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1 **Sex estimation of Early Medieval non-adult individuals through dental enamel**
2 **peptides**

3 **Sex-related morbidity and mortality profiles in non-adult individuals from the Early**
4 **Medieval Italian sample of Valdaro (7th-8th cent): the contribution of dental enamel**
5 **peptides analysis**

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19

20 **Keywords:** sex estimation; LC-MS/MS; amelogenin; proteomics; tooth enamel; non-adults.

21

22 **Abstract**

23 In this work, osteological and paleopathological analyses are combined with liquid-chromatography mass
24 spectrometry to study life and death of 30 non-adult individuals from an Early Medieval Italian funerary context
25 (Valdaro, 7th-8th cent AD). We estimated individual sex by exploiting sexual differences in enamel-bounded
26 peptides. Enamel proteins were extracted through an acid etching of the whole tooth crowns for 4 samples and
27 through a partial digestion of small enamel chunks for the remaining 26 samples. Both protocols were informative
28 on the sex of the individuals through the identification of amelogenin isoforms (AMELX and AMELY). In
29 addition, two low-mineralized tooth germs were analysed and they provided reliable information on the infants'
30 sex. We observed the presence of 13 males and 17 females among the non-adults of Valdaro, not significantly
31 different from a random sample with an equal frequency of males and females. *Cribra cranii* and endocranial
32 lesion occurrence showed an association with sex, with higher frequencies in male individuals.

33

34 1. Introduction

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

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

48 In a broader perspective, survival and living conditions of infants and children offer an assessment of fitness in
49 past populations and of their responsiveness to environmental change (Goodman and Armelagos, 1989; Lewis and
50 Gowland, 2007). Moreover, as highlighted by numerous modern epidemiological studies, living conditions in the
51 course of intrauterine life and during growth influence frailty and mortality during adulthood (Almond and Currie,
52 2011; Barker, 2004, 1990). Recently, the study of non-adults relied on advanced techniques for the analysis of
53 physiological stress indicators and dietary profiles combined with increasingly precise methods for age at death
54 assessment (Nava et al., 2017). On the other hand, these inferences lacked information concerning sex of infants

55 and children. The latter variable can shed light on issues such as possible gender inequality in childcare and feeding
56 practices in past populations. While some insights may originate from retrospective studies on adults (Miller et
57 al., 2019), the younger non-survivor individuals (DeWitte and Stojanowski, 2015) may yield a more
58 comprehensive picture of the actual effects of such inequalities.

59 Along with age-at-death, the other pillar of biological profiling is sex assessment (Acsádi et al., 1970;
60 Schutkowski, 1993). The robust assessment of sex in human skeletal remains is an essential part of any skeletal
61 assemblage study, both in archaeology and forensic anthropology. Nonetheless, it is well-known that sexual
62 dimorphism is not present – or not enough marked – in the human skeleton until the development of secondary
63 sexual characters after puberty (Cardoso, 2008; Loth and Henneberg, 2001). This prevents anthropologists from
64 accurately determining sex of immature skeletal remains (Loth and Henneberg, 2001). However, there is strong
65 evidence suggesting that sex in humans is already established at the moment of conception (Loth and Henneberg,
66 2001; Stull et al., 2017) and, especially during the first year of life, the production of sex hormones is relatively
67 high (Burger et al., 1991; Reinisch et al., 1991). Differences have been found also in body proportions and in
68 biomechanical adaptations during growth (Stull et al., 2017), with males showing e.g. larger diaphyseal breadth
69 than females, and longer bone length after adolescence (Humphrey, 1998). Also, the timing and duration of growth
70 and maturation are different not only between males and females, but also between individuals (Cunningham et
71 al., 2016), making the study of sexual dimorphism even more challenging. Hence, the inaccuracy or the absence
72 of sex information can bias the results of investigation on skeletal growth, health, cultural behaviors, and, more in
73 general, the understanding of the health and well-being of a past population (Mays, 2013). Considering the
74 importance of sex information for the construction of the biological profile and in paleodemographic studies,
75 numerous attempts to design reliable methods were made, with different degrees of success. Several studies
76 showed high accuracy associated with non-adult sex assessment, but the same methods did not achieve robust
77 results in other populations, due to inter-population differences (Stull et al., 2017). Various methods were
78 developed for different parts of the skeleton (Vlak et al., 2008), with a preference for the districts that are less
79 prone to be damaged by taphonomic events, and/or districts with a considerable dimorphism in adults (i.e.,
80 mandible, pelvis and teeth) (Cardoso, 2008; Monge Calleja et al., 2020). Geometric morphometrics methods
81 (GMM) were also employed with varying degree of success, being possibly less dependent on the observer's
82 experience and thus more unbiased (Miller et al., 2019; Wilson et al., 2008, 2017).

83 Despite the considerable amount of research directed towards these questions, sex assessment methods are still
84 highly imprecise and biased, so that crucial details on past childish demography are lost. To date, only molecular
85 investigations, namely ancient DNA, may help to untangle sexing of juvenile individuals with a high degree of
86 confidence. The high costs and limitations of the method (e.g. DNA degradation), however, make them unsuitable
87 for a large number of samples (Tierney and Bird, 2015). **Recent advancements in liquid-chromatography mass
88 spectrometry (LC-MS/MS) allow for effectively estimating individual sex from tooth enamel** (Froment et al.,

89 2020; Lugli et al., 2019; Parker et al., 2019; Stewart et al., 2017, 2016; Wasinger et al., 2019). **In fact**, even if
90 almost entirely mineralized (95-99% wt.), mature enamel averagely contains between 1% and 3% (wt.) organic
91 material, mostly proteinaceous (Castiblanco et al., 2015). Such enamel proteome consists in amelogenins
92 (AMELX and AMELY), enamelin (ENAM), ameloblastin (AMBN), amelotin (AMTN), tuftelin (TUFT1), matrix
93 metalloproteinase 20 (MMP20) and kallikrein 4 (KLK4) (Bansal et al., 2012; Cappellini et al., 2019). These
94 proteins and proteinases are mostly synthesized and secreted by ameloblasts during enamel deposition. Then,
95 throughout enamel secretion and maturation phases, structural proteins are hydrolyzed by proteases (Castiblanco
96 et al., 2015). As enamel maturation proceeds, the organic matrix is in fact resorbed and substituted by minerals.

97 **In particular**, amelogenins have recently demonstrated their pivotal role in sex estimation and taxonomical
98 classification of humans and animals by LC-MS/MS (Cappellini et al., 2019; Lugli et al., 2019; Parker et al., 2019;
99 Stewart et al., 2017; Welker et al., 2019). AMELY is expressed from the Y-chromosome and thus strictly linked
100 to male sex. Previous works demonstrated how AMELY can be easily identified and discriminated from AMELX
101 within high-resolution ion chromatograms of LC-MS/MS analyses, by checking the presence of peptide
102 SM_(ox)IRPPY (monoisotopic $[M+2H]^{+2} = 440.2233$ m/z) and possibly other AMELY-related peptides (Lugli et al.,
103 2019; Parker et al., 2019). Subsequent MS² database searches (e.g. UniProt and NCBI) can be employed to further
104 confirm the sex classification.

105 The high-efficiency of such analytical protocol originates from four main factors: 1) at the end of the maturation
106 process, enamel proteins are physiologically digested by proteases into peptides, obviating laboratory enzymatic
107 digestion (Stewart et al., 2016); 2) amelogenins are relatively abundant within the enamel proteome (Bansal et al.,
108 2012); 3) teeth are generally well represented in the archaeological record (Ogden, 2007); 4) enamel and mineral-
109 bounded peptides showed a high resistance to post-depositional diagenetic alterations (Cappellini et al., 2019;
110 Demarchi et al., 2016; Lugli et al., 2019; Welker et al., 2019).

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

117

118 **2. Materials and Methods**

119 The skeletal record of the Early Medieval cemetery of Valdaro includes 40 skeletons of sub-adults. Overall, the
120 state of preservation and the representation of the anatomical districts were poor. In most cases, the cortical bone
121 was not well preserved, and the skeletal elements were often fragmented or even absent. A subset of the best
122 preserved sub-adult individuals (n = 30) was studied at the Laboratory of Osteoarchaeology and
123 Palaeoanthropology – BONES Lab, of the Department of Cultural Heritage, University of Bologna (Ravenna
124 Campus, Italy).

125

126 **2.1. The archaeological context**

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



139

140 **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
141 in the inset.

142

143 **2.2. Protein extraction and LC-MS/MS**

144 The enamel of 26 specimens (of which two were tooth germs) was manually pre-cleaned and sampled (chunks of
145 ~5 mg) using a drill (Lugli et al., 2019). Four additional specimens were treated following the protocol of Stewart
146 (Stewart et al., 2017), employing an acid etch cleaning and sampling. Thus, a total of thirty teeth pertaining to the
147 same number of individuals were investigated through LC-MS/MS (Table 1).

148 Enamel specimens were demineralized for 45 min - 1 h with 200 μ L of 5% HCl at room temperature. A first batch
149 of acid was discarded before the actual extraction. The supernatant containing both minerals and peptides was
150 transferred in a new Eppendorf tube. Samples were thus desalted and purified by HyperSep SpinTips (Thermo
151 Scientific) with C₁₈ functionalized silica. Resin-bounded peptides were eluted using 20 μ L of 60% acetonitrile in
152 0.1% formic acid. Samples were finally dried down at room temperature under a laminar flow hood (class 100).
153 All the previously described protocols were carried out at the BONES Lab. In total, this extraction protocol
154 required ~10 hours of work of one person.

155 Dry extracted peptides were resuspended in 35 μ L of a mixture of water:acetonitrile:formic acid 95:3:2, before the
156 LC-MS/MS measures. Analyses were conducted using a Dionex Ultimate 3000 UHPLC coupled to a high-
157 resolution Q Exactive mass spectrometer (Thermo Scientific, Bremen, Germany). A total run time between 60 and
158 90 minutes was employed for each sample and blank. Centroided MS and MS² spectra were recorded from 200 to

159 2000 m/z in Full MS/dd-MS² (TOP2) mode, at a resolution of 35000 and 17500 respectively. The two most intense
160 multi-charged ions (TOP2) were selected for MS² nitrogen-promoted collision-induced dissociation. An inclusion
161 list comprising six entries with the m/z and possible charge states of the peptides of interest was included in the
162 method ($[M+2H]^{+2}$ 523.7748; 440.2233; 540.2796; 525.2975; 575.7533; 656.3528 m/z). More details on the
163 analytical protocol are reported in Lugli (Lugli et al., 2019). Raw MS data were deposited in Zenodo
164 (10.5281/zenodo.3774090), including also two blanks as example.

165

166 **2.3. Peak identification and database searches**

167 Ion chromatograms were searched using Xcalibur (Thermo Scientific) with a mass tolerance of 5 ppm. We
168 specifically focused on peptides SM_(ox)IRPPY (AMELY; $[M+2H]^{+2}$ 440.2233 m/z) and SIRPPYPSY (AMELX;
169 $[M+2H]^{+2}$ 540.2796 m/z), previously demonstrated as strong sex biomarkers (Lugli et al., 2019; Stewart et al.,
170 2017) (Figure 2). Additional peptides were also searched to corroborate the presence (or the absence) of AMELY
171 (Lugli et al., 2019). To possibly identify other tooth proteins and unique AMEL peptides, raw data were converted
172 into Mascot generic format (MsConvert v. 3.0.10730, ProteoWizard tools) and simultaneously searched against:
173 1) Swiss-Prot (constrained to *Homo sapiens*); 2) an in-house database downloaded from UniProt & NCBI,
174 including all available mammal amelogenin sequences; 3) cRAP (116 sequences) for contaminants. No proteolytic
175 enzyme was selected in search parameters. Deamidated asparagines/glutamine (NQ) and oxidated methionine (M)
176 were set as variable modifications. Mass tolerances were set at 10 ppm for the precursor ions and 0.05 Da for the
177 fragmented ions. False discovery rate was estimated through an automatic decoy database, with a probability
178 threshold trimmed to an FDR <1%. A specific protein was considered as identified if at least two significant
179 peptides were observed.

180

181 **2.4. Skeletal age assessment and health conditions**

182 The age-at-death of the individuals was assessed using different methods, depending on the developmental stage
183 and the state of preservation. Namely, the dental development and eruption stages (AlQahtani et al., 2010) have
184 been considered as the main indicators. Moreover, the appearance and fusion of the ossification centers and the
185 anthropometric measurements have been used (Black and Scheuer, 1996; Cunningham et al., 2016; Fazekas Gy.
186 and Kosa, 1978; Maresh, 1970). We divided the sample into six age classes, according to Scheuer and Black
187 (Scheuer and Black, 2000) after Knussmann (Knussmann, 1988), and then adapted to our sample as follows:
188 Neonates/Infants 0-1 year; Infants I 1.1-3; Children I 3.1-6; Children II 6.1-10; Adolescent 10.1-15; Post-adolescent
189 >15) (Table 2). All skeletons were macroscopically examined for the presence of non-specific markers of

190 physiological stress (see [Table 3](#), namely *cribra cranii*; *cribra orbitalia*; endocranial lesions; porosity of the hard
191 palate; postcranial porosity) (Ortner, 2003) and **traumas** (Lewis, 2017; Ortner, 2003). We followed the criteria
192 proposed by Ortner and Ericksen (Ortner and Ericksen, 1997) for the assessment of abnormal porosities. For each
193 available anatomical district, these indicators of non-specific stress were recorded as absent, present, or non-
194 observable. We also assessed the presence or absence of Linear Enamel Hypoplasia (LEH) in the permanent
195 dentition.

196

197 **2.5. Statistical analysis**

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

205

206 **3. Results**

207 **3.1. LC-MS/MS**

208 After the search of ion chromatograms, AMELX peptide SIRPPYPSY was found in all the analyzed samples,
209 while AMELY peptide SM(ox)IRPPY in 42% of individuals (13/30; see [Table 1](#)). The ratio between the peak
210 intensities at 440.2233 and 540.2796 m/z resulted equal to 1.05 ± 0.28 (mean \pm SD). Both the tooth germs here
211 analyzed, i.e. T38 (4.5 months) and T85 (9 months), yielded enamel-related peptides, allowing to disclose the sex
212 of the two infants (female and male, respectively). Mascot searches showed the presence of other tooth proteins
213 within some samples, as AMBN and ENAM. CO1A1 (collagen type I α 1), CO1A2 (collagen type I α 2) and DSPP
214 (dentine sialophosphoprotein) related peptides were also detected, possibly due to residual dentine tissue. We have
215 not found any modern contaminant through the cRAP database.

216

217 **3.2. Association between stress indicators and sex/age classes**

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

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

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	LI ¹	•						
42	Rdm ¹	•	•	•				
43	Ldm ¹	•						
46	Rdm ₂	•		•	•			
48	Rd ²	•	•	•				
52	LM ₂	•		•				
53	Rdm ²	•		•				
54	Rdm ¹	•	•	•				
55	Ldc _s	•		•				
59	Rdm ¹	•	•	•				
61	RM ²	•		•		•	•	
64	Rdc _s	•		•				
65	Rdc _s	•	•	•		•	•	•
70 (1)	Rdm ¹	•		•				
70 (2)	Ldm ¹	•		•				
71	Ldc _s	•		•		•	•	
72	Rdm ₁	•	•	•				
75	Ldm ¹	•		•				
76	Rdc _s	•						
79	Rdc _s	•	•	•		•	•	•
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 1 α 1 and α 2 (*H. sapiens* P08123 and P02452); DSPP = dentine sialophosphoprotein (*H. sapiens* Q9NZW4); AMELX and AMELY peptides were identified combining ion chromatograms and the Mascot searches.

231

232

233 **Table 2.** Age-at- death and amelogenin-sex estimation in Valdarò non-adults.

Tomb (Individual)	Mean Age-At-Death (Range) years	Age Class	Tooth Sampled	Sex
T.38	4.5 (\pm 3) months	Neonate/Infant	Rdm ¹	F
T.85	7.5/10.5 (\pm 3) months	Neonats/Infant	Rdc*	M
T.70 (Individual 2)	1.5 (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	LM ₂	M
T.37	2 (1.5-2.5)	Infant	LI ¹	F
T.46	2 (1.5-2.5)	Infant	Rdi ²	F
T.59	2 (1.5-2.5)	Infant	Rdc*	M
T.65	2 (1.5-2.5)	Infant	Ldc*	M
T.70 (Individual 1)	2 (1.5-2.5)	Infant	Rdm ₁	F
T.55	2.5 (2-3)	Infant	Rdm ¹	F
T.79 (Individual 1)	2.5 (2-3)	Infant	Ldm ¹	M
T.75 (Individual 1)	2.75 (2.5-3)	Infant	Rdm ¹	F
T.53	4 (3.5-4.5)	Child I	Rdm ¹	F
T.72	4 (3.5-4.5)	Child I	Rdm ²	M
T.82	4 (3.5-4.5)	Child I	Rdm ²	F
T.124	4 (3.5-4.5)	Child I	Rdc*	M
T.43	5 (4.5-5.5)	Child I	Rdm ₂	F
T.64	5 (4.5-5.5)	Child I	Ldm ¹	F
T.54	5.5 (4.5-5.5)	Child I	Ldc*	M
T.61 (Individual 1)	5.75 (5.5-6)	Child I	Rdm ¹	F
T.42 (Individual 1)	6 (5.5-6.5)	Child I	Ldm ¹	M
T.71 (Individual 1)	6 (5.5-6.5)	Child I	Rdc*	F
T.76	6.5 (6-7)	Child I	RI ₁	F
T.29	9.5 (9-10)	Child II	RI ₂	M
T.123	12.5 (11.5-12.5)	Adolescent	RI ₂	F
T.20 (Individual 1)	13 (12.5-13.5)	Adolescent	LI ²	F
T.52	13 (12.5-13.5)	Adolescent	Rdm ²	F
T.21 (Individual 1)	15.5 (15-16)	Post-Adolescent	LP ³	M
T.22	> 15.5	Post-Adolescent	LM ³	M

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

Stress indicator	N	n	% (n/N)	F	M
<i>Cribrā cranii</i>	28	15	53.6%	6/16	9/12
<i>Cribrā orbitalia</i>	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	23	82.1%	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.

235

236 3.3. Sex vs age-at-death vs stress indicators

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

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

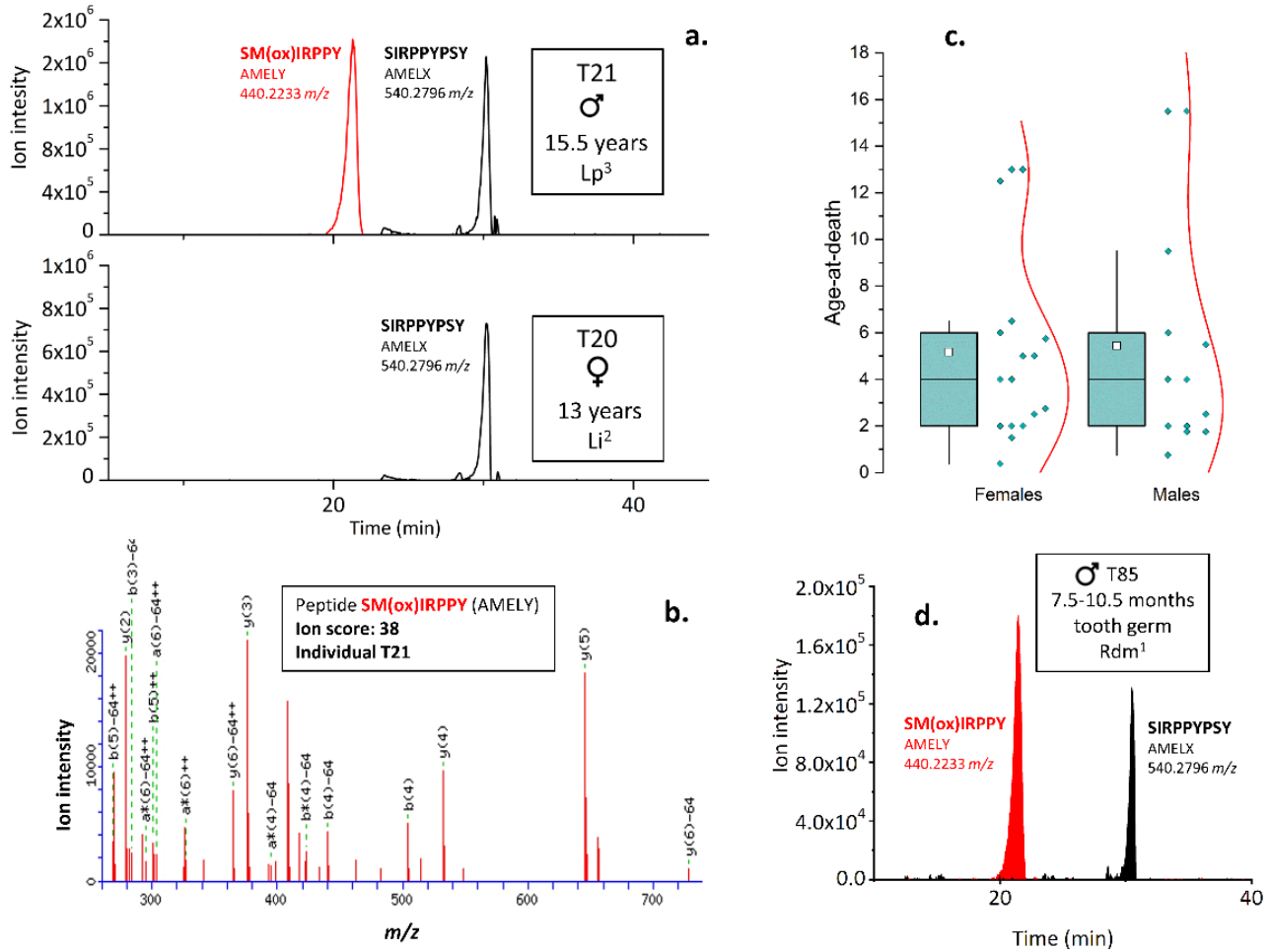
246

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

Stress indicator	Sex (V)	Age class (V)
<i>Cribr</i> <i>a cranii</i>	0.37	0.31
<i>Cribr</i> <i>a orbitalia</i>	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.

247



248

249 **Figure 2.** (a) Sex estimation by ion chromatograms of two individuals (T21 and T20); peaks correspond to $[M+2H]^{+2}$ 440.2233 and
 250 540.2796 m/z . (b) Fragmentation spectrum of peptide SM(ox)IRPPY, with an ion score of 38. (c) Box plots representing age-at-death
 251 distribution of female and male sex. (d) Ion chromatogram peaks of individual T85, showing that sex information can be retrieved from
 252 low-mineralized tooth germs.

253

254 **4. Discussion**

255 Several methods have been developed in the last decade in the attempt to obtain reliable assessments of sex in
 256 juveniles (Cardoso, 2008; Monge Calleja et al., 2020; Stull et al., 2017; Vlak et al., 2008). The protocol proposed
 257 here permitted to unveil the information about sex, leading to a more complete picture of the paleodemography
 258 and paleopathology of the younger cohort of the community that lived (and died) in Valdaro (Northern Italy),
 259 during the 7th-8th centuries A.D. By analyzing enamel peptide of infants, children and juveniles buried in the
 260 necropolis of Valdaro, we showed that thirteen out of thirty individuals retain AMELY peptides within their
 261 enamel tissue, indicating a male sex. Additional peptides were also searched (as in Lugli et al., 2019) to strengthen

262 the sex classification and confirm the presence of AMELY by ion chromatogram query. Moreover, for the first
263 time, to the best of our knowledge, we showed that even tooth germs may possibly provide reliable information
264 about the sex of infants through the analysis of amelogenin. The analysis showed one case of concordance between
265 the archeological gender (the individual T43 was buried with a pair of bronze earrings) and amelogenin-based
266 diagnosis identifying the child as a female; while the other burials have not yielded any gender-specific grave
267 good. We acknowledge that the lower number of males compared to females here observed may relate to a lack
268 of AMELY sequence coverage, and consequently to a misidentification of some female individuals (false positive)
269 (Parker et al., 2019). We observe no remarkable differences in the two extraction protocols (Lugli et al., 2019;
270 Stewart et al., 2017), both able to yield amelogenin peptides. Nevertheless, collagen and/or dentine
271 sialophosphoprotein were identified in all the four acid-etched samples, possibly due to the contact of the tooth
272 root with the acid. Even if this latter protocol is faster and avoid the more destructive drilling-approach, recent
273 work indicates that the presence of collagen and other non-enamel proteins may hamper sex readability (Froment
274 et al., 2020). This, in turn, likely suggests that for e.g. older (fossil) teeth, where protein preservation might be
275 partially compromised due to diagenesis, an extraction through acid-etch of the external enamel surface could
276 potentially impair chromatogram quality. For more recent samples, however, both methods yield easily
277 interpretable results, but with the ‘Stewart’ method requiring less processing time and avoiding the cutting of the
278 specimen.

279 By itself, each sample for amelogenin sex estimation would require ca. 2 hour of work, including pre-treatment
280 and protein extraction through HCl (~80 min through enamel chunks sampling or ~50-60 min through acid etch
281 of the external enamel surface), protein purification (~30 min) and ion chromatograms interpretation (less than 10
282 minutes), but excluding LC-MS/MS time (ca. 60 – 90 min per sample by autosampler plus daily tuning of the
283 instrument). However, considering that samples are processed in batches (~25-30 samples per batch), a proper
284 estimation of time efficiency is on average 30-40 minutes/sample. In terms of laboratory expenses, each sample
285 roughly costs around 10 € (2 € of C₁₈ tip, 1 € of reagents, 5-10 € of LC-MS/MS). Hence, proteomic can be
286 considered a suitable method for sex estimation due to the high efficiency both in terms of work-time and costs,
287 in particular for those contexts that count a large number of individuals (e.g. necropolis).

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

293 The underrepresentation of Valdaro neonates may be partially linked to post-depositional diagenetic processes, as
294 claimed by Guy (Guy et al., 1997) for other archeological contexts; an alternative explanation relies on specific

295 funerary choices, excluding the very young individual from the burial ground of the community. In this regard, it
296 should be mentioned the growing archeological evidence of differential burial treatment of newborns, disposed in
297 non-conventional burial places (for Italy see for instance (Amoretti et al., 2018; Sperduti et al., 2018b)). Usually,
298 juvenile mortality is higher during the first year of life (Novak et al., 2017; Sperduti et al., 2018b), while gradually
299 decreasing from infancy onwards. Lewis (Lewis, 2007) highlights the presence of a second peak of non-adult
300 mortality occurring during the weaning phase, when children's diet may not guarantee an adequate intake of
301 fundamental nutrients, ultimately undermining the immune system (Katzenberg et al., 1996; Pearson et al., 2010).
302 While early age-at-death is suggestive of poor maternal health (Newman et al., 2019), post-neonatal mortality is
303 related to exogenous factors (e.g. infectious diseases, undernourishment, parasitism) after the first month of life
304 (Gowland, 2015; Lewis, 2007; Lewis and Gowland, 2007; Ządzińska et al., 2015).

305 Concerning juvenile mortality rate between sexes, modern demographic studies indicate that males tend to have
306 higher mortality rates compared to females (Barford et al., 2006). However, as pointed out by Tierney & Bird
307 (Tierney and Bird, 2015), there are limited possibilities of comparison with archaeological populations concerning
308 young individuals, due to the aforementioned limits of sex assessment through morphological and morphometrics
309 analyses, DNA costs/problems, and relatively paucity of modern known skeletal collections. Still, the lack of
310 information about sex rate at birth in past human groups limits our interpretation. In our study, we were able to
311 determine the sex of all the individuals, observing 13 males and 17 females. This indicates a slight imbalance,
312 **even if not statistically significant**, of the mortality rate toward females, even though the neonatal mortality sex
313 ratio is missing. As for the health status of the juvenile community of Valdaro, we observed indicators of non-
314 specific stress in all the individuals, with a higher frequency in the third class (1.1-3 years, i.e. one of the most
315 represented age classes). Porous lesions of the cranial bones may have multifactorial causes (as, for example,
316 vitamin A, C and D deficiencies, infections, parasitism, anemia, neoplastic conditions, and traumas) (Brickley,
317 2018; Ortner, 2003). Scurvy can cause the formation of porous lesions, as a consequence of the hemorrhage and
318 inflammatory processes (Brickley, 2018; Ortner et al., 1999). Interestingly, one probable case of scurvy is detected
319 in an adolescent female. This individual displayed the porosity of the great wing of the sphenoid bone (i.e.,
320 pathognomonic of scurvy, according to (Ortner, 2003; Snoddy et al., 2018)) and the co-presence of other indicators
321 of non-specific stress (namely, *cribra cranii*, *cribra orbitalia*, and LEH). The possibility of co-occurrence of more
322 than one condition has to be considered. Scurvy and anemia frequently co-occur, and it is known that anemia-
323 related porous lesions are strongly correlated with the age of the individual, i.e., to the distribution of red and
324 mixed marrow (Brickley, 2018). In our sample, *cribra cranii* showed a relatively high number of observations
325 (Table 3) and a slight association with sex (Table 4), with higher frequencies in male individuals. Still, the high
326 co-presence of CC and CO may be linked to anemia, as the most plausible cause (Stuart-Macadam, 1985). In fact,
327 iron-deficiency anemia, likely caused by dietary variability or dissimilar nutrient absorption and subsequent low
328 levels of iron and/or B₁₂ vitamin (Walker et al., 2009) may have a sex-related frequency. Studies on pre-adolescent

329 children and infants indicate that prevalence of anemia and iron deficiency are significantly higher in males than
330 in females (Marino et al., 2011; Woodhead et al., 1991), possibly corroborating the idea that *cribra cranii* may
331 occur with higher frequencies in children males. Similarly, previous work suggests that vitamin B₁₂ deficiency is
332 more common in males than in females, with a higher incidence of severe cases in men (Margalit et al., 2018).
333 Similarly, endocranial lesions are more frequent in males (5/11) than in females (0/13), likely indicating an
334 averagely worst health status for male children. These lesions may have different etiology, and are commonly
335 linked to inflammation or hemorrhage of the meninges (Lewis, 2004). Nevertheless, considering the relatively low
336 number of individuals of our study, the low preservation rates of some districts, and the multi-factorial etiology of
337 both *cribra cranii* and endocranial lesions, prevented us from making accurate differential diagnoses. Further work
338 on larger samples is needed to precisely understand the link between sex and non-specific stress
339 indicators/metabolic diseases in children. Nutritional deficiencies and diffuse stress status are also highlighted by
340 high frequency of LEH manifestation, potentially caused by several interlinked factors as: 1) changes in the quality
341 and quantity of the diet (Ash et al., 2016); 2) weaning-linked stress events (Moggi-Cecchi et al., 1994); 3)
342 infections (Ford et al., 2009); 4) multiple environmental stresses (Blakey et al., 1994).

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

346

347 **5. Conclusions**

348 LC-MS/MS analyses of amelogenin is a rapid and easy way to accurately determine the sex of non-adults. This
349 method has revolutionized the sexing of ancient human (and non-humans) (Cappellini et al., 2019) skeletal
350 remains. The technique, originally proposed by Stewart (Stewart et al., 2017), can be extrapolate to several
351 archaeological and possibly forensic contexts, becoming a newly routine method in bio-anthropology due to its
352 relatively low-costs and high-reliability. Here we showed how proteomics, when combined with canonical
353 osteological and paleopathological analyses, may overcome the lack of information about sex assessment. When
354 applied to large number of individuals, this method can profoundly impact our knowledge on sex representation
355 of e.g. infants and juveniles in the archaeological record, offering new insights on our view of burial practices and
356 demographic evolution from prehistory to modern era. In terms of paleodemography, this work will open the way
357 to new line of studies, such as (selective) infanticide, parental care, prevalence of metabolic diseases by sex and it
358 will also improve the reliability of age-estimation methods based on sex.

359

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