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He or she? The Use of an Integrated Approach for Sex Determination in the Bioarcheological Research

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Abstract

Sex determination in non-adult human remains

This research aims to determine the sex of non-adult skeletal remains from two archaeological sites dating to the Roman Imperial and Medieval periods by using morphological, morphometrics and molecular approaches. The determination of

sex from non-adult remains is a debated issue in the literature even though it has important implications in the palaeodemographic reconstruction of past populations.
Concordance between the different approaches was obtained in the 63% of cases (N=19). The obtained results although preliminary are encouraging even though much research is needed for enlarging the sample size and for applying the cutting-edge High Throughput Sequencing (HTS) technologies.

Keywords: Palaeodemography - Non-adults - Morphometrics - Molecular sex assessment

Introduction

One of the major concerns of bioarchaeology is the disentangling of the characteristics of population groups whose sources are reticent[1,2,3]. In that context, despite a recent revival of scientific interest, childhood is frequently unexplored in past human population studies. Although we are aware children played an active role in drawing up the past population dynamics, they remain understudied in the archaeological record partly because their poor mineralization and their small size, make them more prone to taphonomic damages[4].
Bioarcheologists, by contrast with their archaeological counterparts, have readier to concede to children a more prominent place within historical communities[5] frequently seeking to establish age at death from the skeleton[6,7]. This estimate determines the years an individual lived on the basis of the skeletal and tooth development, and it thus represents an index of skeletal maturity, backed up by pediatric radiology and endocrinology[8,9]. Age at death estimation can be performed by using different methods and it involves several factors - gender, nutrition, metabolic, genetic, social, and medical. Bioarchaeological studies should shed more light on history's children, including health and life-style[10] contributing to reducing the differences between skeletal morphology age at death and cultural identities of infancy, childhood, and adulthood[11].
An already existing literature has touched on the many factors influencing the accuracy of evaluation of non-adult skeletons leveraging living community, highlighting methodological concerns related to reference and inter- and intra-individual variation[12,13,14,15].
Overall, multiple non-adult age-estimation methods are generally considered reliable as they consider relatively short intervals between stages of developmental growth instead of degeneration patterns of adult skeletal remains[16]. However, as they reflect developmental stages, multiple confounders as hormonal changes can act on developing both teeth and bones[17,18].
Gender represents a confounding factor for the reliable estimation of the skeletal age of a child, so the accurate identification of the biological sex for a sample should be mandatory; unfortunately, however, only a few reliable techniques exist for estimating the sex of children in skeletal individuals. The morphological sex assessment becomes reliable – with a not negligible accuracy bias[19] – beyond the puberty; even though sev-

eral authors pointed out that some differences between males and females can also be accounted for in the pre-pubertal phase, right from the first years of life[20,21,22].
Specifically, Schutkowski[23] takes into consideration some differences that, right after birth, can be found in the mandible and ilium. At birth the newborns' mandible consists of two different bone portions that merge medially during the first year of life, from the external and inferior surfaces proceeding towards the internal and superior ones[24]. After birth, the mandible is the skull bone that undergoes the greatest number of shape and size variations, as it must grow in harmony with the development of the deciduous and then permanent dentition and with the skull growth[25]. Although sexual dimorphism at the skeletal level becomes more evident during puberty, since craniofacial development occurs almost completely before this period, it is possible to find differences in the growth of the bones of the skull, and therefore also at the level of the jaw, of males and females already in childhood[26]. Also, each coxal bone is originally made up of three different bony elements, ilium, ischium, and pubis which unify during the individual's development. Although the morphology of these three elements changes little in the first years after birth, each of them shows rapid growth in the first 3 months of life, which slows down slightly until the age of 2-3 years, and further decreases until puberty when, concomitantly with adolescent growth, secondary changes related to sexual development appear[27]. At birth the ilium possesses most of the characteristics of adult bone, with the anterior and posterior superior iliac spines already well developed[28].
Rogers[29] instead proposes the observation of four different features of the distal epiphysis of the humerus, which appearing at a young age are also found in the adult skeleton. Finally, Stull and collaborators[30,31] developed a method based on 18 different measurements of the diaphyses of the main long bones (humerus, ulna, radius, femur, tibia, and fibula), presenting ranges thanks to which it is possible to distinguish between male and female even in pre-pubertal age.
Currently, the analysis of the genetic characteristics of ancient individuals is increasing, and the identification of the molecular markers in the sex chromosomes (chromosome X and chromosome Y) is useful when the preservation status of the DNA molecules is detectable[32,33,34,35].The *AMEL* locus of the amelogenin gene encodes the main protein of the extracellular matrix involved in the amelogenesis and development of dental enamel, it consists of 7 exonic units[36] and the coding portion extends from exon 2 to 6[37,38]. The gene exists in two distinct forms characterized by an 89% of homology[39]: *AMELX*, located on the X chromosome in the region p22.1-p22.3 (chrX: 11,311,533- 11,318,881 (GRCh37 / hg19)) and *AMELY* located on the Y chromosome in position p11.2 (chrY: 6,733,959-6,742,068 (GRCh37 / hg19)). It is possible, indeed, to characterize by amplification the homologous regions according to the presence of a deletion of 6 bp in intron 3 of *AMELX*, which is absent in *AMELY*[40,41,42,43,44,45].

Primer sets that generating small amplicons have found wide application in analyses on degraded DNA, and for this reason the amplification of portions of the amelogenin gene has proved particularly effective even in the case of ancient DNA (aDNA). Since Sullivan's (1993) work[46], several primer sets that can be used for the determination of length polymorphism in the *AMEL* locus, have been developed. Haas-Rochholz and Weiler[47] developed primer sets flanking a 3 bp deletion in *AMELX* exon 5 capable of generating 80 and 83 bp amplicons for *AMELX* and *AMELY* respectively. More recently, Codina et al.[48] designed a primer pair that targets the same deletion, but leads to the production of shorter amplicons, 55 and 58 bp respectively, thus being particularly suitable for aDNA.

The Sex determining Region Y (*SRY*, chrY: 2,654,896-2,655,792 (GRCh37 / hg19)), located on the short arm of the Y chromosome in position p11.31, near the pseudo-autosomal region (PAR), is a further genomic marker which plays a key role in the molecular determination of sex. *SRY* is an intron-free locus whose coding sequence (845 bp long)[49] encodes for the Testis Determination Factor (TDF), a transcription factor binding the HMG-box type (High Mobility Group) domain[50]. The *SRY* gene has been shown to be directly involved in the development of the male reproductive organs since the expression of TDF induces the male differentiation of the somatic cells of the primordial gonads[51]. Unlike *AMEL*, *SRY* is a sexual genetic marker whose products are an integral part of the sexual development of the individual. The close association between *SRY* and the maturation of sexually dimorphic features make this marker the most accurate for predicting male sex and it is often used as an element of validation of the results obtained with the amelogenin amplification products.

Identifying the reliable *sex ratio* for deceased children certainly represents a considerable asset for evaluating the funeral practices and lifestyles of past societies. Indeed, the representativeness of a skeletal population cannot exclude the role of the children when seeking to understand living conditions, also taking into consideration cultural variables influencing the resting place of the deceased, impacting on the differential burial for social status or even geographic origin of the individuals[52].

Palaeodemographic evaluation could still benefit from the reliable sex determination of the child fraction of the samples to reconstruct past human populations' structure and dynamics, to estimate essential parameters such as life expectancy, fertility, and mortality patterns[53] by sex or at the population level.

This research aims to attempt at sexing non-adult individuals from Central Italy populations by applying morphological, morphometric [54,55,56,57] and molecular approaches[58,59,60,61].

Materials and methods

The whole sample consists of 64 individuals, 52 from the archaeological site of Leopoli-Cencelle[62] and 12 individuals from Quarto Cappello del Prete[63]. Both

the samples refer to burial areas where there was a remarkable presence of child burials, making the sex-determination of the buried individuals critical for further evaluations.

Age at death estimation

As non-adult individuals ranging from perinatal to adolescents were analyzed, general age at death estimation methods were used. In particular, tooth formation and eruption[64], the measurement of the ilium[65], diaphyseal length of the long bones of the limbs[66], of the clavicle[67], and the development and fusion of the main ossification centers[68] were applied. In the case of perinatal individuals, the measurements were compared with those proposed by Fazekas and Kòsa[69].

Sex assessment: morphological approach

Three different approaches were merged for achieving a reliable sex determination. Specifically, the assessment was performed following the protocols proposed by Schutkowski[70], Rogers[71], and Stull et al.[72,73]. The method by Schutkowski[74] analyzes morphological features of the mandible and the ilium; the protocol by Rogers[75] observes four different characters at the level of the distal epiphysis of the humerus; finally, the protocol of Stull and collaborators[76,77], is based on 18 different measurements of the diaphysis of the main long bones (humerus, ulna, radius, femur, tibia, and fibula).

Following the indications of Schutkowski's study[78], three different traits were observed on the mandible:

- chin protrusion: not very prominent, with a tapered shape and a smooth surface in females; more prominent, broad and angular in males;
- shape of the anterior dental arch: parabolic in females; anteriorly wider, U-shaped in males;
- eversion of the gonial region: the *gonion* is aligned with the external surface of the horizontal branch of the mandible in females; in frontal view, the *gonion* is slightly everted with respect to the rest of the mandibular body in males.

For each trait a value from -1 to +1, was assigned. The negative value corresponds to a purely feminine morphology while the positive corresponds to a masculine one; the traits showing an intermediate morphology were recorded as zero (0).

On the basis of the study proposed by Schutkowski[79], the following characteristics were observed on the ilium:

- the angle of the greater sciatic notch: the bone is observed ventrally and positioned so that the anterior part of the greater sciatic notch is vertically

aligned, the angle is greater than 90° in females, approximately equal to 90° in males;
- "arch criterion": the bone is observed ventrally, the arch represents an extension of the vertical side of the greater sciatic notch; this crosses the auricular surface in females while it runs along the medial margin in males;
- depth of the greater sciatic notch: the bone is placed with the dorsal surface towards the operator. The posterior inferior iliac spine and the edge of the acetabular region point downwards and are aligned with each other. The notch is shallow in females and deeper in males;
- the curvature of the iliac crest: looking at the bone from above, the iliac crest appears flattened in females, in the shape of a clear "S" in males.

In this case the morphology of each trait was also scored by assigning a value from -1 (feminine) to +1 (masculine), marking with 0 the intermediate morphology.
The fusion of the distal epiphysis of the humerus with the diaphysis begins at about 11.5 years in females and about a year later in males and is completed around the age of 15[80].
For this reason, the indications reported by Rogers[81] were applied exclusively to adolescents and non-infantile individuals. Observing the posterior surface of the humerus the following traits were observed:

- trochlear constriction: the trochlea is flattened, in the shape of a "bow tie", with the distal margin drawing a distinct angle in the females; less flattened, with the medial margin taking on a gradual curvature in males;
- trochlear symmetry: the trochlea is symmetrical in females and asymmetrical in males;
- shape and depth of the olecranon fossa: deep and oval in females, less deep and triangular in males;
- the angle of the medial epicondyle: placing the eyes at the height of the work surface, the medial epicondyle is higher than the rest of the distal epiphysis in females, parallel to the plane in males.

Also in this case, a value from -1 (feminine) to +1 (masculine) has been assigned to the observed features; the undefined characters and intermediate morphology were also in this case recorded by the value 0.
Diaphyseal length was measured according to the protocol by Stull et al.[82,83]. Each measurement was repeated five times and the mean value was used for sex assessment.
For some individuals, mainly aged between 13 and 18 years, the methodologies described above were integrated with others routinely used for determining sex in adult individuals.

The morphological approach is based on the observation of a series of features at the level of the skull, jaw, and pelvis.

The male skull is generally more robust with marked muscular insertions, compared to those present on the female skull, which is frailer. The male skull is characterized by pronounced and protruding glabella and superciliary arches, by a large and voluminous mastoid process, a generally broad and very developed nuchal plane and occipital protuberance, and by a receding forehead, all characteristics that appear less marked in the female skulls. By contrast, the frontal and parietal tuberosities, almost absent in males, are more evident in the female skull, giving it a more rounded shape at the back[84].

The mandible is more robust and squared in males, with the *gonion* (mandibular angle) presenting often retroverse roughness, and a prominent chin[85]. The female mandible, on the other hand, is slender, with a rounded chin, and a smooth or only slightly wrinkled *gonion*[86].

The pelvis, composed of two *os coxae* and the sacrum, is analyzed both at the level of the individual bones and as a whole. The male coxal bone is usually tall and narrow, with marked muscular insertions, and the greater sciatic notch is narrow and V-shaped. In female individuals, on the other hand, the coxal bone is low and wide, with underdeveloped muscle insertions, and the greater sciatic notch is wide and U-shaped. Overall, the upper and lower circumferences of the pelvis (*pelvis major* and *minor*) are narrow in males and very large in females, in order to facilitate pregnancy and childbirth[87].

The metric approach of Stull and collaborators[88,89] was used along with methods by other authors for older individuals as Martin and Saller[90], Dwight[91], Pearson[92,93], Olivier[94], Black[95], Di Bernardo and Taylor[96], Stewart[97], Berrizbeitia[98] and Borrini[99,100].

The measurements, differentiated according to the bone elements to which they refer, should be recorded on different days and at different times of the day, with the use of osteometric board, calipers, and metric tape.

Sex assessment: molecular approach

Ancient DNA (aDNA) was extracted from 9 samples recovered in the archaeological site of Leopoli-Cencelle. and ten samples from Quarto Cappello del Prete[101] aiming at analyze molecular markers useful for sex determination.

For all the selected specimens, a micro-sampling of the petrous bone was performed. This portion of the temporal bone is particularly suitable for aDNA studies showing high yield of endogenous DNA due to its location and to the high density of its bone matrix[102,103,104].

In order to reduce the levels of contaminants by cross-linking of exogenous DNA molecules, the remains were UV-irradiated before proceeding to pulverization and DNA extraction. For each specimen 1 mL of Yang-Urea DNA extraction buffer (0.5 M EDTA pH 8.0, 1M Urea), and 20 µL of proteinase K (100 µg/ml) were added to 0.5 g of bone powder[105]. The obtained solution was then incubated at 37° C overnight.

At the end of the incubation phase, necessary for a partial dissolution of the cell membranes and for the denaturation of the histone proteins, the samples were spinned at 4,000 rpm for 5 minutes. The supernatant was then transferred into Amicon® Ultra-4 Centrifugal filter (30 kDa) and further centrifuged at 4,000 rpm for a minimum of 15 minutes, until obtaining an unfiltered volume of 100 µL. The latter was then transferred into Eppendorf tubes with the addition of 500 µL of PB buffer (QIAGEN) and purified by MinElute kit (QIAGEN) and eluted in 55µL of EB buffer (QIAGEN) after an incubation of 10 minutes at 37°C.

Samples were subjected to amplification of different genomic traits used for the determination of sex[106]. For each sample, a reaction mix (total volume of 25 µL) consisting of a PCRBIO Mix (Reaction buffer 1x, 25 mM $MgCl_2$, and dNTPs 25 mM), a primer pair, ddH_20, TaqPol, and DNA was prepared. The first primers used for the amplification of this region were designed by Sullivan et al.[107] and generated 106 bp amplicons for *AMELX* marker or 112 bp amplicons for *AMELY* marker. Subsequently, the samples along with a negative and a positive control (two in case of polymorphisms associated with the amelogenin gene) were amplified (Bio-Rad T100TM Thermal Cycler). Moreover, SRY amplicons were also produced and analyzed[108].

The proper amplification of the fragments was verified through an electrophoretic run carried out on 12% polyacrylamide gel (for *AMEL*, *AMEL1,AMEL2,* and *SRY* markers, Table 1).

MARKERS	Primer sequences	Length PCR fragments	References
AMEL	F 5'-CCCTGGGCTCTGTAAAGAA-3' R 5'-ATCAGAGCTTAAACTGGGAAGCTG-3'	106 and 112 bp	Sullivan et al. (1993)[109]
AMEL1	F 5'- ACCCCTTTGAAGTGGTACCAGAGCAT-3' R 5'- GAACAAAATGTCTACATACYGGTGG-3'	80 e 83 bp	Haas-Rochholz e Weiler (1997)[110]
AMEL2	F 5'-CCCTTTGAAGTGGTACCAGAGCA-3' R 5'- GCATGCCTAATATTTTCAGGGAATA-3'	55 e 58 bp	Codina et al. (2009)[111]
SRY	F 5'-GAGTGAAGCGACCCATGAAC-3' R 5'-TTTCGCATTCTGGGATTCTC-3'	85 bp	Su e Lau 1993[112]

Table 1. Primer sequences and length of the amplicons

Results and discussion

The sample consists of 64 non-adult individuals: 52 from the archaeological site of Leopoli-Cencelle (9th-15th centuries CE) and 12 from Quarto Cappello del Prete (1st-

3rd centuries CE). For some individuals from Leopoli-Cencelle none of the methods could be applied due to remains' preservation status, so they were excluded from the analysis. All the other individuals were analyzed by morphological and morphometric approaches, and a subgroup of 19 individuals was subjected to molecular analyses.

Age at death assessment

Due to the lack and/or to the high fragmentation of some skeletal elements it was not possible to apply all the described methodologies for all the analyzed individuals.
The obtained age at death estimates for the individuals from Leopoli-Cencelle are reported below:

- Perinatal (<1 year) = 12 individuals
- Infant I (1-6 years) = 16 individuals
- Infant II (7-12 years) = 12 individuals
- Adolescents (13-18 years) = 12 individuals

For all the individuals it was possible to evaluate the fusion degree of the main skeletal ossification centers; for 29 individuals, however, it was not possible to observe the degree of dental eruption as the teeth were absent.
For 8 individuals aged between 13 and 18 years it was not possible to estimate the age by measuring the long bones as, although the epiphyses are not completely welded to the diaphysis, the average length was greater than those of the reference standard[113].
Age at death estimates for the 12 individuals from Quarto Cappello del Prete are reported below:

- Perinatal (<1 year) = 6 individuals
- Infant I (1-6 years) = 6 individuals

The obtained results show that the highest number of individuals (considering both the populations) are in the Infant I age group (1-6 years). This age group coincides with the weaning phase that causes a drastic change in the eating habits of infants[114]. This period is particularly challenging as the start of weaning may also lead to malabsorption problems and, at times, to gastrointestinal diseases to which children are particularly susceptible also because the immunity acquired by the mother during breastfeeding is interrupted[115,116]. The number of perinatal individuals is also high, as infants in this period, in addition to the stress related to birth itself, are extremely prone to virulent infectious diseases such as measles, pertussis, smallpox, and gastrointestinal infections which can often be fatal[117].

Sex assessment by morphological and osteometric approach

Sex determination by morphological approach was performed following the indications of Schutkowski[118], Rogers[119] and Stull et al.[120,121].

However, due to the brittleness of the non-adult skeletal elements, the absence and/or fragmentation of the informative areas prevented, in some cases, proper observation. In particular, the mandible is the least preserved element, especially in younger individuals. Notwithstanding, it was decided to include it in the protocol so as to be able to have more information when present. In the case of missing of some skeletal remains sex was determined by the osteometric analysis of the long bone length[122,123,124,125,126,127,128,129,130,131,132,133].

The approach proposed by Schutkoski[134] was applied to the entire sample for which the skeletal elements were preserved (51 individuals). However, for 6 individuals, aged between 13 and 18 years, the complete fusion of the *os coxae* hindered the analysis, so in these cases sex was assessed by the methods proposed by Acsádi and Nemeskéri[135] and by Ferembach et al.[136].

For 8 individuals sex could not be assessed by using the abovementioned method nor the methods for adult sex determination due to the fragmentation and/or absence of the informative skeletal elements but also due to the presence of an intermediate morphology for some of the observed traits which led to ambiguous results.

For the individuals for whom it was possible to analyze both the skeletal elements, sex assessment was concordant except from the individual SU 11604 (female on the basis of morphology of the mandible, and male for that of the ilium). However, this individual also showed discrepancies in age at death estimation protocols. We can therefore hypothesize that these differences found both in the estimate of the age at death and in the determination of sex may be linked to problems in the individual's skeletal development.

As regards the protocol proposed by Rogers[137] this is aimed at determining the sex in adolescents and was therefore applied to individuals from Leopoli-Cencelle belonging to the age groups infant II (7-12 years) and adolescents (13-18 years). Unfortunately, the humerus was recovered only in 17 individuals.

The measurement protocol of Stull et al.[138,139] concerning the diaphysis lengths of the long bones, was applied to a large number of samples. However, this method also presented some limitations: for perinatal individuals, it was not always possible to compare the results obtained from the measurements with those present in the standard proposed by Stull and collaborators[140,141], since they were lower than those considered (these were therefore recorded as ND, not determinable). Only for three individuals, aged between 13 and 18 years, sex was determined by osteometric analysis performed according to the standards for adult individuals[142,143,144,145,146,147,148,149,150,151,152]. Overall, the application of the morphological and morphometric approached led to the determination of sex of 55 individuals out of 64.

The obtained results are reported in Table 2.

LEOPOLI-CENCELLE						
PERINATAL (<1 year)						
Individual	**Mandible**[153]	**Ilium**[154]	**Long bones measurements**[155]	**Distal epiphysis of the humerus**[156]	**Adults morfological protocol**[157,158,159]	**Adults measu rement**[160,161,162, 163,164,165,166,167,168 ,169,170]
5696	ND	M	ND	-	-	-
5702	-	-	-	-	-	-
5703	A	ND	ND	-	-	-
5728	A	F	ND	-	-	-
5773	ND	M	ND	-	-	-
5960	M	M	M	-	-	-
9031	A	ND	ND	-	-	-
9035	A	F	ND	-	-	-
10116	A	M	ND	-	-	-
11615	A	ND	ND	-	-	-
11633	A	M	ND	-	-	-
11831	A	F	ND	-	-	-
INFANT I (1-6 years)						
5691	ND	ND	F	-	-	-
5738	M	M	M	-	-	-
5932	A	F	F	-	-	-
5946	A	M	M	-	-	-
5959	ND	M	ND	-	-	-
9115	M	M	M	-	-	-
9133Sb	M	M	M	-	-	-
10046	M	M	M	-	-	-
10086	ND	A	M	-	-	-
10093	ND	F	F	-	-	-
10100	M	M	M	-	-	-
10111	ND	M	M	-	-	-
11568	A	A	F	-	-	-
11737	A	F	ND	-	-	-
11870	-	-	-	-	-	-
14040	ND	M	M	-	-	-
INFANT II (7-12 years)						
11134	A	A	F	A	-	-
11166	F	F	F	F	-	-
11566	-	-	-	-	-	-
11591	ND	ND	F	F	-	-
11604	F	M	M	M	-	-
11698	M	M	ND	M	-	-
11861	A	M	ND	M	-	-
11908	A	M	F	M	-	-
11944	A	ND	F	F	-	-
14021	M	M	ND	M	-	-
14043	ND	A	M	M	-	-
14136	A	A	M	A	-	-

ADOLESCENTS (13-18 years)						
11586	A	ND	M	M	-	-
11620	-	-	-	A	M	F
11647	M	M	M	M	-	-
11734	A	A	F	A	-	-
11815	-	-	-	A	F	F
11821	A	ND	ND	M	F	-
11837	A	A	F	A	-	-
11846	A	M	M	M	-	-
14032	M	M	M	M	-	-
14055	-	-	-	M	M	M
14089	-	-	ND	M	M	ND
14121	-	-	ND	M	M	-
QUARTO CAPPELLO DEL PRETE						
PERINATAL (<1 year)						
T8A	A	M	A	-	-	-
T14	A	M	A	-	-	-
T16	A	F	A	-	-	-
T20A	M	M	A	-	-	-
T47A	A	M	A	-	-	-
T47B	A	F	A	-	-	-
INFANT I (1-6 years)						
T5	A	M	A	-	-	-
T6	A	F	A	-	-	-
T8	A	M	A	-	-	-
T36	M	M	A	-	-	-
T56	A	F	A	-	-	-
T59	A	M	A	-	-	-

Table 2. Results of the sex assessment by morphological and osteometric approach. For each method the sex determination was reported. M indicates males, F females, ND not determinable, A absent. The symbol "-was used for indicating the method was not applicable

As shown in Table 2 in the cases in which multiple methods could be applied (58 individuals) the sex assessment was consistent with a few exceptions (SU 11604, SU 11908, SU 11620, SU 11821).

As regards the adolescents, for six individuals sex was determined following the methodologies by Acsádi and Nemeskéri[171] and Ferembach et al.[172]. In one case (SU 11821) there was a discrepancy between the results obtained by the analysis of the *os coxae*[173,174,175] and that obtained by the method by Rogers et al.[176]. In one other case (SU 11620) a discrepancy was noted between the methods (morphological and morphometric) for adults but this is not surprising as the individual could be weak due to his young age.

Sex assessment by molecular approach

In order to confirm the results obtained from the morphological and osteometric analyses, for 19 individuals, sex was also determined by aDNA analysis analyzing sepa-

rately the amplified products for each of the four markers (*AMEL*, *AMEL1*, *AMEL2*, *SRY*). The obtained results are reported in Table 3.

Individual	AMEL **106/112 bp**	AMEL1 **55/58 bp**	AMEL2 **80/83 bp**	SRY **85 bp**	**Sex**
LEOPOLI-CENCELLE					
US 5691	M	/	/	M	M
US 5696	F?	/	F	/	F
US 5773	M	/	/	M	M
US 9035	F	/	/	M?	F
US 9115	M	/	/	M	M
US 10100	/	M	/	M	M
US 11591	M?	/	/	M	M
US 14032	M	M	/	/	M
US 14043	/	/	M	/	M
QUARTO CAPELLO DEL PRETE					
T5	M	M?	/	M	M
T6	F	M	M	/	M
T8	F	M?	F	/	F?
T8A	F	M?	M?	M	M
T14	M	M	M	M	M
T16	M	/	M	/	M
T20A	M	F?	F	M	M
T36	/	M	/	/	M
T47A	/	F	F	/	F
T56	F	?	F	M	M

Table 3. Results of the aDNA analysis for sex determination of the analyzed individuals

The heterogeneity of aDNA preservation did not allow the amplification of all the genetic markers (Table 3). This may be attributable to the degradation of the genomic pairing regions of the probes or to the fragmentation of the aDNA molecules. However, it is worth noticing that for almost all of the analyzed individuals (N= 13) at least two markers are consistent.

Despite the larger size of the *AMEL* amplicon, this allowed the typing of 13/19 individuals, while the *AMEL1* and *AMEL2* markers returned positive results only in 10/19 assays. This evidence appears in partial disagreement with the analytical hypotheses that envisaged the generation of short amplicons to overcome diagenetic problems.

In the evaluation of the discordant results (N=6) sex was preferentially resolved according to the concordance of multiple markers. However, the *SRY* determination was substantially critical for what concerns the masculine sex; as it is chromosome-specific and free from potential diagenetic biases that could modify the regions flanking the markers, resulting in a putative secondary allelic drop out[177].

As previously stated, the heterogeneity highlighted during the analysis is attributable to the characteristics of the ancient DNA, whose fragmentation and small quantity can

result in failures of the oligonucleotide annealing in the amplification reactions, making it sometimes impossible to amplify the markers. Nevertheless, these characteristics could generate false positives hints where the template molecule is represented by contaminating modern molecules, which cannot be determined through a target approach such as that based on selective amplification.
Table 4 shows the results of the combined morphological, morphometric and molecular approaches applied in the present research.

Individual	Sex determination by morphological and osteometric approach	Sex determination by aDNA analysis	Individual	Sex determination by morphological and osteometric approach	Sex determination by aDNA analysis
LEOPOLI-CENCELLE			QUARTO CAPELLO DEL PRETE		
US 5691	F	M	**T5**	M	M
US 5696	M	F	**T6**	F	M
US 5773	M	M	**T8**	M	F
US 9035	F	F	**T8A**	M	M
US 9115	M	M	**T14**	M	M
US 10100	M	M	**T16**	F	M
US 11591	F	M	**T20A**	M	M
US 14032	M	M	**T36**	M	M
US 14043	M	M	**T47A**	M	F
			T56	F	F

Table 4. Sex assessment for the morphological, osteometric and molecular approaches

A 63% match (12 out of 19 individuals) was observed in the estimates, 10 males and 2 females. Only one (SU 11591) out of the 7 individuals for whom it is possible to highlight an analytical discrepancy in the diagnosis of sex, belongs to the age group infant II (7-12years), while all the others fall into the perinatal (<1 year) and infantile I (1- 6 years) age groups. It should also be noted that in some discordant cases only one morphological/osteometric approach allowed to determine sex.
This evidence seems to suggest that although many of the sexually distinctive traits are present from birth, some characteristics become more marked during growth[178,179,180]. Accordingly, it could be hypothesized that this discrepancy may be attributable to erroneous individual assessments deriving from the incomplete development of the dimorphic features.

Conclusions

Sex was determined by applying several morphological and osteometric approaches described in the literature, which resulted in an overall concordance assignment but for four individuals. Otherwise, it was possible to confirm the obtained results by molecular sex determination only for the 63% of the 19 analyzed individuals.

Although preliminary and limited to a small analyzed sample, the obtained results show the prospective use of an adjunct simple and cost-effective practice to improve our knowledge about sexing of non-adult ancient individuals[181,182]. So far, the data are encouraging, especially when the resources for more sensitive approaches as those implemented in the cutting-edge genomic and proteomic evaluations are not feasible[183,184,185,186]. Further research is needed for enlarging the analyzed sample size to gain a more complete evaluation of paleodemography in skeletally immature individuals especially for the Roman Imperial communities[187,188].

Bibliography and notes

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