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Published Version:
Marino, R., Tanganelli, V., Pietrobelli, A., Belcastro, M.G. (2021). Evaluation of the auricular surface method for subadult sex estimation on Italian modern (19th to 20th century) identified skeletal collections. AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY, 174(4), 792-803 [10.1002/ajpa.24146].

Availability:
This version is available at: https://hdl.handle.net/11585/809618 since: 2024-11-12

Published:
DOI: http://doi.org/10.1002/ajpa.24146

(Article begins on next page)

Roberta Marino, Viola Tanganelli, Annalisa Pietrobelli, Maria Giovanna Belcastro (2021). Evaluation of the auricular surface method for subadult sex estimation on Italian modern (19th-20th c.) identified skeletal collections.

The final published version is available online at: https://doi.org/10.1002/ajpa.24146

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Running Title: Evaluation of the auricular surface method for subadult sex estimation

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ABSTRACT

Objectives: Sex estimation in subadult skeletal remains is still considered highly problematic. The aim of this research is to test the reliability of the method of subadult sex assessment proposed by Luna and co-workers in 2017, based on the analysis of the auricular surface of the ilium.

Materials and Methods: Seven ratios and three morphological traits were recorded for 127 subadult individuals (63 males and 64 females), aged between 0 and 17 years, from several Identified Skeletal Collections of the University of Bologna. Nonparametric Mann Whitney test, Kolmogorov Smirnov test, and the Pearson correlation coefficient were used for continuous variables, whereas the Cramer Coefficient was calculated for qualitative variable. A Principal Component Analysis was also performed on ratio values. The statistic η was taken into account for both types of variables.

Results: None of the ratios presented significant dimorphic results. Two qualitative variables show statistically significant differences between sexes. The overall morphology proved to be an accurate sex predictor among children aged ≥ 4 years (78% - 86%) and meets the minimum accuracy standard (75%) for subadult sex estimation for individuals between 1 and 17 years of age. The morphology of the Retroauricular End of the Superior Demiface (MRS) can be used with a high level of accuracy for sexing individuals from 1 to 12 years (77% - 81%).

Conclusions: The metric variables did not replicate the accuracy values originally obtained by Luna and co-workers. Otherwise, the evaluation of the morphological variables proposed by the authors yielded promising results as a reliable sexing technique for individuals who died before puberty.

KEYWORDS

Sex estimation, subadults, ilium, documented skeletons, sexual dimorphism

INTRODUCTION

Sex estimation from skeletal remains is a key point in the reconstruction of the biological profile, which is one of the primary goals for both physical and forensic anthropology. The assessment of sex is fundamental, as the estimation of age, stature and ancestry are considered sex dependent, according to most existing methods (White et al., 2012; Krishan, 2016). While there are several reliable methods for sexing adult remains, many sexually dimorphic features commonly used in adults perform less well in subadult skeletal remains, as they develop during puberty up to adulthood (Scheuer et al., 2000). Sexual dimorphism is a consequence of the differences in hormonal secretion between males and females. However, the process is also influenced by socio-environmental variables during growth and development (Humphrey, 1998; Scheuer et al., 2000; Scheuer and Black, 2000; Stinson, 2000; Cardoso, 2005; Cardoso, 2008; Luna et al., 2017; Zadzińska et al., 2008; Klales, 2017; Stull et al., 2017). Variations in morphology, size and robustness occur during puberty, which begins between 10 and 14 years, and it generally starts earlier in females than males (Scheuer et al., 2000; Scheuer and Black, 2000; Sutter, 2003; Vlak et al., 2008; Wilson et al., 2008; Rogers, 2009; Luna et al., 2017; Klales, 2017). Nevertheless, sex differences in hormones have been documented from early stages of foetal development and after birth, influencing long bone dimensions and body proportions throughout growth (Cardoso, 2005; Knickmeyer and Baron-Cohen, 2006; Stull et al., 2017).

Sex differences begin around the eighth week after conception, when the embryo goes through a rise in hormonal levels. By the twelfth week, sexual differentiation is almost complete (Fechner, 2003; Knickmeyer, 2006; Stull et al., 2013). The next period of increased hormone production, with median levels equal to the pubertal hormonal surge, is referred to as the neonatal hormonal surge (Fechner, 2003; Knickmeyer, 2006). During this particular stage of life, which lasts through the first year of life, levels of estrogen and testosterone are triggered by gonadotropin levels (Fechner, 2003; Knickmeyer, 2006). In males, hormone production increases between the third and fourth month after birth, while females have increased estradiol production, which fluctuate until 6 months of age (Loth and Henneberg, 2001; Fechner, 2003; Knickmeyer, 2006; Stull et al., 2013, Lanciotti et al., 2018). When puberty starts, the levels of gonadotropins rise, causing the gonads to enlarge, mature, and secrete increased amounts of gonadal steroids. The pubertal surge allows the development of secondary sexual characteristics, which provide a basis for sex estimation of adults (Fechner, 2003; Knickmeyer, 2006).

Difficulties in developing and testing methods of sex diagnosis in juveniles are also attributable to the scant number of subadults in identified human skeletal collections (Schutkowski, 1993; Loth and Henneberg, 2001; Rogers, 2009; Irurita, 2016). Nevertheless, during the last few decades numerous studies developed and implemented reliable techniques for sexing immature remains (Mittler and Sheridan, 1992; Holcomb and Konigsberg, 1995; Scheuer, 2002; Vlak, 2008; Cardoso and Saunders, 2008; Wilson et al., 2008; Irurita et al., 2016; Krishan et al., 2016; Luna et al., 2017; Campo et al., 2018).

As a general criterion, 85% accuracy is usually considered the minimum standard for adult sex estimation methods. However, according to DiGangi and Moore (2013, p. 107) "the goal for accuracy should be at least 75% (which is 50% better than chance)" for subadult sex estimation.

As most craniofacial growth occurs before puberty (Scheuer and Black, 2004), several studies aimed to find evidence of sexual dimorphism in facial bones, especially in the mandible (Schutkowski, 1993; Molleson et al., 1998; Loth and Henneberg, 2001; Scheuer, 2002; Sutter, 2003; Irurita et al., 2016) and orbital morphologies (Molleson et al., 1998). Other studies focused on sexing immature skeletal remains through measurements on deciduous dentition (Black, 1978; Rösing, 1983; De Vito and Saunders, 1990; Zadzińska et al., 2008; Cardoso, 2008). Long bone dimensions are also considered useful variables for subadult sex diagnosis (Choi and Trotter, 1970; Humphrey, 1998; Stull and Godde, 2013; Stull et al., 2017). As the pelvis is considered the skeletal area that express the highest degree of sexual dimorphism (Scheuer and Black, 2004), many studies have focused on this skeletal region. Various features were analysed, such as the greater sciatic notch (Boucher, 1957; Weaver, 1980; Schutkowski, 1993; Holcomb and Konigsberg, 1995; Sutter, 2003; Vlak, 2008, Wilson et al., 2008, 2011; Irurita et al., 2016), the subpubic concavity (Klales and Burns, 2017), the auricular surface elevation (Weaver, 1980; Mittler and Sheridan, 1992; Sutter, 2003), the arch criterion (Schutkowski, 1993; Sutter, 2003; Cardoso and Saunders, 2008; Irurita et al., 2016), the curvature of the iliac crest (Schutkowski, 1993; Sutter, 2003, Wilson et al., 2008, 2011; Irurita et al., 2016) and the auricular surface morphology (Wilson et al., 2008, 2011, Luna et al., 2017). Recently, a successful approach has been carried out through geometric morphometrics analysis of the ilium (Wilson et al., 2008; Wilson et al., 2011; Estevèz et al., 2017), while the application of advanced morphometric techniques in order to investigate the presence of sexual dimorphism of the immature pubis and ischium was less successful (Campo et al., 2018). Frequently, these proposed methods achieved very high sexing accuracies in the original study, while other validation studies obtained worse results. This issue is generally attributed to the size and composition of the sample or differences among populations (Scheuer, 2002; Sutter, 2003; Vlak, 2008; Cardoso and Saunders, 2008; Irurita et al., 2016). Several authors have previously attempted to explore the sexual dimorphism of the subadult auricular surface using different morphological and metric methods and by studying various features (Weaver, 1980; Mittler and Sheridan, 1992; Sutter, 2003; Wilson et al., 2008; 2011; Luna et al., 2017).

Recently, Luna and co-workers (2017) proposed a new method of sex diagnosis based on morphological and metric features of the auricular surface of the subadult ilia, using a juvenile sample (7-18 years of age) from the Coimbra Identified Skeletons Collection (Portugal) that offered promising results for sex allocation. Villotte (2018) applied this method on a Prehistoric Ligurian (Italy) immature individual, while Bonczarowska and co-workers (2019) tested exclusively the morphological variables on 194 adult individuals of Greek origin from a documented modern collection, with unsatisfactory results. This method has been newly validated by Monge Calleja and co-workers (2020) on a subadult sample ranging between 0-18 years of age (N=61), from the identified skeletal collection of Lisbon (Portugal), confirming the usefulness of the auricular surface for subadult sexual estimation, although offering better results for morphological rather than metric variables. As the authors suggest additional validation tests in documented individuals from other geographic regions, the aim of the present study is to test and validate the reliability of the morphological and metric variables of this method, by analysing a larger sample of identified individuals coming from other geographical areas. For this purpose, we selected an identified Italian subadult sample (N=127), representing several subadult age classes ranging from 0 to 17 years of age.

MATERIALS AND METHODS

A sample of 127 subadult individuals of known sex (females= 64; males= 63) and age (0-17 years) was considered in this study (Figure 1; Table S1). They belong to seven identified human skeletal collections housed at University of Bologna (Italy), coming from the Emilia Romagna (Bologna, Parma, Faenza) and Sardinia (Sassari, Cagliari) cemeteries. These collections refer to a time period spanning from 1899 to 1937. Most skeletons are associated with demographic and social information (full name, date and place of birth and death, cause of death, cf. Belcastro et al., 2017). Due to the high rate of infant mortality in the first months of age, the dataset is slightly skewed toward younger individuals. The sample was analysed according to the age classes proposed by Buikstra and Ubelaker (1994): infant (0-3 years), child (4-12 years) and adolescent (13-20 years), with the maximum age within the sample being 17 years.

The analysis of the auricular surface of the ilium implicates that the observer would be able to unambiguously identify specific reference points. As this task proved to be too challenging when the preservation status was assessed as poor or mediocre, only individuals with well-preserved ilia were selected for the purpose of this study (White and Folkens, 2005; Luna et al., 2017).

The analysis of the auricular surface was carried out using the metric and morphological variables proposed by Luna and collaborators (2017). First, photographs of the posterior area of the left ilia were taken for each individual of the sample, with a standardised distance of 20 cm between the camera and the bone. In the absence of the left ilium, digital images of the right ilia were considered for seven individuals. The bone was positioned with the inferior border placed vertically and a measuring grid was digitally superimposed (Figure 2) identifying the most inferior point of the border (α), where a vertical line along the inferior border was drawn. The point in the central area of the auricular surface coincident with the angle of the auricular edge (β) and the most superior point of the anterior area of the joint (γ), where used to draw two parallel lines. Three parallel lines were then outlined, orthogonal to the segments previously drawn. The first line should touch the anterior point of the auricular surface (δ), the second should touch the posterior point (ε), and the last line should include the angle of the auricular edge (β). Angle vertices were finally labelled with capital letters from A to I, in order to record various measurements, using an image analysis software (Digimizer), that are used to measure seven ratios, AC/CI, FI/CF, AB/BC, HI/EH, EF/CF, DE/AD and DE/EH. As originally proposed by Luna and colleagues (2017) and tested by Monge Calleja and colleagues (2020), we tested a discriminant function ($X = -3.470 + 0.865[DE/AD] + 2.739[FI/CF]$; section point = 0.078) and a logistic regression (Psex = 1/[(1 + e-(−25,819 + 37,723*(DE/AD) + [−2,320*(FI/CF)])]; section point = 0.5), utilizing selected measurements (DE/AD and FI/CF). Values higher that the section points indicate a male individual, while lower ones indicate female individuals.

No difficulty was experienced when attempting to follow the guidelines proposed by Luna et al. (2017) in order to correctly position the photograph of the auricular surfaces before drawing the grid. Three morphological variables of the auricular surface were considered: overall morphology (OM), morphology of the retroauricular end of the superior demiface (MRS, called Apex Morphology in Luna et al., 2017) and inflection (IN) (Figure 3). According to the authors who proposed the method, males are associated with an overall morphology of the auricular surface, which could be described as a lying V-shape; the anterior and inferior edges have a similar length, and the angle between them (εβγ) is obtuse. In females, the auricular surface resembles an inverted L-shape, the inferior margin is longer than the anterior margin, and these margins are perpendicular, also, the angle (εβγ), is approximately 90 degrees. The morphology of the retroauricular end of the superior demiface (Figure 3, “MRS”), has a morphology that could be defined as angular in males, while females shows a more rounded morphology (Luna et al., 2017). Furthermore, Luna and co-workers (2017) proposed that the

inflection, which can be defined as an indentation located in the area of the angle εβγ, is absent or attenuated in males, while females show an easily distinguishable and very clearly defined inflection.
The statistical analysis was performed using SPSS 23, PAST 3 and Minitab 18, following the same procedures of Luna and colleagues (2017). Intra- and inter-observer errors were evaluated on a selected sub-sample of 20 individuals, calculating the Cohen kappa coefficient (κ) for qualitative variables and the intraclass correlation coefficient (ICC) for the quantitative variables, normally distributed in this sample-subset. To evaluate intra- and inter-observer bias, each variable was recorded twice, without prior knowledge of sex and age. Each set of observations was carried out at least one week apart. The grids on the auricular surface were also drawn twice in order to evaluate the precision with which each reference point can be identified by a trained observer. After determining the minimum (Min), maximum (Max), mean and standard deviation (SD) values for all the ratios by sex, a section point (SP) for each ratio, the mid-point between male and female means, was calculated, separating female (below section point) from male features (above section point) (Albanese et al., 2005; Cardoso, 2008; Luna et al., 2017). The percentages of a correct discrimination and the likelihoods of a correct allocation were calculated for males, females and for the whole sample. The likelihood of a correct allocation indicates the ratio of individuals in which a certain dimorphic feature matches the known sex vs. the overall number of individuals exhibiting the same feature regardless of known sex. This allows for understanding the likelihood of correct sex prediction of a new individual (Mittler and Sheridan, 1992; Irurita et al., 2016; Luna et al., 2017).
For the whole sample, the normal distribution of our metric variables was assessed with a Shapiro-Wilks normality test, which revealed that our metric variables differ from normal distribution (p-values < 0.05). For subsequent statistical analysis, we opted to closely follow the statistical procedures that were originally adopted in the study by Luna and co-workers (2017). For continuous metric variables (AC/CI, FI/CF, AB/BC, HI/EH, EF/CF, DE/AD and DE/EH), both the nonparametric Mann-Whitney (U) and the Kolmogorov Smirnov (Z) tests were used to evaluate the degree of significance of the observed differences between sexes (Bryman and Cramer, 2004), while the Pearson correlation coefficient (r) was used to evaluate the influence of age for each metric variable. We opted for the Pearson correlation coefficient despite the non-normal distribution of our data, since this statistic is considered robust against deviations from normality of the tested variables (Edgell and Noon, 1984). The Coefficient of Cramer (V) was used for the qualitative variable, in order to assess the association between the morphological variables and sex (Bryman and Cramer, 2004). The statistic eta (η) was considered for both metric and morphological variables and was used to measure the degree of association between sex and the different values for each ratio, and the correlation between age at death and the morphological features (Siegel, 1956). Then, a principal component

analysis (PCA) was realized on ratios values (James et al., 2013). Changes in hormone levels in children during development have been taken into account in order to assess if sexual dimorphism is present in younger ages. To evaluate the impact of very young (<1 years) individuals on the application of the method, some analyses were also carried out excluding those individuals, in order to verify if this precaution yielded better results, as the auricular surface show very high variation during the first year of life (Reynolds, 1945; Cunningham, 2009; Yusof, 2013).

RESULTS

Intra- and inter-observer errors results are shown in Table 1, indicating good/substantial to very high replicability of the recording of the features, except for inflection, for which the replicability is low. Descriptive statistics are shown in Table 2, which indicates that most of the ratios exhibit very similar minimum, maximum, mean and standard deviation values, however, FI/CF, AB/BC/ and DE/AD show more variation between sexes. No ratios show percentages of correct assignment considered acceptable (≥75%) (DiGangi and Moore, 2013) for the whole sample, with values ranging between 46% and 54% when taking into consideration both males and females together (Table 3). When males and females are grouped separately, the values of the correctly assigned cases ranged between 23% and 57%, with the only exception being FI/CF, which reached a correct assignment score of 75 % in males, while females reached only 23%. A similar situation is observed when considering the three age classes separately. The values of the correctly assigned cases, taking into consideration both sexes, ranged between 40% and 66%. When males and females are grouped separately, in both the 4-12 and 13-17 age classes, females performed better, with values ranging between 45% and 80%, and between 0% and 50% in males. In the 13 - 17 age class, for HI/EH and EF/CF males performed better, with correct assignment scores of 83% and 75% respectively, while females reached only 30% (HI/EH) and 45% (EF/CF) (Table 3). As regards the tested discriminant function (DF) and logistic regression (LG) formulas, males obtained correct assignment scores ranging from 53-83%, generally higher than in females (13%-37%). However, when males and females are grouped together, both functions correctly assign only 41% to 61%, below the acceptable threshold of 75% (DiGangi and Moore, 2013). This trend is observable both within different age classes and within the whole sample. On the contrary, for the morphological features, the overall morphology reached a percentage of global correct assignment as high as 73% (Table 3). When males and females were grouped separately, the latter performed better, with a correct assignment score of 78%, while males reached only 68%. In the 4-12 age class, overall morphology correctly assigned 86% of the cases in the whole sample and 66% and 100% for males and females, respectively. Similar values emerged from the analysis of the 13–17 age class, were 78% of the individuals were sexed correctly in the whole sample. The MRS reached a good global result of 81% in the 4-12 age class, with a correct assignment score of 83% in females and 78% in males. For the inflection, the percentages of the overall correct assignation for the whole sample and the three different age ranges are all close to 50% (Table 3).

The likelihood of a correct allocation for all the ratios, discriminant function and logistic regression, taking into consideration the whole sample and the age at-death categories, are generally close to 50%, reaching maximum 62% likelihoods of a correct allocation based on the logistic regression in males aged 0-3 years (Table 4). For the morphological features, there is a 73% probability of estimating correctly the sex considering overall morphology in the whole sample; the likelihood of an individual with an inverted L-shape auricular surface to be female is 71%, while the probability of an individual with a lying V-shaped auricular surface morphology to be male is 75%. For both the 4-12 and 13-17 age classes, the global likelihood is higher (78%-86%). The probability of correctly estimate the sex considering MRS is high for individuals between 4 and 12 years with a global value of 81%. Likelihood values for the inflection are much lower, all close to 50%.

With the exclusion of individuals <1 year, the ratios (FI/CF and DE/AD) which offered the most dimorphic results in the study by Luna and co-workers (2017), yielded almost the same results as those obtained considering the complete sample (Tables S2 and S3).

For the morphological variables, the overall morphology showed a better performance, correctly assigning 76% of all cases. When males and females were grouped separately, females reached a correct assignment score of 84% and males only of 66%. Our results did not show any significant difference in the auricular surface between male and female morphology during the first year of life (Tables S2 and S3). MRS performed better for 1-2-year individuals with a global result of 76% and the general likelihood of correct sex estimation was 75%. This trait appears to be more diagnostic for males than females. In comparison, OM did not show a marked improvement in accuracy; 50% of males and 86% of females were accurately identified for individuals 1-2 years old. For the inflection, the results mirrored the results obtained taking into consideration the whole sample.

The coefficients of Cramer V for the overall morphology and the morphology of the retroauricular end of the superior demiface show a strong level of association between the obtained results and sex (overall morphology: V = 0.466, p<0. 001; MRS: V=0.323, p<0.001), while the level of association between inflection and sex is not significant (inflection: V = 0.125, p = 0.158) (Table S4). The measure of association obtained with η between the results and age-at-death is very weak for the three features (overall morphology: η = 0.112, MRS: η = 0.116, inflection: η = 0.055). The coefficients of Cramer V estimated excluding the individuals < 1 year are higher for both overall morphology and morphology of the retroauricular end of the superior demiface (Table S4). The Mann-Whitney (U) and Kolmogorov Smirnov (Z) tests showed no significant statistical difference for all the ratios between sexes (Table 5). The measure of association obtained with η between sex and the different values for each ratio is very weak. Pearson correlation coefficient (r) values implies that the metric data were not conditioned by the age-at-death (Table 5). The PCA on ratios values showed no sex discrimination (Figure 4). The first two components explain 93.8 % of the total variance, with the first principal component accounting for 88.3 % of the variability. There is not a substantial separation between female and male data.

DISCUSSION

Over the last few decades, various studies suggested that changes in the shape of the subadult pelvis are evident beginning in early childhood (Holcomb and Konigsberg, 1995; Scheuer and Black, 2000; Vlak et al., 2008; Wilson et al., 2008; Cardoso and Saunders, 2008; Irurita and Alemán, 2016). This research attempts to apply and validate a method of subadult sex assessment based on the analysis of the auricular surface of the ilium proposed by Luna and co-workers in 2017. In the current study, the use of a larger Italian sample (N=127), distributed over a wider age range and a sex ratio of almost 1:1, allowed for a broad testing in a population of different geographical context than the one in which the method was implemented and validated (Luna et al. 2017; Monge Calleja et al., 2020). The reproducibility of the method is confirmed by the low interobserver errors in the Portuguese samples (Luna et al. 2017; Monge Calleja et al., 2020) and in the current research, where the replicability of the recording procedures of the features ranged from good/substantial to very high, except for inflection, for which the agreement was low. This result can be attributed to difficulties in visually assessing this trait, as the irregular edges of the joint surface could be erroneously identified as presence of an inflection. The same consideration was made by Bonczarowska and co-workers (2019). The application of Luna and coworkers' (2017) method is affected by the state of preservation of the auricular surface, as this area is generally subject to post mortem modification. In cases where the preservation of the skeleton might be an issue, such as in archaeological settings, the use of this method is not without difficulties. The overall poor state of bone preservation in an archaeological context might prevent the construction of the grid and possibly lead to inconsistent evaluation of the proposed features.

It is often suggested that metric methods may provide a more objective approach. Rmoutilová and co-workers (2017) estimated the sexual dimorphism of the posterior ilium on three European osteological adult samples, comparing the accuracy of geometric morphometric (GM) and traditional morphological methods. Their results indicated that the visual evaluation of the auricular surface was affected by a large inter-observer error compared to when the metric assessment of the features was carried out. Luna and co-workers (2017) have shown that two ratios (DE/AD and FI/CF) yielded promising results. Nevertheless, in our study the metric variables offered unsatisfactory results, as none of the ratios emerged as dimorphic enough to allow a high probability of correct assignations. Sporadically high correct assignment was likely due to sampling factors. In fact, our testing of the discriminant function (DF) and the linear regression formula (LG), revealed low percentages of correct allocation and likelihood and correct estimation for the whole sample, as well as within different age classes, as expected since the two selected ratio present insufficient discriminatory power. It is interesting to note, though, that when sexes are grouped separately, both DF and LG formulas yielded better accuracy values for males, up to 83 %. This is in fact mirrored by the results of Monge Calleja and colleagues (2020), in which accuracy percentages for DF an LG in males are found to be higher than in females in most cases.

On the contrary, the outcomes of the morphological features in our study lead to some encouraging results, with the highest accuracy percentages ranging between 73%-86%. Among the three qualitative variables analysed, overall and retroauricular end of the superior demiface morphologies proved to be dimorphic, showing statistically significant differences between sexes, while the inflection did not demonstrate a dimorphic pattern. This confirms the results of Luna and co-workers (2017), which correctly assigned 73.3% of the cases for both overall and retroauricular end of the superior demiface morphologies. A similar trend was also observed by Monge Calleja and co-workers (2020), as morphological features provided better results than the metric variables on the Lisbon Identified Collection, stressing that the application of the metric variables must be done with caution. However, Bonczarowska and co-workers (2019) found that the morphological variables failed to produce satisfactory results on adult Greek individuals, with overall accuracies of 36%, 50% and 53%. This raises the question on whether this method can be used with accuracy on adults.

In the current study, overall and retroauricular end of the superior demiface morphologies performed better in the 1-17 years subset, as the high variability of the ilium, and consequently of the auricular surface, during the first year of life seems to have a negative impact on the performance of this method, in contrast with the trend identified by Monge Calleja and co-workers (2020), which highlighted higher accuracies and probabilities of correct sex estimation for the 0–2 years age group, the range of age within which hormone production levels are higher, compared to later ages (Lanciotti, 2018). The result of the peak of the neonatal hormone surge (3 – 4 months), if detectable in the auricular surface metric and morphological features, should have resulted in a sexual dimorphic difference in the corresponding age cohort (0 - 1 years). However, in this study it appears that the level of success is less marked during this period than the one achieved in the older age ranges (Table S2 and S3). Even though the possible effect of hormonal difference is not evident in our results, this is not to say that this event does not affect the auricular surface's features, or would be prevalent in different samples, as shown by Monge Calleja and co-workers (2020). Larger sample sizes may allow for a clearer insight into true growth differences between males and females within this age group. Overall, it is recommended that this method should be used with caution when assigning sex to human remains of this age class.

Our findings indicate that the morphology of the retroauricular end of the superior demiface can be used with a high level of accuracy for sexing individuals between one and 12 years (77% - 81%), despite its performance seeming to be age-dependent. Indeed, MRS appears to be more diagnostic for males than females for individuals between 1 and 2 years old (80%), while in the 4-12 age range, it gives better results in females than males (83%). This is in contrast to the results of Luna and collaborators (2017) but is analogous to the results obtained by Monge Calleja and co-workers (2020). The performance of MRS exhibited a marked improvement in accuracy for individuals 1-2 years old, which may be derived from the hormonal process. The overall morphology also demonstrated to be a reliable tool for sex estimation for children > 4 years. For individuals aged between one and 17 years, this feature scores over 75% accuracy, which is usually considered the minimum standard for subadult sex estimation methods (DiGangi and Moore, 2013). For the individuals >13 years, this variable performed better in males (83%), while for individuals <13 years, it performed better in females (78%-100%). A similar trend was identified by Monge Calleja and co-workers (2020). However, this finding contrasts with the results produced by Luna and co-workers (2017), which highlighted an opposite, inverse trend, where in individuals aged < 13 and > 13 years the overall morphology performed better for males and females, respectively. This difference could be attributed to the sex distribution of the Coimbra sample (21 females and 13 males).

The percentages and probabilities of correct sex estimation, considering both metric and morphological variables, are not systematically better for the older age group, as it would be expected considering the sex variation in the levels of pubertal hormonal secretion (Tables 3 and 4). Our findings indicate that OM can be used with a high level of accuracy for sexing individuals between 13 and 17 years. Nevertheless, MRS correctly assigned only 59% of the cases in the same age cohort (Table 3). The relatively small sample size of this age group (13 - 17 years; N=32) may have had implications on the application of the method, possibly concealing any trends that may be associated with the pubertal hormonal surge (Table 3 and 4). Continued growth through adolescence could also possibly affect the appearance of this area of the auricular surface (Rogers, 2009). It should also be considered that the onset of puberty could significantly vary among different populations or even between individuals of the same population (Scheuer and Black, 2000; Klales, 2017). Thus, the reduced performance may also be due to the fact that in the last century the onset of puberty progressively shifted towards younger ages in several European countries, with a levelling off in the last decades. Studies on Italian populations (e.g. Benso et al., 1986, Castellino et al., 2005), demonstrated that children living in Italy begin pubertal development at a younger ages (on average 1 year earlier) than suggested in Tanner's percentiles (Marshall and Tanner, 1969; 1970), which are generally used as reference in clinical practice, with the mean age at onset of puberty being 10.3 years in females and 11.2 years in males (Castellino et al., 2005). Hence, it is possible that the results obtained for the older age group may have been influenced by this factor.

Divergence between the accuracies of our study and that of Luna and co-workers (2017), especially for the metric variables and both discriminant function and logistic regression formulas, could be related to the different sample size as in the latter the sample (34 individuals) was smaller than the sample used for this study, ranging in age from 7 to 18 years. This is consistent with the findings of Monge Calleja and co-workers (2020), as with the application of the method in a larger sample (61 individuals) the metric features showed a reduced accuracy. Genetic, environmental and sociocultural factors (such as malnutrition, diseases, and lifestyle) may play a very important role in establishing sex-related differences among populations (Hall, 1978; Johnston and Zimmer, 1989; Scheuer and McLaughlin-Black, 1994; Belcastro et al., 2019). Actually, differences between Italian and Portuguese populations already emerged in previous studies focused on other postcranial skeletal features in adult samples (e.g. sacral vertebral body fusion, variations in epiphyseal fusion) (Belcastro et al., 2008; Belcastro et al., 2019). Several studies observed inter-population variation in the degree of sexual dimorphism of various pelvic features (Steyn et al., 2004; Patriquin et al., 2003; Walker, 2005). Furthermore, the study of Rmoutilová and collaborators (2017) mentioned above confirmed the presence of differences in the shape of the auricular surface of the ilium among English, French and Portuguese populations, adding new data to the previous observation that sexual dimorphism of the pelvis may show high level of variation due to inter-population differences (Walker, 2005). It is important to acknowledge that the development of biological sex is intricate, involving not only anatomy but also a complex choreography of genetic and chemical factors that unfolds over time (Hobbs, 2017). Understanding this complexity is critical. It is argued that biological sex is not a dichotomous feature and should be considered as a continuum or spectrum (Johnson and Repta, 2012). There is a range of variation in the expression of sex characteristics at both genotypic and phenotypic levels. By birth, a baby has five layers of sex: chromosomal sex, differentiated fetal gonadal sex, fetal hormonal sex, fetal internal reproductive sex and genital sex (Fausto-Sterling, 2012). Despite the fact that standards for recording human remains advise the use of five categories: female, probably male, unknown or indeterminate sex, male and probably male (e.g. Buikstra & Ubelaker, 1994), suggesting a wider non-binary range of sex variation, the majority of studies continue to place human remains into one of the two categories. It should be useful to present the data for the sex-attribution counting also the uncertain attributions that may reflect a biological feature. According to Fausto-Sterling (1993, p.21) “Sex is a vast infinitely malleable continuum that defies the constraints of even five categories”. Thus, it is plausible to assume that in subadults, the boundary between the sexes becomes even more blurred, related to the sex hormone surges, and forcing individuals in terms of the binary categories of male and female can account for the lower reliabilities of this method or any subadult sex estimation method.

CONCLUSIONS

The current study represents the first validation study in subadults of Luna and coworkers’ method (2017) conducted to demonstrate the reliability of both metric and morphological variables outside of Portugal. The metric approach of Luna and co-workers (2017) proved to be less effective in the Italian sample utilized in this study, partially confirming the validation study of Monge Calleja and co-workers (2020). In light of our results, we suggest that the use of the metric variables may be problematic and should be cautiously applied to other skeletal populations, whereas the morphological approach yielded promising results. The present findings indicate that two qualitative variables of the auricular surface of the ilium, the overall morphology and the morphology of the retroauricular end of the superior demiface , can be considered as suitable indicators for sex estimation in subadult individuals from an early age and are reliable enough for forensic and archaeological cases, as Monge Calleja and co-workers previously highlighted. Although, it must be taken into account that poor preservation status could make the application of the method challenging.

Our results showed that the overall morphology does meet the minimum acceptable accuracy for subadult sex estimation for individuals from 1 to 17 years (75%), while the MRS can be considered an accurate sex predictor among children from 1 to 12 years (77% - 81%). Future research should focus on the improvement of the metric approach, and on the development of more clearly identifiable landmarks on the auricular surface. Moreover, the results of the inter-observer error call for a more detailed definition of the dimorphic features that would decrease the ambiguity of diagnosis among different researchers. The morphological approach proposed by Luna and co-workers seems to be replicable in populations from different geographical areas, even though it would be useful to test the procedure on other identified samples outside the Mediterranean area. As already suggested in previous studies, in order to improve the accuracy of subadult sex estimation, it will be useful to incorporate the results of the morphology of the auricular surface into a multifactorial approach, combining several pelvic variables with other osseous and tooth features (Mittler and Sheridan, 1992;

Schutkowski, 1993; Sutter, 2003; Cardoso, 2008; Cardoso and Saunders, 2008; Zadzińska, 2008; Wilson et al., 2008; Irurita et al., 2016; Estevèz et al., 2017; Stull et al., 2017) .

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article

TABLE S1 Age and sex composition of the subadult sample

TABLE S2 Frequencies of cases correctly assigned for all the variables, for sex, considering two different age groups and the total sample with the exclusion of individuals younger than one year of life, calculated to evaluate the impact of very young (<1 years) individuals on the application of the method. The highest values (≥75%) are in bold.

TABLE S3 The likelihoods of a correct allocation of all the variables, for sex and considering two different age groups and the total sample with the exclusion of individuals younger than one year of life, calculated to evaluate the impact of very young (<1 years) individuals on the application of the method. The highest values (≥75%) are in bold.

TABLE S4 Results of the Coefficient of Cramer V test and statistic eta (η), applied to the morphological variables to assess their association with sex. *Statistically significant *p* values

TABLE S5 Coefficient of Cramer V test's results, applied to the morphological variables, calculated excluding individuals under one year of life from the sample. * Statistically significant *p* values

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TABLE 3 Frequencies of cases correctly assigned for all the variables, for sex and considering the whole sample and three different age groups. The highest values (≥75%) are in bold

TABLE 4 The likelihoods of a correct allocation of all the variables, for sex and considering the whole sample and three different age groups. The highest values (≥75%) are in bold

TABLE 5 Results of the statistical tests applied to metric variables to evaluate the degree of significance of the observed differences between sexes (Mann Whitney U and Kolmogorov Smirnov Z), to measure the degree of association between sex and the different values for each ratio (eta η) and to evaluate the influence of age of death for each metric variable (Pearson *r*)

FIGURE 1 Distribution of the individuals of the sample, by age and sex

FIGURE 2 Example of the measuring grid drawn on the auricular surface of the ilium of CA2 - 32B individual (10-year-old female). MRS: indicates the morphology of the retroauricular end of the superior demiface. In: indicates the location of the inflection

FIGURE 3 Examples of sexual dimorphic characteristics of the auricular surface. Left: 8-year-old female (BO82-28B) right: 4-year old male (BO4-11A)

FIGURE 4 PCA space plots of the metric variables. The first two components together explain 93.8 % of the variability. Females: circles. Males: squares

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.