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

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Abstract

Evidence of maternal care and childbirth events in the past are rare in the archaeological record and are difficult to recognize. To combat this, we analyzed thirteen double burials potentially related to childbirth death events, thereby containing an adult and a perinate. The specimens were excavated from the archaeological area identified as "Forlì Campus" (Forlì, Italy), that dated to 17th-18th centuries AD and was adjacent to a hospital in use at that time. This period witnessed the development of medical techniques and novel approaches in obstetrics in Europe, with the introduction of lying-in hospitals and maternity wards. We here tested if the double burials were ascribable to childbirth death events and thus represent the first reported cases of the hospitalization of childbirth in the history of medicine. A multidisciplinary analysis was undertaken to achieve this aim, combining anthropology, archaeology, paleopathology and archaeogenetics.

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

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

1. Introduction

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

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

Attempts to identify maternal events in past populations have mainly focused on burials of females which appear to have died during pregnancy, childbirth, or soon after. Such evidence is rare, due to the fragility and damage of fetal and perinatal bones that are often missed or misidentified during excavations. In addition, cause of death surrounding childbirth is often unclear because this event can be related to numerous maternal health issues or be archaeologically invisible (Buschmann et al., 2013; Willis and Oxenham, 2013). The position of the fetus can offer clear insights because, when a women died during pregnancy or delivery, the fetus is usually found at her pelvis cavity (Buschmann et al., 2013) or expelled post-mortem due to decomposition processes (Augias et al., 2015; Viva et al., 2020). On the contrary, interpretations are difficult if the mother and fetus died at different times or in the case of differential mortuary treatments (Buschmann et al., 2013; Willis and Oxenham, 2013). Certainly, in recent years, the interest for fetuses and perinates in forensic and bioarchaeological fields has been growing because they can provide information about health of fetuses, and potentially, the health of the mother and population as well (Satterlee Blake, 2018). Thus, several papers have been recently published, with an increasing use of multidisciplinary, innovative and holistic approaches (Le Roy and Murphy, 2020; Rebay-Salisbury, 2018).

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

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

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

2. Materials and methods

2.1. The archaeological and historical context

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

Since 1223 the site was part of a complex including the charity hospital *Domus Dei*, probably founded in the 13th century, and Saint James church. It was located just outside the walls in the south-east area of the town (Gori and Tramonti, 2004). As a charity hospital, the institution acted for helping poor, invalid people and orphans. In the 14th century the town walls were extended to include the site. During the 16th century, *Domus Dei* became the most important sanatorium in Forlì and the presence of a surgeon was documented in 1612 (Gori and Tramonti, 2004). Since then, the number of resident surgeons progressively increased, present in four units by 1800 (Matteucci, 1842). Over time, the hygienic conditions of the structure drastically worsened (Gori and Tramonti, 2004). As a consequence, during the first half of the 18th century, the community rebuilt the hospital. According to the *Décret Impérial sur les Sépultures* issued by Napoleon in 1804, all cemeteries were moved outside city walls, and the graveyard was abandoned. The hospital was definitely closed at the beginning of the 20th century (Scalise et al., 2018). The Gregorian Cadastre, dated to the 19th century, still attributed the property of this area to the hospital.

2.2 The samples and the anthropological analyses

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

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

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

A systematic analysis protocol was followed, divided into different steps. First, sex determination of the adult individuals was made by observing the morphological characteristics of skull and pelvic girdle (Acsádi and Nemeskéri, 1970; Bruzek, 2002). The individuals considered female (Table 1) underwent further analysis. Due to the incompleteness of many individuals, in which not all skeletal elements were represented, age-at-death was assessed through the combined application of different methods to obtain the smallest possible range. Dental attrition was evaluated by Lovejoy's approach (1985); Meindl and Lovejoy's (1985) and Acsadi and Nemeskéri's (1970) methods were used for judging cranial sutures degree of closure; the auricular surface was evaluated employing Schmitt's methodology (2005) and pubic symphysis was compared with standards suggested by Brooks and Suchey (1990) and Kimmerle et al. (2008).

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

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

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

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

2.3. Ancient DNA analyses

2.3.1. Criteria of authenticity

DNA investigations were carried out in the Laboratory of ancient DNA of the Department of Cultural Heritage, University of Bologna, Ravenna Campus. DNA extractions and PCR set-up were performed in rooms reserved for the analysis of degraded DNA. The clean-lab area of the Laboratory of ancient DNA is physically separated from the other areas of the Department and is equipped with positive air pressure with HEPA filters and laminar flow cabinets reserved for the different phases of the work. All steps were conducted under strict guidelines for contamination control, detection, and reproducibility of data (c.f. Cooper & Poinar, 2000; Gilbert et al., 2005; Deguillox et al., 2011; Llamas et al., 2017).

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

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

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

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

2.3.2 Samples preparation and DNA extraction

Based on the availability of skeletal elements and looking to perform multiple extractions, teeth, petrous or long bones (a tibia) were collected from the adult individuals, instead, from the perinates, only petrous bones were sampled (Table 2). At the Laboratory of ancient DNA in Ravenna, samples preparation (bone surface decontamination, drilling and powder collection) was conducted in a dedicated room of the laboratory, where the superficial layer of the samples (1-2 mm) was removed by means of a drill and then exposed to UV light for 20 min. Thereafter the samples (teeth, petrous and long bones) were subsampled to collect powder material for the DNA extraction. Teeth were cut transversely at the cemento-enamel junction before sampling the inner layers of the root canal with a diamond drill-bit. Instead, from the petrous bones, using a sterile dentistry drill, we took the denser inner part, which was reduced to fine powder using pestle and mortar. For the tibia, holes were drilled into the compact bone to gain access to the internal matrix and collect bone powder. Between 22 to 311 mg of bone powder was used for each DNA extraction, following a silica-based protocol (Serventi et al., 2018), modified from Dabney et al. (2013). In Verona, a slightly modified version of this protocol was performed (c.f. Angelici et al., 2019). Protocol details are listed in the Supplementary Information.

2.3.3. Mitochondrial HVR-I analysis

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

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

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

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

2.3.4. Mitochondrial coding region SNPs genotyping

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

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

3. Results

3.1. The anthropological analyses

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

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

3.2. Mitochondrial DNA haplogroup assignment and maternal kinship evaluation

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

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

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

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

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

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

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

4. Discussion and conclusions

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

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

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

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

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

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

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

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

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

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

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

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Author contributions

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

Supplementary Information

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

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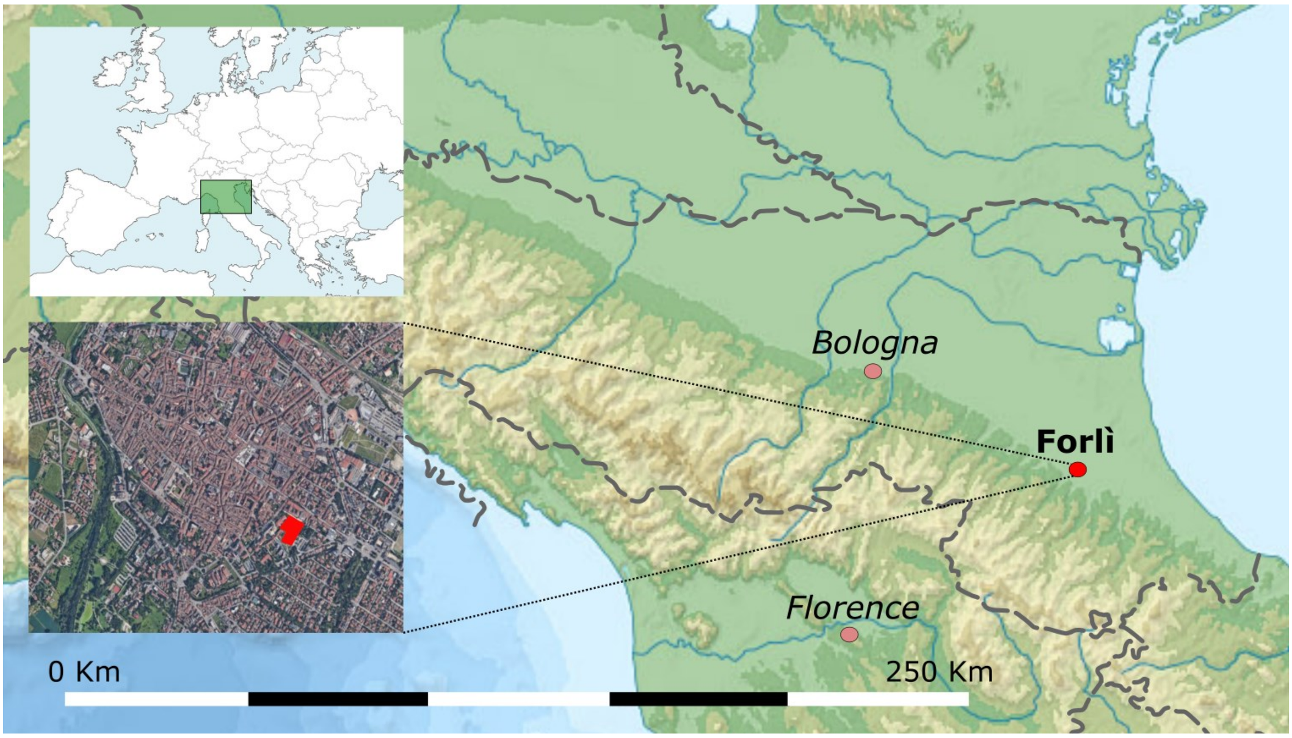

Figure 1. Geographical location of the cemetery of Forlì Campus.

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

	id.1			**id.2**	
	Adult individuals			**Perinatal individuals**	
Burial	*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.
	age at death in years			*age at death in weeks*	

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

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

		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%

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