo (10.5281/zenodo.3774090), including also two blanks as example.

### 2.3. Peak identification and database searches

Ion chromatograms were searched using Xcalibur (Thermo Scientific) with a mass tolerance of 5 ppm. We specifically focused on peptides $SM_{(ox)}$IRPPY (AMELY; $[M+2H]^{+2}$ 440.2233 *m/z*) and SIRPPYPSY (AMELX; $[M+2H]^{+2}$ 540.2796 *m/z*), previously demonstrated as strong sex biomarkers (Lugli et al., 2019; Stewart et al., 2017) (Figure 2). Additional peptides were also searched to corroborate the presence (or the absence) of AMELY (Lugli et al., 2019). To possibly identify other tooth proteins and unique AMEL peptides, raw data were converted into Mascot generic format (MsConvert v. 3.0.10730, ProteoWizard tools) and simultaneously searched against: 1) Swiss-Prot (constrained to *Homo sapiens*); 2) an in-house database downloaded from UniProt & NCBI, including all available mammal amelogenin sequences; 3) cRAP (116 sequences) for contaminants. No proteolytic enzyme was selected in search parameters. Deamidated asparagines/glutamine (NQ) and oxidated methionine (M) were set as variable modifications. Mass tolerances were set at 10 ppm for the precursor ions and 0.05 Da for the fragmented ions. False discovery rate was estimated through an automatic decoy database, with a probability threshold trimmed to an FDR <1%. A specific protein was considered as identified if at least two significant peptides were observed.

### 2.4. Skeletal age assessment and health conditions

The age-at-death of the individuals was assessed using different methods, depending on the developmental stage and the state of preservation. Namely, the dental development and eruption stages (AlQahtani et al., 2010) have been considered as the main indicators. Moreover, the appearance and fusion of the ossification centers and the anthropometric measurements have been used (Black and Scheuer, 1996; Cunningham et al., 2016; Fazekas Gy.

and Kosa, 1978; Maresh, 1970). We divided the sample into six age classes, according to Scheuer and Black (Scheuer and Black, 2000) after Knussmann (Knussmann, 1988), and then adapted to our sample as follows: Neonates/Infants 0-1 year; Infants 1.1-3; Children I 3.1-6; Children II 6.1-10; Adolescent 10.1-15; Post-adolescent >15) (Table 2). All skeletons were macroscopically examined for the presence of non-specific markers of physiological stress (see Table 3, namely *cribra cranii*; *cribra orbitalia*; endocranial lesions; porosity of the hard palate; postcranial porosity) (Ortner, 2003) and traumas (Lewis, 2017; Ortner, 2003). We followed the criteria proposed by Ortner and Ericksen (Ortner and Ericksen, 1997) for the assessment of abnormal porosities. For each available anatomical district, these indicators of non-specific stress were recorded as absent, present, or non-observable. We also assessed the presence or absence of Linear Enamel Hypoplasia (LEH) in the permanent dentition.

### 2.5. Statistical analysis

Significant departure from the null hypothesis of a prior probability of 0.5 assigned to males and females was assessed via a binomial test. Due to small sample size and contingency tables with values <5, association among variables was quantified by calculating Cramer's V between each stress indicator and sex/age class respectively (using the function assocstats in the package vcd) (Meyer et al., 2020). Only individuals for which sex, age, and pathology could be identified were considered in each case. We also tested for differences between sexes in the distribution of age-at-death assessments through a two-tailed Mann-Whitney U test because either sample failed to meet assumptions of parametric tests. All analyses were run in R 3.6.2.

## 3. Results

### 3.1. LC-MS/MS

After the search of ion chromatograms, AMELX peptide SIRPPYPSY was found in all the analyzed samples, while AMELY peptide SM(ox)IRPPY in 42% of individuals (13/30; see Table 1). The ratio between the peak intensities at 440.2233 and 540.2796 m/z resulted equal to 1.05 ± 0.28 (mean ± SD). The tooth germs here analyzed, e.g. T38 (4.5 months) and T85 (9 months), yielded enamel-related peptides, allowing to disclose the sex of the infants. Mascot searches showed the presence of other tooth proteins within some samples, as AMBN and ENAM. CO1A1 (collagen type I α1), CO1A2 (collagen type I α2) and DSPP (dentine sialophosphoprotein) related peptides were also detected, possibly due to residual dentine tissue. We have not found any modern contaminant through the cRAP database.

### 3.2. Association between stress indicators and sex/age classes

Overall, females were slightly more represented than males (Male/Female ratio = 0.76), even if the presence of 17 females over 30 individuals is consistent with a random sampling with a prior probability of 0.5 (P = 0.58). The age-at-death ranged from 4.5 months to 15.5 years (Table 2; Supplementary Table 1), with a peak recorded in the Infant (n = 11, 36.7%) and Children I (n = 10, 33.3%) age groups, followed by Neonate/Infant (n = 2, 6.7 %). Several individuals showed indicators of non-specific metabolic stress (Table 3): 15 individuals exhibited signs of *cribra cranii* (CC), while 15 individuals displayed evidence of postcranial porosity. Abnormal porosity was also recorded in the hard palate (n = 16), and 8 individuals were affected by *cribra orbitalia* (CO). One individual, i.e. Tomb 20 (Adolescent), showed abnormal porosity on the greater wing of the sphenoid bone, along with porosities in other districts, (i.e. *cribra crani, cribra orbitalia*, mastoid process, hard palate, postcranial porosity). No traumas were recorded with the exception of a case of greenstick fracture of ulna and radio in an individual in the "Post-adolescent" age class (Tomb 21). Dental enamel hypoplasia was present in 23 individuals with permanent teeth. LEH was present in almost all the oldest sub-adults, except for an individual in the children I cohort, while it was less frequent in the infant's subset (5/11), due to the still forming crowns and the shorter life time.

### 3.3. Sex vs age-at-death vs stress indicators

The distribution of age-at-death does not differ between males and females (Mann-Whitney U = 116, P = 0.83; Figure 2c).

Cramer's V test (Table 4) shows that both sex and age classes exhibit differential association with the examined stress-indicators, although just few of them are based on enough observations to infer an actual relationship, based on the current dataset. For example, sex is strongly associated with endocranial lesions (V = 0.56) but less with *cribra cranii* (V = 0.37) (see Table 4) with males showing higher rate of these pathological markers. The smallest association (V = 0.1) was recorded between sex and postcranial porosity. On the other hand, age is associated with endocranial lesions (V = 0.61), *cribra orbitalia* (V = 0.47) and postcranial porosity (V = 0.39). LEH is weakly associated with sex (Cramer's V = 0.17).

## 4. Discussion

Several methods have been developed in the last decade in the attempt to obtain reliable assessments of sex in juveniles (Cardoso, 2008; Monge Calleja et al., 2020; Stull et al., 2017; Vlak et al., 2008). The protocol proposed here permitted to unveil the information about sex, leading to a more complete picture of the paleodemography and paleopathology of the younger cohort of the community that lived (and died) in Valdaro (Northern Italy), during the $7^{th}$-$8^{th}$ centuries A.D. By analyzing enamel peptide of infants, children and juveniles buried in the necropolis of Valdaro, we showed that thirteen out of thirty individuals retain AMELY peptides within their enamel tissue, indicating a male sex. Additional peptides were also searched (as in Lugli et al., 2019) to strengthen the sex classification and confirm the presence of AMELY by ion chromatogram query. Moreover, for the first time, to the best of our knowledge, we showed that even tooth germs may possibly provide reliable information about the sex of infants through the analysis of amelogenin. The analysis showed one case of concordance between the archeological gender (the individual T43 was buried with a pair of bronze earrings) and amelogenin-based diagnosis identifying the child as a female; while the other burials have not yielded any gender-specific grave good. We acknowledge that the lower number of males compared to females here observed may relate to a lack of AMELY sequence coverage, and consequently to a misidentification of some female individuals (false positive) (Parker et al., 2019). We observe no remarkable differences in the two extraction protocols (Lugli et al., 2019; Stewart et al., 2017), both able to yield amelogenin peptides. Nevertheless, collagen and/or dentine sialophosphoprotein were identified in all the four acid-etched samples, possibly due to the contact of the tooth root with the acid. Even if this latter protocol is faster and avoid the more destructive drilling-approach, recent work indicates that the presence of collagen and other non-enamel proteins may hamper sex readability (Froment et al., 2020). This, in turn, likely suggests that for e.g. old (fossil) teeth, where protein preservation might be partially compromised due to diagenesis, an extraction through acid-etch of the external enamel surface could potentially impair chromatogram readability. Thus, sampling a small enamel chunk would allow a thorough manual and chemical cleaning of the specimen, in order to remove potential exogenous contaminants and dentine residues. For more recent samples, however, both methods yield easily interpretable results, but with the acid-etch protocol requiring less processing time and avoiding the cutting of the specimen.

By itself, each sample for amelogenin sex estimation would require ca. 2 hour of work, including pre-treatment and protein extraction through HCl (~80 min through enamel chunks sampling or ~50-60 min through acid etch of the external enamel surface), protein purification (~30 min) and ion chromatograms interpretation (less than 10 minutes), but excluding LC-MS/MS time (ca. 60 – 90 min per sample by autosampler plus daily tuning of the instrument). However, considering that samples are processed in batches (~25-30 samples per batch), a proper estimation of time efficiency is on average 30-40 minutes/sample. In terms of laboratory expenses, each sample roughly costs around 10 € (2 € of $C_{18}$ tip, 1 € of reagents, 5-10 € of LC-MS/MS). Hence, proteomic can be considered a suitable method for sex estimation due to the high efficiency both in terms of work-time and costs, in particular for those contexts that count a large number of individuals (e.g. necropolis).

The age-at-death profile of our sample shows very few individuals in the youngest class, e.g. two neonates. Ancient communities, as Valdaro, are expected to show high mortality rates at birth and within the first year of life (Coale et al., 2013; Weiss and Wobst, 1973); actually, in Italy, few archeological samples reflect this mortality pattern (see e.g. (Gnes et al., 2018; Sperduti et al., 2018a; Vassallo, 2015)), while most of them are affected by strong biases (Baldoni et al., 2016; Catalano et al., 2001; Goodson et al., 2016; Manzi et al., 1995; Paine et al., 2009).

The underrepresentation of Valdaro neonates may be partially linked to post-depositional diagenetic processes, as claimed by Guy (Guy et al., 1997) for other archeological contexts; an alternative explanation relies on specific funerary choices, excluding the very young individual from the burial ground of the community. In this regard, it should be mentioned the growing archeological evidence of differential burial treatment of newborns, disposed in non-conventional burial places (for Italy see for instance (Amoretti et al., 2018; Sperduti et al., 2018b)). Usually, juvenile mortality is higher during the first year of life (Novak et al., 2017; Sperduti et al., 2018b), while gradually decreasing from infancy onwards. Lewis (Lewis, 2007) highlights the presence of a second peak of non-adult mortality occurring during the weaning phase, when children's diet may not guarantee an adequate intake of fundamental nutrients, ultimately undermining the immune system (Katzenberg et al., 1996; Pearson et al., 2010). While early age-at-death is suggestive of poor maternal health (Newman et al., 2019), post-neonatal mortality is related to exogenous factors (e.g. infectious diseases, undernourishment, parasitism) after the first month of life (Gowland, 2015; Lewis, 2007; Lewis and Gowland, 2007; Ządzińska et al., 2015).

Concerning juvenile mortality rate between sexes, modern demographic studies indicate that males tend to have higher mortality rates compared to females (Barford et al., 2006). However, as pointed out by Tierney & Bird (Tierney and Bird, 2015), there are limited possibilities of comparison with archaeological populations concerning young individuals, due to the aforementioned limits of sex assessment through morphological and morphometrics analyses, DNA costs/problems, and relatively paucity of modern known skeletal collections. Still, the lack of information about sex rate at birth in past human groups limits our interpretation. In our study, we were able to determine the sex of all the individuals, observing 13 males and 17 females. This indicates a slight imbalance, even if not statistically significant, of the mortality rate toward females, even though the neonatal mortality sex ratio is missing. As for the health status of the juvenile community of Valdaro, we observed indicators of non-specific stress in all the individuals, with a higher frequency in the third class (1.1-3 years, i.e. one of the most represented age classes). Porous lesions of the cranial bones may have multifactorial causes (as, for example, vitamin A, C and D deficiencies, infections, parasitism, anemia, neoplastic conditions, and traumas) (Brickley, 2018; Ortner, 2003). Scurvy can cause the formation of porous lesions, as a consequence of the hemorrhage and inflammatory processes (Brickley, 2018; Ortner et al., 1999). Interestingly, one probable case of scurvy is detected in an adolescent female. This individual displayed the porosity of the great wing of the sphenoid bone (i.e., pathognomonic of scurvy, according to (Ortner, 2003; Snoddy et al., 2018)) and the co-presence of other indicators of non-specific stress (namely, *cribra cranii, cribra orbitalia,* and LEH). The possibility of co-occurrence of more

than one condition has to be considered. Scurvy and anemia frequently co-occur, and it is known that anemia-related porous lesions are strongly correlated with the age of the individual, i.e., to the distribution of red and mixed marrow (Brickley, 2018). In our sample, *cribra cranii* showed a relatively high number of observations (Table 3) and a slight association with sex (Table 4), with higher frequencies in male individuals. Still, the high co-presence of CC and CO may be linked to anemia, as the most plausible cause (Stuart-Macadam, 1985). In fact, iron-deficiency anemia, likely caused by dietary variability or dissimilar nutrient absorption and subsequent low levels of iron and/or $B_{12}$ vitamin (Walker et al., 2009) may have a sex-related frequency. Studies on pre-adolescent children and infants indicate that prevalence of anemia and iron deficiency are significantly higher in males than in females (Marino et al., 2011; Woodhead et al., 1991), possibly corroborating the idea that *cribra cranii* may occur with higher frequencies in children males. Similarly, previous work suggests that vitamin $B_{12}$ deficiency is more common in males than in females, with a higher incidence of severe cases in men (Margalit et al., 2018). Similarly, endocranial lesions are more frequent in males (5/11) than in females (0/13), likely indicating an averagely worst health status for male children. These lesions may have different etiology, and are commonly linked to inflammation or hemorrhage of the meninges (Lewis, 2004). Nevertheless, considering the relatively low number of individuals of our study, the low preservation rates of some districts, and the multi-factorial etiology of both *cribra cranii* and endocranial lesions, prevented us from making accurate differential diagnoses. Further work on larger samples is needed to precisely understand the link between sex and non-specific stress indicators/metabolic diseases in children. Nutritional deficiencies and diffuse stress status are also highlighted by high frequency of LEH manifestation, potentially caused by several interlinked factors as: 1) changes in the quality and quantity of the diet (Ash et al., 2016); 2) weaning-linked stress events (Moggi-Cecchi et al., 1994); 3) infections (Ford et al., 2009); 4) multiple environmental stresses (Blakey et al., 1994).

Archaeologically, the fact that only a little sex-bias was observed for the individuals buried in the necropolis of Valdaro may suggest that, during Early Middle Ages in northern Italy, juveniles were likely handled in the same way in terms of funerary rituals, regardless of their sex.

## 5. Conclusions

LC-MS/MS analyses of amelogenin is a rapid and easy way to accurately determine the sex of non-adults. This method has revolutionized the sexing of ancient human (and non-humans) (Cappellini et al., 2019) skeletal remains. The technique, originally proposed by Stewart (Stewart et al., 2017), can be extrapolate to several archaeological and possibly forensic contexts, becoming a newly routine method in bio-anthropology due to its relatively low-costs and high-reliability. Here we showed how proteomics, when combined with canonical osteological and paleopathological analyses, may overcome the lack of information about sex assessment. When

applied to large number of individuals, this method can profoundly impact our knowledge on sex representation of e.g. infants and juveniles in the archaeological record, offering new insights on our view of burial practices and demographic evolution from prehistory to modern era. In terms of paleodemography, this work will open the way to new line of studies, such as (selective) infanticide, parental care, prevalence of metabolic diseases by sex and it will also improve the reliability of age-estimation methods based on sex.

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**Table 1**
Protein identification (significant peptides n > 2) in human teeth from Valdaro.

| Tomb | Tooth element | AMELX | AMELY | AMBN | ENAM | CO1A1 | CO1A2 | DSPP |
|---|---|---|---|---|---|---|---|---|
| 16 | $Rdm_1$ | • | • | • | | | | |
| 20 | $LI^2$ | • | | • | | | | |
| 21 | $LP^3$ | • | • | • | • | | | |
| 22 | $LM^3$ | • | • | • | • | | | |
| 29 | $RI_2$ | • | • | • | | | | |
| 37 | $RI_2$ | • | | • | | • | • | • |
| 38 | $Ldi^1$ | • | | | | | | |
| 42 | $Rdm^1$ | • | • | • | | | | |
| 43 | $Ldm^1$ | • | | | | | | |
| 46 | $Rdm_2$ | • | | • | • | | | |
| 48 | $Rdi^2$ | • | • | • | | | | |
| 52 | $LM_2$ | • | | • | | | | |
| 53 | $Rdm^2$ | • | | • | | | | |
| 54 | $Rdm^1$ | • | • | • | | | | |
| 55 | $Ldc_{\|}$ | • | | • | | | | |
| 59 | $Rdm^1$ | • | • | • | | | | |
| 61 | $RM^1$ | • | | • | | • | • | |
| 64 | $Rdc_{\|}$ | • | | • | | | | |
| 65 | $Rdc_{\|}$ | • | • | • | | • | • | • |
| 70 (1) | $Rdm^1$ | • | | • | | | | |
| 70 (2) | $Ldm^1$ | • | | • | | | | |
| 71 | $Ldc_{\|}$ | • | | • | | • | • | |
| 72 | $Rdm_1$ | • | • | • | | | | |
| 75 | $Ldm^1$ | • | | • | | | | |
| 76 | $Rdc_{\|}$ | • | | | | | | |
| 79 | $Rdc_{\|}$ | • | • | • | | • | • | • |
| 82 | $Rdm^2$ | • | | • | • | | | |
| 85 | $Rdm^1$ | • | • | • | | | | |
| 123 | $RI_1$ | • | | • | • | • | • | |
| 124 | $Ldm^1$ | • | • | • | | | | |

AMELX = amelogenin X isoform 1 (*H. sapiens* Q99217); AMELY = amelogenin Y isoform 2 (*H. sapiens* Q99218 or *Pan troglodytes* Q861X8); AMBN = ameloblastin (*H. sapiens* Q9NP70); ENAM = enamelin (*H. sapiens* Q9NRM1); CO1A1 and CO1A2 = collagen type I α1 and α2 (*H. sapiens* P08123 and P02452); DSPP = dentine sialophosphoprotein (*H. sapiens* Q9NZW4); AMELX and AMELY peptides were identified combining ion chromatograms and the Mascot searches.

Table 2
Age-at- death and amelogenin-sex estimation in Valdaro non-adults.

| Tomb (Individual) | Mean Age-At-Death (Range) years | Age Class | Tooth Sampled | Sex |
|---|---|---|---|---|
| T.38 | 0.375 (±0.25) [4.5 (±3) months] | Neonate/ Infant | *$Ldi^1$ | F |
| T.85 | 0.75 (±0.25) [7.5/10.5 (±3) months] | Neonate/ Infant | *$Rdm^1$ | M |
| T.70 (Id 2) | 1.25 (1–1.5) | Infant | $Ldm^1$ | F |
| T.16 | 1.75 (1.5–2) | Infant | $Rdm_1$ | M |
| T.48 | 1.75 (1.5–2) | Infant | $Rdi^2$ | M |
| T.37 | 2 (1.5–2.5) | Infant | *$RI_2$ | F |
| T.46 | 2 (1.5–2.5) | Infant | $Rdm_2$ | F |
| T.59 | 2 (1.5–2.5) | Infant | $Rdm^1$ | M |
| T.65 | 2 (1.5–2.5) | Infant | Rdc | M |
| T.70 (Id 1) | 2 (1.5–2.5) | Infant | $Rdm^1$ | F |
| T.55 | 2.5 (2–3) | Infant | $Ldc_]$ | F |
| T.79 (Id 1) | 2.5 (2–3) | Infant | $Rdc^]$ | M |
| T.75 (Id 1) | 2.75 (2.5–3) | Infant | $Ldm^1$ | F |
| T.53 | 4 (3.5–4.5) | Child I | $Rdm^2$ | F |
| T.72 | 4 (3.5–4.5) | Child I | $Rdm_1$ | M |
| T.82 | 4 (3.5–4.5) | Child I | $Rdm^2$ | F |
| T.124 | 4 (3.5–4.5) | Child I | $Ldm^1$ | M |
| T.43 | 5 (4.5–5.5) | Child I | $Ldm^1$ | F |
| T.64 | 5 (4.5–5.5) | Child I | $Rdc_]$ | F |
| T.54 | 5.5 (5–6) | Child I | $Rdm^1$ | M |
| T.61 (Id 1) | 5.75 (5.5–6) | Child I | *$RM^1$ | F |
| T.42 (Id 1) | 6 (5.5–6.5) | Child I | $Rdm^1$ | M |
| T.71 (Id 1) | 6 (5.5–6.5) | Child I | $Ldc_]$ | F |
| T.76 | 6.5 (6–7) | Child I | $Rdc_]$ | F |
| T.29 | 9.5 (9–10) | Child II | $RI_2$ | M |
| T.123 | 12 (11.5–12.5) | Adolescent | $RI_1$ | F |
| T.20 (Id 1) | 13 (12.5–13.5) | Adolescent | $LI^2$ | F |
| T.52 | 13 (12.5–13.5) | Adolescent | $LM_2$ | F |
| T.21 (Id 1) | 15.5 (15–16) | Post-Adolescent | $LP^3$ | M |
| T.22 | > 15.5 | Post-Adolescent | $LM^3$ | M |

Samples marked by '*' are low-mineralized tooth germs.

Table 3
Presence of indicators of non-specific stress in Valdaro.

| Stress indicator | N | n | % (n/N) | F | M |
|---|---|---|---|---|---|
| *Cribra cranii* | 28 | 15 | 53.6% | 6/16 | 9/12 |
| *Cribra orbitalia* | 10 | 8 | 80% | 5/5 | 3/5 |
| Endocranial lesions | 24 | 5 | 20.8% | 0/13 | 5/11 |
| Porosity of the hard palate | 20 | 16 | 80% | 10/14 | 6/6 |
| Postcranial porosity | 29 | 15 | 51.7% | 9/16 | 6/13 |
| LEH | 28 | 21 | 75% | 13/16 | 8/12 |

N = considered; n = observed; F = observed cases in females over the total females for the stress marker; M = observed cases in males over the total males for the stress marker.

Table 4
Association between stress indicators and sex/age classes by Cramer's V test.

| Stress indicator | Sex (V) | Age class (V) |
|---|---|---|
| *Cribra cranii* | 0.37 | 0.31 |
| *Cribra orbitalia* | 0.5 | 0.47 |
| Endocranial lesions | 0.56 | 0.61 |
| Porosity palate | 0.33 | 0.29 |
| Postcranial porosity | 0.1 | 0.39 |

V = 0 no association; V = 1 complete association.


**Figure 1.** Photographic record of tombs 43 and 70, showing the earring found in tomb 43. The location of the Valdaro site is also reported in the inset.

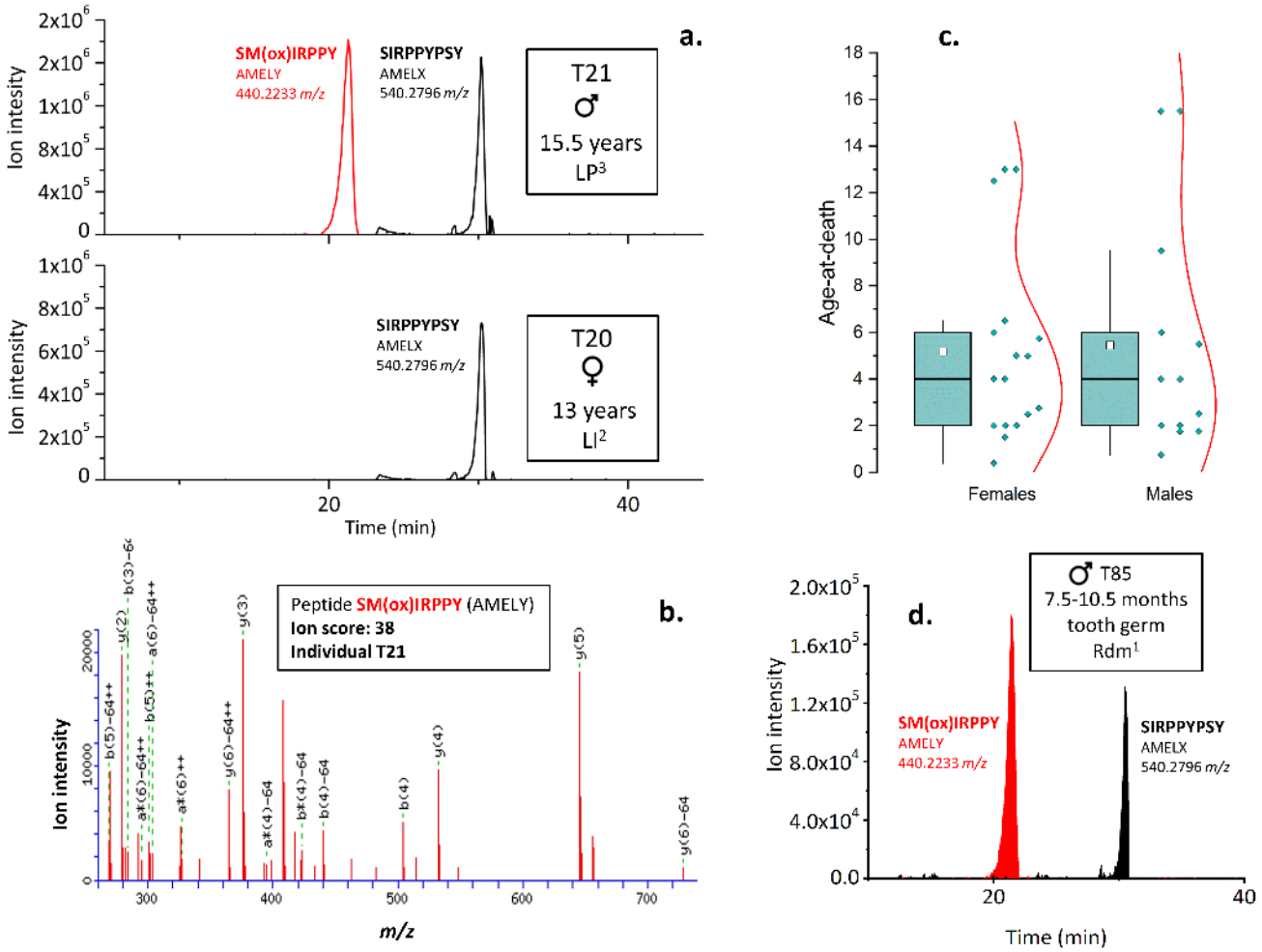


**Figure 2.** (a) Sex estimation by ion chromatograms of two individuals (T21 and T20); peaks correspond to $[M+2H]^{+2}$ 440.2233 and 540.2796 *m/z*. (b) Fragmentation spectrum of peptide SM(ox)IRPPY, with an ion score of 38. (c) Box plots representing age-at-death distribution of female and male sex. (d) Ion chromatogram peaks of individual T85, showing that sex information can be retrieved from low-mineralized tooth germs.