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A multifaceted approach towards investigating childbirth deaths in double burials: Anthropology, paleopathology and ancient DNA

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Elisabetta Cilli, G.G. (2020). A multifaceted approach towards investigating childbirth deaths in double burials: Anthropology, paleopathology and ancient DNA. JOURNAL OF ARCHAEOLOGICAL SCIENCE, 122, 1-9 [10.1016/j.jas.2020.105219].

Availability:

This version is available at: <https://hdl.handle.net/11585/769609> since: 2024-07-12

Published:

DOI: <http://doi.org/10.1016/j.jas.2020.105219>

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1 **A multifaceted approach towards investigating childbirth deaths in double burials:**
2 **Anthropology, paleopathology and ancient DNA**

3
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23
24 **Keywords**

25 ancient DNA, anthropology, maternal kinship, mitochondrial DNA, childbirth deaths, double
26 burials, Early Modern

27
28 **Running title:** Investigating childbirth deaths in double burials

29
30 **Abstract**

31 Evidence of maternal care and childbirth events in the past are rare in the archaeological record and
32 are difficult to recognize. To combat this, we analyzed thirteen double burials potentially related to
33 childbirth death events, thereby containing an adult and a perinate. The specimens were excavated
34 from the archaeological area identified as "Forlì Campus" (Forlì, Italy), that dated to 17th-18th

35 centuries AD and was adjacent to a hospital in use at that time. This period witnessed the
36 development of medical techniques and novel approaches in obstetrics in Europe, with the
37 introduction of lying-in hospitals and maternity wards. We here tested if the double burials were
38 ascribable to childbirth death events and thus represent the first reported cases of the hospitalization
39 of childbirth in the history of medicine. A multidisciplinary analysis was undertaken to achieve this
40 aim, combining anthropology, archaeology, paleopathology and archaeogenetics.

41 In five burials the adult individual was recognized as a female in fertile age and the non-adult
42 individual was assigned as perinate. Mitochondrial DNA analysis highlighted different haplotypes
43 among the individuals of these burials, and these results, combined with the archaeological and
44 anthropological data do not support a possible maternal relationship between them.

45 This study is novel in testing the hypotheses of childbirth deaths, through a reliable approach in the
46 interpretation of these archaeological contexts. The analysis of ancient DNA in this particular
47 application proves a useful strategy to support and complete the interpretation of archaeological and
48 anthropological data, showing that a general assumption of mother/child relations within such
49 burials can be misleading.

50

51 **1. Introduction**

52 Osteological remains offer an important insight to the past, able to develop our understanding of the
53 biological and cultural changes that affected and influenced human populations and of course their
54 dynamics. As is the case today, pregnancy and childbirth represented important events in women's
55 lives in the past, controlling population dynamics and driving changes in culture and society.

56 Global efforts to improve maternal health and thus reduce maternal mortality were established by
57 world leaders in the year 2000, in Action 5 of the Millennium Development Goals (Alkema et al.,
58 2016). Today in developing countries the World Health Organization estimates an average of 239
59 deaths per 100,000, compared to only 12 in developed countries (WHO, 2018). However, despite
60 the assumption that maternal and infant mortality was much higher in the past, evidence of this is
61 lacking in the archaeological literature (e.g. Appleby et al., 2014; Cruz and Codinha, 2010; Dulias
62 et al., 2019; Lieverse et al., 2015; Malgosa et al., 2004; Pasini et al., 2018; Rebay-Salisbury, 2018;
63 Willis and Oxenham, 2013; Zhou et al., 2019).

64 Attempts to identify maternal events in past populations have mainly focused on burials of females
65 which appear to have died during pregnancy, childbirth, or soon after. Such evidence is rare, due to
66 the fragility and damage of fetal and perinatal bones that are often missed or misidentified during
67 excavations. In addition, cause of death surrounding childbirth is often unclear because this event
68 can be related to numerous maternal health issues or be archaeologically invisible (Buschmann et

69 al., 2013; Willis and Oxenham, 2013). The position of the fetus can offer clear insights because,
70 when a women died during pregnancy or delivery, the fetus is usually found at her pelvis cavity
71 (Buschmann et al., 2013) or expelled post-mortem due to decomposition processes (Augias et al.,
72 2015; Viva et al., 2020). On the contrary, interpretations are difficult if the mother and fetus died at
73 different times or in the case of differential mortuary treatments (Buschmann et al., 2013; Willis
74 and Oxenham, 2013). Certainly, in recent years, the interest for fetuses and perinates in forensic and
75 bioarchaeological fields has been growing because they can provide information about health of
76 fetuses, and potentially, the health of the mother and population as well (Satterlee Blake, 2018).
77 Thus, several papers have been recently published, with an increasing use of multidisciplinary,
78 innovative and holistic approaches (Le Roy and Murphy, 2020; Rebay-Salisbury, 2018).

79 We here applied a multifaceted approach to analyze double burials retrieved in an archaeological
80 excavation in Forli (Northern Italy, Fig. 1), dated approximately to the 17th and 18th centuries AD,
81 which in total returned 271 burials. During the excavation, several double burials were unearthed,
82 thirteen of which contained a perinate buried in the same grave with an adult. The location of this
83 archaeological site (identified as “Forli Campus”), near the area of the ancient city hospital, implied
84 that this site was the hospital cemetery at that time. This particular period witnessed the introduction
85 of novel techniques, practices and manuals for surgeons and doctors who had a new approach in
86 obstetrics (Cosmacini, 1989; Pancino, 1984). In the 18th century maternal issues and childbirth
87 attracted more attention within the medical field, through the introduction of lying-in hospitals and
88 maternity wards (Scotti, 1984; Stone, 2016). However, prior to the 20th century, child-birth often
89 occurred at home (Stone, 2016), being considered as women’s work since prehistoric times, thus
90 not engaged by males (Allotey, 2011; Leavitt, 1986; Versluisen, 1981).

91 Thanks to continual innovations in the medical treatments and hospitalization procedures, maternal
92 mortality rates have decreased in the last few centuries. In England and Wales, these rates
93 significantly decreased though centuries, from 1700 to 1935, as reported by Chamberlain (2006)
94 and Loudon (2000), thanks to the improvements in maternal care (McFadden and Oxenham, 2019).
95 Similar data are not available for ancient Italy, but only for modern times (Donati et al., 2018),
96 therefore a detailed study in this field is needed. Hence, our study of the double burials, containing
97 an adult and a perinate, coupled with the period to which they date and with their location into a
98 possible hospital cemetery, is of particular interest. This work has the potential to further the
99 knowledge of maternal issues, the challenges during pregnancy and childbirth, and also the
100 diffusion of medical practices in the obstetric field. These burials possibly represent the first
101 reported cases of hospitalization of childbirth in the history of medicine, therefore these samples

102 offer a rare opportunity to apply a holistic approach in the analysis of maternal events and further
103 their inference.

104 To the best of our knowledge, the current study is the first to use a multidisciplinary approach to
105 analyze a considerable number of double burials of the same archaeological context, where
106 perinates were buried in the same grave with an adult, with the aim to identify potentially childbirth
107 deaths. Archaeological and historical data were analyzed to contextualize the area, the historical
108 period and all the features of the burials. Then, anthropological investigations were conducted to
109 identify the sex of the individuals, to estimate the age at death and also to evaluate possible
110 disorders, pathologies, cause of death, and health conditions. Moreover, to evaluate the possible
111 maternal relationship between individuals buried together, ancient DNA (aDNA) analysis was
112 applied, focusing on the mitochondrial DNA (mtDNA), passed down through generations only
113 along the maternal line. In particular, in this study the hypervariable region I (HVR-I) of the
114 mtDNA was analyzed, combined with single nucleotide polymorphisms (SNPs) in the coding
115 region of mtDNA. Indeed, several studies demonstrate the usefulness of this genomic region to
116 identify maternal relationships of archaeological or forensic interest (e.g. Coble et al., 2009;
117 Deguilloux et al., 2018, 2014; Le Roy et al., 2016; Mooder et al., 2005).

118

119 **2. Materials and methods**

120 2.1. The archaeological and historical context

121 The archaeological excavation, which took place in 2014, identified 271 single and multiple graves,
122 recovering a total of 405 individuals, plus six burials that were used as an ossuary (Fig. S1). These
123 were pit burials, comprised of a majority of primary depositions and depositions damaged by
124 unintentional anthropic events. However, there were also secondary depositions in the six ossuaries
125 and some skeleton reduction. Based on the stratigraphic data and on the dating of recovered
126 ceramics and findings, the use of this area for funeral purposes has been dated to two centuries,
127 from 17th-18th century until the Napoleonic Age. The burials were disposed in rows, subdivided into
128 two groups defined by orientation: East to West in the more ancient phase and North to South in the
129 most recent phase. There was no internal division in the graveyard, and in several cases the burials
130 were very poor.

131 Since 1223 the site was part of a complex including the charity hospital *Domus Dei*, probably
132 founded in the 13th century, and Saint James church. It was located just outside the walls in the
133 south-east area of the town (Gori and Tramonti, 2004). As a charity hospital, the institution acted
134 for helping poor, invalid people and orphans. In the 14th century the town walls were extended to
135 include the site. During the 16th century, *Domus Dei* became the most important sanatorium in Forlì

136 and the presence of a surgeon was documented in 1612 (Gori and Tramonti, 2004). Since then, the
137 number of resident surgeons progressively increased, present in four units by 1800 (Matteucci,
138 1842). Over time, the hygienic conditions of the structure drastically worsened (Gori and Tramonti,
139 2004). As a consequence, during the first half of the 18th century, the community rebuilt the
140 hospital. According to the *Décret Impérial sur les Sépultures* issued by Napoleon in 1804, all
141 cemeteries were moved outside city walls, and the graveyard was abandoned. The hospital was
142 definitely closed at the beginning of the 20th century (Scalise et al., 2018). The Gregorian Cadastre,
143 dated to the 19th century, still attributed the property of this area to the hospital.

144

145 2.2 The samples and the anthropological analyses

146 During the analysis of hospital cemetery burials, the presence of thirteen double and multiple
147 burials was noted, comprising a perinate and at least one adult (burials 24, 62, 88, 94, 106, 119, 126,
148 162, 174, 176, 185, 271, and 295) (Table 1 and Fig. S1). All burials were randomly located, the
149 majority of them in the N-E of the site. The burials were all pit burials, and their orientation vary
150 from N-S (burials 62, 106, 119, 174, 176, 185, and 295) to W-E (burials 24, 88, 94, 126, 162, and
151 271). The adult individuals were mostly in supine position (burials 24, 94, 106, 119, 162, 174, 176,
152 185, and 271), but also lateral position was present (burials 62, 88, 126, and 295). In some of the
153 burials were grave goods, i.e. small bronze medals (burials 24 and 126), rosary beads (burial 88) or
154 glass paste (burial 271).

155 In all thirteen burials the position of the perinate was clearly outside the pelvic girdle of the adult
156 (Fig. 1). In all but one case there was no systematic placement of the perinate, which was located
157 beside the upper body of the adult, right (burials 24, 106, 126, and 271) or left (burials 94, 174, and
158 185), next to the chest (burial 94), near the pelvic girdle (burial 62) or beside the lower body of the
159 adult, right (burials 119 and 295) or left (burials 162 and 176). In burial 88 the perinate was placed
160 near the arms of the adult, on the right side of her (Fig. 1).

161 The remains of the adult individuals were generally well preserved, although in none of the cases
162 was the skeleton been completely preserved because of damage from modern anthropic events (e.g.
163 burial 106). The non-adult individuals were less preserved than the adults, and most were
164 represented by long bones and iliac bones.

165 A systematic analysis protocol was followed, divided into different steps. First, sex determination
166 of the adult individuals was made by observing the morphological characteristics of skull and pelvic
167 girdle (Acsádi and Nemeskéri, 1970; Bruzek, 2002). The individuals considered female (Table 1)
168 underwent further analysis. Due to the incompleteness of many individuals, in which not all skeletal
169 elements were represented, age-at-death was assessed through the combined application of different

170 methods to obtain the smallest possible range. Dental attrition was evaluated by Lovejoy's approach
171 (1985); Meindl and Lovejoy's (1985) and Acsadi and Nemeskéri's (1970) methods were used for
172 judging cranial sutures degree of closure; the auricular surface was evaluated employing Schmitt's
173 methodology (2005) and pubic symphysis was compared with standards suggested by Brooks and
174 Suchey (1990) and Kimmerle et al. (2008).

175 To estimate health conditions, the remains were examined through macroscopic observation, on
176 which the prevalence of the pathologies observed was then calculated. Findings were compared to a
177 reference atlas (Ortner, 1985) (Table 1). Prevalence is the number of cases in the group being
178 studied, no time based, according to the formula:

$$179 \quad P = \frac{n}{N}$$

180 where P= prevalence, n=number of cases and N=number in the study group. For non-adult
181 individuals, it was primarily distinguished whether the subject was pre or post-natal, evaluating the
182 appearance of ossification centers, according to the indications of Black and Scheuer (2009).
183 Subsequently, the anthropometric analysis protocol for pre-natal (1st-40th week) individuals
184 suggested by Fazan and Kòsa (1978) was applied, while for post-natal individuals the Scheuer and
185 Black (2009) and Figus et al. (2016) protocol were used (Table 1). In the absence of dental elements
186 on which to evaluate dental development, as in our case, metric assessment of fetal, perinatal and
187 infant remains is the most commonly used method for estimating chronological age-at-death in this
188 young individuals. Also taking into account the fact that some bone districts are more susceptible to
189 inaccuracy of the estimate (i.e. tibia), measures such as the diaphyseal length of the femur, the
190 maximum iliac length and width were selected, which undergo fewer variations from pathological
191 processes (Gowland and Halcrow, 2019; Han et al., 2018; Sherwood et al., 2000).

192 Additional information on the so called parturition scars in pelvis were initially collected following
193 the study of Capasso and Di Tota (1991), but more recent studies have pointed out that these
194 changes have been observed also in male individuals (Maass and Friedling, 2016; Praxmarer et al.,
195 2020). Therefore, their presence was not taken in account to infer information about delivery and
196 childbirth.

197

198 2.3. Ancient DNA analyses

199 2.3.1. *Criteria of authenticity*

200 DNA investigations were carried out in the Laboratory of ancient DNA of the Department of
201 Cultural Heritage, University of Bologna, Ravenna Campus. DNA extractions and PCR set-up were
202 performed in rooms reserved for the analysis of degraded DNA. The clean-lab area of the
203 Laboratory of ancient DNA is physically separated from the other areas of the Department and is

204 equipped with positive air pressure with HEPA filters and laminar flow cabinets reserved for the
205 different phases of the work. All steps were conducted under strict guidelines for contamination
206 control, detection, and reproducibility of data (c.f. Cooper & Poinar, 2000; Gilbert et al., 2005;
207 Deguillox et al., 2011; Llamas et al., 2017).

208 When possible, multiple samples from different skeletal elements (e.g. petrous bone and tooth) were
209 collected and analyzed from the same individual (Table 2). The sampling was carried out before the
210 anthropological analyses, by a laboratory researcher (researcher_1), with all the necessary
211 precautions to minimize the occurrence of contamination from exogenous human DNA (Fortea et
212 al., 2008; Llamas et al., 2017). PCR and post-PCR laboratory procedures were carried out in a
213 separate room, physically separated from the clean-lab area.

214 All analyses were independently replicated in the clean rooms reserved to ancient DNA analysis at
215 the Paleomicrobiology laboratory of the Department of Diagnostics and Public Health,
216 Microbiology Section, University of Verona, Italy, in order to evaluate the authenticity of the
217 results obtained in all the samples included in this project (Table 2).

218 All the amplifications and reaction sequencing were replicated at least twice in each laboratory in
219 order to authenticate the results and carefully check the mutations.

220 Moreover, buccal swab samples from all the researchers involved in this study (anthropologists and
221 paleobiologists) were analyzed to monitor potential sources of contamination (Supplementary
222 Information).

223

224 *2.3.2 Samples preparation and DNA extraction*

225 Based on the availability of skeletal elements and looking to perform multiple extractions, teeth,
226 petrous or long bones (a tibia) were collected from the adult individuals, instead, from the perinates,
227 only petrous bones were sampled (Table 2). At the Laboratory of ancient DNA in Ravenna, samples
228 preparation (bone surface decontamination, drilling and powder collection) was conducted in a
229 dedicated room of the laboratory, where the superficial layer of the samples (1-2 mm) was removed
230 by means of a drill and then exposed to UV light for 20 min. Thereafter the samples (teeth, petrous
231 and long bones) were subsampled to collect powder material for the DNA extraction. Teeth were
232 cut transversely at the cemento-enamel junction before sampling the inner layers of the root canal
233 with a diamond drill-bit. Instead, from the petrous bones, using a sterile dentistry drill, we took the
234 denser inner part, which was reduced to fine powder using pestle and mortar. For the tibia, holes
235 were drilled into the compact bone to gain access to the internal matrix and collect bone powder.
236 Between 22 to 311 mg of bone powder was used for each DNA extraction, following a silica-based
237 protocol (Serventi et al., 2018), modified from Dabney et al. (2013). In Verona, a slightly modified

238 version of this protocol was performed (c.f. Angelici et al., 2019). Protocol details are listed in the
239 Supplementary Information.

240

241 *2.3.3. Mitochondrial HVR-I analysis*

242 The HVR-I portion of the mitochondrial control region was amplified in both laboratories using
243 three couples of primers (L15995-H16132, L16107-H16261, L16247-H16402 (Caramelli et al.,
244 2003), in order to obtain three overlapping fragments of 179, 197 and 156 bp, respectively
245 (Supplementary Information). For each reagent mixture, a negative control without DNA was
246 carried out to detect possible contamination. Sanger sequencing of amplicons using both forward
247 and reverse amplification primers was conducted in separated reactions.

248 For each sample the sequences of the three overlapping fragments and multiple amplifications were
249 edited and aligned to the revised Cambridge Reference Sequence (rCRS) (Andrews et al., 1999)
250 with BioEdit v7.2.5 (Hall, 1999). Consensus haplotypes were compared between the individuals of
251 the same burial, but also between all the sequences here obtained. The haplotypes were also
252 searched on the BLASTn database (Altschul et al., 1997).

253 A database of mtDNA control region sequences was created with a large number (n=865) of
254 unrelated individuals from continental Italy, Sicily and Sardinia, obtained from Boattini et al.
255 (2013). In this database the samples were collected with the standard ‘grandparents’ criterion (only
256 those individuals whose four grandparents were born in the same macro-area were sampled).

257 The software DnaSP v.5.10.01 (Librado and Rozas, 2009) was used to identify identical sequences
258 between the database and to collapse them into unique haplotypes. The search for shared haplotypes
259 between the samples here analyzed and the mtDNA Italian population database (Boattini et al.,
260 2013) was conducted with Arlequin software version 3.5.1.2 (Excoffier et al., 2005).

261

262 *2.3.4. Mitochondrial coding region SNPs genotyping*

263 To better evaluate the haplogroup assignment of each sample and ameliorate the detection of
264 possible matrilinear correlations, the samples were also checked for the polymorphisms in the
265 coding region of the mitochondrial DNA. The protocol consisted of two different multiplex PCRs
266 coupled with the SNaPshot method, where the first multiplex included variants that define the most
267 common non-H European lineages, whereas the second contains variants of H sub-lineages
268 (Bertoncini et al., 2012) (Supplementary Information).

269 Mitochondrial haplogroups were determined based on the PhyloTree mtDNA phylogeny, build 17
270 (www.phyloree.org) (Oven and Kayser, 2009) and Haplogrep2 software (Kloss-Brandstätter et al.,
271 2011), based on coding SNPs, but also taking into account the results of HVR-I region.

272

273 **3. Results**

274 3.1. The anthropological analyses

275 We found that five out of thirteen double burials randomly distributed across the necropolis area
276 (Fig. S1) were occupied by a female adult individual in fertile age (samples 88_1, 106_1, 174_1,
277 176_1, and 295_1) buried with a non-adult perinate (Table 1). In 4 cases, the perinate demonstrated
278 an estimate age-at-death compatible with the end of gestation (samples 106_2, 174_2, 176_2, and
279 295_2). In one case, the perinate showed a premature age-at-death not compatible with the term of
280 gestation (88_2), and the adult female individual (88_1) was the only one in the samples here
281 analyzed to show evident pathological stigmata (Table 1).

282 The pathologies detected are related to degenerative aspects, often attributable to an intense and
283 chronic use over time of the body (i.e. vertebral spondylarthrosis, Schmorl's nodes, tibial
284 periostitis), or to deficiencies due to a poor or invariable diet (i.e. cribra cranii, cribra orbitalia);
285 dentoalveolar pathologies are also typical of the historical series (i.e. dental caries, alveolar
286 resorptions) (Table 3). In only one case (tomb 88) was a pathological aspect inconsistent with the
287 individual's age at death. Despite an estimated age of 30-39 years, this individual displayed a severe
288 osteoporosis (Table 1). No pathological aspect was detected for prenatal individuals. The
289 differential diagnosis conducted on the adult individual of the burial 88, considered both primary
290 and secondary osteoporosis. Juvenile idiopathic osteoporosis and imperfect osteogenesis belong to
291 the first type. Secondary osteoporosis is defined as low bone mass with microarchitectural
292 alterations in bone leading to fragility fractures in the presence of an underlying disease or other
293 situations. Considering the age of this individual and the absence of bone curvatures, fractures or
294 bone calluses always due to past fractures, the most consistent hypothesis is that relating to a
295 secondary form of osteoporosis (i.e. large numbers of childbirths).

296

297 3.2. Mitochondrial DNA haplogroup assignment and maternal kinship evaluation

298 Genetic analyses were performed on the five burials selected from the anthropological study (Fig. 2
299 and Table 1), based on the criterion of a double burial containing a female in fertile age and a
300 perinate. The strict criteria followed in this study allowed us to exclude any modern DNA
301 contamination and confirm the reliability of the aDNA results. No contamination was observed in
302 any of the blank extractions or negative controls included in each reaction, both for extraction
303 (Qubit High Sensitivity quantification = too low) and also for amplification steps. The data were
304 consistent between replicates of extractions and also amplifications. Moreover, the data were

305 confirmed between overlapping regions amplified by different couples of primers, and also between
306 the results obtained by the two laboratories involved.

307 We report the absence of a recurrent haplotype, which could be due to contamination by modern
308 exogenous DNA, belonging for example to the researchers who came into contact with the remains.
309 Moreover, all but one sequences obtained from ancient samples were different from those of the
310 researchers involved in the project that worked on the samples (Table S1). We detected the same
311 HVR-I haplotype, specifically the rCRS sequence, in one sample (176_2) and in one of the
312 laboratory researchers (researcher_4) based in Ravenna. This haplotype is very common in the
313 European population, and thus in Italy. In fact, in the database of Boattini et al. 2013 it occurs in
314 about 15% of the samples. However, we proceeded with all possible tests to recognize any possible
315 contamination by exogenous DNA. The same result was independently obtained in Verona, which
316 received samples directly from the subsampling made from the researcher_1, with no contribution
317 of researcher_4, however this sample was independently re-extracted and amplified to confirm the
318 data obtained.

319 We were able to obtain the complete HVR-I region and genotyping of SNPs from all the samples
320 analyzed (Table 2).

321 No maternal relationship was highlighted within double burials. For each tomb tested, the two
322 individuals buried together (adult female and alleged child), displayed different polymorphisms in
323 the HVR-I region and also different mutations in the SNPs of the mtDNA coding region (Table 2).

324 Moreover, no similarities were identified between the individuals of the different burials analyzed,
325 since among the ten sequences obtained, we highlighted ten different haplotypes (Table 2).

326 Considering the results of the coding region SNPs, the samples were assigned to seven different
327 mtDNA haplogroups (including sublineages) (H1, H5, H*, J* and U*), corresponding to typical
328 lineages of the West Eurasian area (Richards et al., 2000).

329 Some haplotypes obtained from Forlì Campus are rare or unique in the Italian population. In
330 particular, four haplotypes are unique respect to the 865 samples of the Italian database considered
331 (Boattini et al., 2013). Among them, two haplotypes provided a match in the NCBI nucleotide
332 database through the BLASTn algorithm. The two remaining haplotypes of the samples 88_1 and
333 295_1 were not retrieved by BLASTn, although their mutations are confirmed both by the
334 alignment of the multiple fragments amplified, which enabled coherent lineage attribution through
335 SNPs and HVS-I typing. The six other haplotypes here obtained are more frequent in the Italian
336 database, in particular the haplotypes of the samples 176_2 and 174_1, detected in 131 and 17
337 Italians, respectively.

338

339 **4. Discussion and conclusions**

340 In this study, a multifaceted approach was applied to thirteen double burials where an adult was
341 buried in the same grave of a perinate. These burials potentially represent the first reported cases of
342 hospitalization of childbirth in the history of medicine. Therefore, these samples constitute a rare
343 opportunity both to apply a multidisciplinary approach in the analysis of maternal events and to
344 establish a good practice to interpret potential cases of childbirth deaths. Thus, the aim of this
345 project was to ascertain if a maternal kinship existed between the individuals buried together.
346 Anthropological analyses hypothetically confirmed this interpretation in five burials where adult
347 individuals were recognized as a female in childbearing age and infants were evaluated of perinatal
348 age. In four of these burials, the perinates have an estimated age compatible with the term of
349 gestation and were associated with adult individuals with no macroscopic pathological evidence. In
350 one case (burial 88), the adult was clearly ill, showing obvious signs of osteoporosis, and was
351 associated with a preterm infant of 28-30 weeks. Osteoporosis during pregnancy is a rare event, it
352 happens in general because the maternal body is called to supply the fetus with a large amount of
353 body calcium, which is consequently removed from the process of renewal of the maternal bones.
354 This physiological process, if not balanced by specific body adaptation mechanisms (e.g., increase
355 in estrogen hormones and vitamin D), leads to aggravate the health of the maternal skeleton,
356 promoting as much as possible the formation of the skeletal system of the fetus, which will tend to
357 maintain more or less normal development (Yun et al., 2017). The data obtained from individual
358 88_1 and individual 88_2 could therefore lead to suggest the situation of a mother probably
359 suffering from a primary form of osteoporosis, whose health has worsened once pregnant, perhaps
360 even to the point of leading her to death along with the preterm fetus.

361 However, the contextual presence of eight burials of male individuals associated with perinates, has
362 posed several questions which led us to investigate the hypothetical mother-child bonds with
363 genetic analyses, despite the archaeological data and the anthropological study seemed to
364 hypothesize and support this argument.

365 Nevertheless, through genetic analyses, no maternal relationships were highlighted for the
366 individuals buried together in the five double burials, nor between all the individuals here analyzed.
367 In fact, ten mitochondrial haplotypes were retrieved from the ten different samples.

368 In many species, including humans, mitochondrial DNA are inherited through maternal line
369 (Hutchison et al., 1974). Claims about possible paternal inheritance of mitochondrial DNA in
370 humans were published in two papers (Kraytsberg et al., 2004; Luo et al., 2018), of which the latter
371 was criticized for the methodology used and inconsistency of the data (Lutz-Bonengel and Parson,
372 2019). Until now, despite efforts from several independent groups, the evidence for paternal

373 inheritance of mtDNA in humans is controversial and has been shown only in one case (Kraytsberg
374 et al., 2004).

375 However, since only the maternal genetic inheritance was investigated, we cannot dismiss another
376 type of familial relationships (e.g. half-siblings, cousins), though this is less likely. Moreover,
377 adoption events also cannot be tested and dismissed, but the age of the perinates and the
378 simultaneity of the burials, leads us to rule out this possibility.

379 The absence of a matrilinear kinship is contrary to a standard archaeological interpretation, which
380 assumes that women retrieved in these burials most likely died during or soon after the childbirth
381 and were buried together with their fetuses or perinates. Similar results were obtained in a recent
382 study that investigated the deposition of a fetus and perinate close to an adult female (Dulias et al.,
383 2019). In the study, Dulias and colleagues demonstrated that the adult female was not the mother of
384 the perinate buried alongside her, but DNA analysis could not exclude the possibility that the
385 female was the mother of, or maternally related to, the fetus (because of a single different mutation
386 recognized in the whole mtDNA) (Dulias et al., 2019). At present, ancient DNA is poorly applied to
387 test maternal kinship in such archaeological contexts. To best of our knowledge, only this study and
388 the recent one from Dulias and colleagues (2019) applied archaeogenetics analyses for this aim.

389 This study constituted a possibility to define a multidisciplinary approach in the study of double
390 burials potentially correlated to childbirth death, like that recently applied by Rebay-Salisbury
391 (2018), for which, however, the aDNA analyses had not been carried out yet. The joint
392 interpretation between historical, archaeological, anthropological paleopathological data examined
393 here is essential in such inferences. Moreover, the key evidence about possible maternal kinship
394 was obtained from the application of ancient DNA analysis.

395 Mitochondrial DNA was selected for the purposes of this study for several reasons. It is often
396 preferred as target of choice in ancient remains because mtDNA is more likely to be preserved due
397 to its higher copy number than nuclear DNA (Rizzi et al., 2012). Usually kinship is detected with
398 high degree of confidence by typing of nuclear short tandem repeats (STRs), as today in forensic
399 cases. However, since both the failures reported in typing STRs in ancient remains (Deguilloux et
400 al., 2018, 2014; Serventi et al., 2018) and because we just wanted to test the maternal inheritance,
401 only the mitochondrial portion of the genome was examined. In cases of positive matches between
402 the female and the perinate in the same burials, it would have been possible to affirm only that they
403 belonged to the same maternal line. However, the archaeological and anthropological evidence,
404 supporting a contemporary burial of both individuals, would have led to a mother-son relationship.
405 Moreover, in case of positive maternal matches but dubious archaeological evidences, the
406 amplification of STRs is suggested.

407 From a technical point of view, the mitochondrial DNA appeared well preserved in the remains of
408 Forlì Campus, indeed complete HVR-I sequences were obtained from all the ten samples here
409 analyzed. Moreover, thanks to the strict criteria of authentication here applied, no contamination
410 was observed in our analyses. The strength of this assertion is also supported by our independent
411 replicate tests in different laboratories.

412 Certainly, with the advancement of methodologies and protocols and the continuous and
413 simultaneous lowering of costs, the possibility to analyze genomes and mitogenomes through next-
414 generation sequencing (NGS) technologies is becoming accessible to all types of studies, and future
415 projects and analyses must go in this direction.

416 This study highlighted the relevance of a multidisciplinary approach where archaeogenetic data are
417 precisely applied to test arguments provided by archaeology and anthropology. This approach can
418 yield valuable results for a better understanding of ancient burial patterns, funerary practices and
419 ancient population social structure. In this case, the archaeogenetic analyses applied suggests that a
420 general assumption of mother/child relations within such burials can be misleading.

421

422 **Acknowledgements**

423 The authors wish to express their gratitude to Dr. Sveva Savelli and Dr. Claudia Tempesta of the
424 Superintendence of Archaeology, Fine Arts and Landscape for the provinces of Ravenna, Forlì-
425 Cesena and Rimini for allowing the study of the osteological remains presented in this work. We
426 thank the anonymous reviewers for their insightful comments and suggestions. Finally, we would
427 like to thank Adam Jon Andrews for improving the use of English in the manuscript.

428

429 **Funding**

430 This work was supported by MIUR-PRIN action assigned to DL (AGED-1000 Ancient Italian
431 Genomes: Evidence from ancient biomolecules for unravelling past human population Dynamics.
432 Grant ID: 20177PJ9XF). GiG was supported by RFO grants of the University of Bologna.

433

434 **Author contributions**

435 MT GaG, EC and DL conceived the study; MMC, AB, SDF GF and AF performed the genetic
436 analyses; EC, SS, SDF and PS analyzed genetic data; GaG and MT performed the anthropological
437 and paleopathological analyses; DL, GiG and GC provided the funds to support the research; EC,
438 GaG and MT wrote the manuscript; all the authors revised the paper.

439

440 **Supplementary Information**

441 Supplementary materials contains information about ancient DNA protocols, Figure S1 and Table
442 S1.

443

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671 Figure 1. Geographical location of the cemetery of Forlì Campus.

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 Perinatal individual

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688 Figure 2. The double burials containing a female in childbearing age and a perinate, analysed
 689 in this study by means of anthropological, paleopathological and archaeogenetic analysis. The
 690 position of the perinates in the burials is highlighted by means of a red shade.

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Burial	id.1			id.2	
	Sex	Age at death	Pathologies	Age at death	Pathologies
24	M	-	-	-	-
	M	-	-	-	-
62	M	-	-	-	-
88	F	30-39	Dental caries, alveolar resorptions, vertebral spondylarthrosis, Schmorl's nodes, osteoporosis	28-30	n.r.
94	M	-	-	-	-
106	F	20-29	Schmorl's nodes	40	n.r.
119	M	-	-	-	-
	M	-	-	-	-
126	M	-	-	-	-
162	M	-	-	-	-
	M	-	-	-	-
174	F	17-20	Schmorl's nodes	40	n.r.

176	F	30-39	Dental caries, alveolar resorptions, periodontitis, vertebral spondylarthrosis, Schmorl's nodes	38-40	n.r.
185	M	-	-	-	-
271	M	-	-	-	-
295	F	30-39	Cribra cranii, cribra orbitalia, dental caries, alveolar resorptions, vertebral spondylarthrosis, Schmorl's nodes, vertebral fracture, tibial periostitis	36-38	n.r.
<i>age at death in years</i>				<i>age at death in weeks</i>	

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705 Table 1. Anthropological results about estimation of sex and age of all the individuals. Burial
706 119 contains also a sub-adult non perinatal. Burials with adult male individuals were not
707 considered for anthropological and archaeogenetic analyses. (–) means not tested.

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721 Table 2. Mitochondrial data from ancient human remains. The consensus HVR-I haplotype
722 was obtained from multiple amplification from different skeletal elements or different
723 extractions, in addition to the repetition of all the analyses in the second laboratory. The
724 column "SNPs" contains the mutated SNPs of the multiplex assays used (see Supplementary
725 Information for details about the two multiplex amplifications). The haplogroup (Hg-SNPs)
726 was inferred from SNPs results.

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		pat/obs	%
Pathology observed	Dental caries	3/5	60%
	Alveolar resorptions	3/5	60%
	Periodontitis	1/5	20%
	Cribra crania	1/5	20%
	Cribra orbitalia	1/5	20%
	Vertebral spondylarthrosis	3/5	60%
	Vertebral fracture	1/5	20%
	Schmorl's nodes	5/5	100%
	Osteoporosis	1/5	20%
	Tibial periostitis	1/5	20%

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742 Table 3. Prevalence of pathologies observed in the adult female record
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