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Sex determination of human remains from peptides in tooth enamel

Nicolas Andre Stewart¹,², Raquel Fernanda Gerlach³, Rebecca L. Gowland⁴, Kurt J. Gron⁵, and Janet Montgomery⁶

¹School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton BN2 4AT, United Kingdom; ²Department of Morphology, Physiology, and Basic Pathology, School of Dentistry of Ribeirão Preto, University of São Paulo, FORP/USP, 807 São Paulo, Brazil; and ³Department of Archaeology, Durham University, Durham DH1 3LE, United Kingdom

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The assignment of biological sex to archaeological human skeletons is a fundamental requirement for the reconstruction of the human past. It is conventionally and routinely performed on adults using metric analysis and morphological traits arising from postpubertal sexual dimorphism. A maximum accuracy of ~95% is possible if both the cranium and os coxae are present and intact, but this is seldom achievable for all skeletons. Furthermore, for infants and juveniles, there are no reliable morphological methods for sex determination without resorting to DNA analysis, which requires good DNA survival and is time-consuming. Consequently, sex determination of juvenile remains is rarely undertaken, and a dependable and expedient method that can correctly assign biological sex to human remains of any age is highly desirable. Here we present a method for sex determination of human remains by means of a minimally destructive surface acid etching of tooth enamel and subsequent identification of sex chromosome-linked isoforms of amelogenin, an enamel-forming protein, by nanoflow liquid chromatography mass spectrometry. Tooth enamel is the hardest tissue in the human body and survives burial exceptionally well, even when the rest of the skeleton or DNA in the organic fraction has decayed. Our method can reliably determine the biological sex of humans of any age using a body tissue that is difficult to cross-contaminate and is most likely to survive. The application of this method will make sex determination of adults and, for the first time, juveniles a reliable and routine activity in future bioarchaeological and medico-legal science contexts.

Significance

The ability to assign biological sex to human skeletal remains is a fundamental requirement in archaeology, paleoanthropology, and medico-legal sciences. While DNA sequencing can be used, it is expensive, time-consuming, and often fails due to the poor quality of the remaining DNA. An easier, more reliable, and consistently applicable method is needed. We present a method for sex determination of human remains using peptides retrieved from tooth enamel. Amelogenin is an enamel-forming protein encoded for by both chromosomes X and Y, with slight differences in their amino acid sequences. Peptides with these differences were identified by nanoflow liquid chromatography mass spectrometry and found to correctly assign sex to archaeological human remains of various chronological ages, from hundreds to thousands of years old.


The authors declare no conflict of interest.

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Fig. 1. A representative base peak chromatogram (300–1,600 m/z) produced from Fewston sample SK339. (Inset) Amino acid sequences of the two dimorphic peptides of amelogenin: AMELY-(58-64) peptide and AMELX-(44-52) peptide. The reconstructed ion chromatograms (to 4 ppm) for each of these are shown in red and blue, respectively, with full-scan MS and corresponding MS/MS below.
are processed by proteases, resulting in peptides of varying lengths, and remain in the mature enamel (9, 11, 12).

For amelogenin, peptides from the central portion of the protein are absent, but peptides from the N and C termini remain and have been identified (PRIDE identifier PXD007856). The dimorphic differences between amelogenin X and Y are found in these regions, and one such peptide identified is the AMELY-(58-64) peptide, which possesses an additional methionine compared

Fig. 2. Reconstructed ion chromatograms for the AMELY-(58-64) peptide (440.2233 m/z) and AMELX-(44-52) peptide (540.2796 m/z) (4 ppm mass tolerance) for the seven 19th century Fewston samples. Peaks corresponding to these are shown in red and blue, respectively. Known sex and age at death are indicated.
Fig. 3. Reconstructed ion chromatograms for the AMELY-(58-64) peptide (440.2233 m/z) and AMELX-(44-52) peptide (540.2796 m/z) (4 ppm mass tolerance) for three male/female pairs of archaeological samples from St. Guthlac’s Priory, 12th–16th century AD (A); Whitwell, ca. 5,700 BP (B); and Seaham, 7th–9th century AD (C) (previously published). Osteological age and sex determinations are indicated.
with the aligned sequence of AMELX (Fig. 1, Inset). In one of the samples (SK130), the AMELX peptide seemed to be relatively lower in abundance compared with the AMELY peptide. This most likely reflects a higher relative amount of the AMELY peptide, as this peptide contains a methionine, and it may be oxidized in greater amount in this sample. Therefore, the oxidized version of the AMELY peptide was chosen for Y sex confirmation, as it is expected to predominate in old samples, as opposed to the unoxidized peptide, which is anticipated to be of low abundance or absent.

A peptide from amelogenin X, AMELX-(44-52) peptide, with similar ion intensity to the AMELY-(58-64) peptide, was used to clearly assign sex based on the presence or absence of AMELY-(58-64) from the seven Fewston samples (Fig. 2) and the three sets of sex-paired samples (Fig. 3).

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**Table 1. Details of samples used in this study**

<table>
<thead>
<tr>
<th>Site location</th>
<th>Period</th>
<th>Type of burial</th>
<th>Skeleton no.</th>
<th>Age and sex*</th>
<th>Methods used to determine sex</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whitwell, Derbyshire, UK</td>
<td>Neolithic ca. 5,700 BP</td>
<td>Fragmentary, disarticulated cranium (SK485) and articulating mandible (SK219)</td>
<td>SK219</td>
<td>Adult female</td>
<td>Morphological traits of the mandible of SK219 and the articulating maxilla of SK485 (3, 14); marked sexual dimorphism</td>
<td>(15)</td>
</tr>
<tr>
<td>Whitwell, Derbyshire, UK</td>
<td>Neolithic ca. 5,700 BP</td>
<td>Fragmentary, disarticulated mandible</td>
<td>SK53</td>
<td>Adult male</td>
<td>Morphological traits of the mandible only (3, 14); marked sexual dimorphism</td>
<td>(15)</td>
</tr>
<tr>
<td>Seaham, County Durham, UK</td>
<td>7th–9th century AD</td>
<td>Inhumation cemetery</td>
<td>FFS SK15</td>
<td>Female 26–45 y</td>
<td>Morphological traits of the pelvis and skull (3, 14, 16)</td>
<td>(17)</td>
</tr>
<tr>
<td>Seaham, County Durham, UK</td>
<td>7th–9th century AD</td>
<td>Inhumation cemetery</td>
<td>FFS SK3</td>
<td>Male 36+ y</td>
<td>Morphological traits of the pelvis and skull (3, 14, 16)</td>
<td>(17)</td>
</tr>
<tr>
<td>St Guthlac’s Priory, Hereford, UK</td>
<td>12th–16th century AD</td>
<td>Inhumation cemetery</td>
<td>SK 9503</td>
<td>Female old adult</td>
<td>Morphological traits of the pelvis and skull (3, 14, 16)</td>
<td>(18)</td>
</tr>
<tr>
<td>St Guthlac’s Priory, Hereford, UK</td>
<td>12th–16th century AD</td>
<td>Inhumation cemetery</td>
<td>SK 9515</td>
<td>Male old adult</td>
<td>Morphological traits of the pelvis and skull (3, 14, 16); significant grave goods: chalice</td>
<td>(18)</td>
</tr>
<tr>
<td>Fewston, North Yorkshire, UK</td>
<td>19th century AD</td>
<td>Inhumation cemetery</td>
<td>SK363</td>
<td>Female 54 y</td>
<td>Documented age and sex: coffin plate; morphological traits of the pelvis and skull (2, 3, 16)</td>
<td>(13)</td>
</tr>
<tr>
<td>Fewston, North Yorkshire, UK</td>
<td>19th century AD</td>
<td>Inhumation cemetery</td>
<td>SK378</td>
<td>Female 34 y</td>
<td>Documented age and sex: coffin plate; morphological traits of the pelvis and skull (2, 3, 16)</td>
<td>(13)</td>
</tr>
<tr>
<td>Fewston, North Yorkshire, UK</td>
<td>19th century AD</td>
<td>Inhumation cemetery</td>
<td>SK366</td>
<td>Male 76 y</td>
<td>Documented age and sex: coffin plate; morphological traits of the pelvis and skull (2, 3, 16)</td>
<td>(13)</td>
</tr>
<tr>
<td>Fewston, North Yorkshire, UK</td>
<td>19th century AD</td>
<td>Inhumation cemetery</td>
<td>SK119</td>
<td>Male 38 y</td>
<td>Documented age and sex: coffin plate; morphological traits of the pelvis and skull (2, 3, 16)</td>
<td>(13)</td>
</tr>
<tr>
<td>Fewston, North Yorkshire, UK</td>
<td>19th century AD</td>
<td>Inhumation cemetery</td>
<td>SK339</td>
<td>Male 41 y</td>
<td>Documented age and sex: coffin plate; morphological traits of the pelvis and skull (2, 3, 16)</td>
<td>(13)</td>
</tr>
<tr>
<td>Fewston, North Yorkshire, UK</td>
<td>19th century AD</td>
<td>Inhumation cemetery</td>
<td>SK130</td>
<td>Male 66 y</td>
<td>Documented age and sex: coffin plate; morphological traits of the pelvis and cranium (2, 3, 16)</td>
<td>(13)</td>
</tr>
<tr>
<td>Fewston, North Yorkshire, UK</td>
<td>19th century AD</td>
<td>Inhumation cemetery</td>
<td>SK351</td>
<td>Male 63 y</td>
<td>Documented age and sex: grave stone; morphological traits of the pelvis and skull (2, 3, 16)</td>
<td>(13)</td>
</tr>
</tbody>
</table>

*As previously determined by osteological, epigraphic, and grave goods.
results agree with the assignment of sex by either coffin plates or standard osteological methods.

It should be noted that this method identifies the presence of peptides originating from sex chromosome-linked isoforms of amelogenin and currently cannot identify polymorphisms of multiple copies of these chromosomes (e.g., aneuploid 47, XXX or 47, XXY). Quantitation of these peptides may allow for this in these rare cases and warrants further investigation.

The ability to determine the sex of infant and juvenile remains completely revolutionizes studies of growth, child care, epidemiology, and demography in the past. For the first time, it will allow osteologists to examine sex-specific cultural treatment and differentiate between the health of boys and girls, as well as sex-specific growth trajectories and past developmental milestones, such as age of puberty and subsequent repercussions for fertility.

Sites with poor preservation are common in archaeological contexts, and at such sites teeth generally survive better than bone, and thus sex can be established for adults as well as juvenile skeletons in the absence of key skeletal identifiers. In addition, the dimorphic peptide sequence is identical in apes (Fig. S1) and so should be present in all hominins. Finally, this technique will also have a transformative effect on human identification in medicolegal contexts, such as mass disasters and war graves, allowing sex to be established both reliably and cost-effectively.

Materials and Methods

Fewston is a small village located in the Washburn Valley, near Harrogate in North Yorkshire, United Kingdom. The skeletal assemblage was excavated from the parish churchyard in advance of building work in 2009–2010 and was reburied in September 2016. Twenty-one of the excavated individuals were confidently identified based on coffin plates and grave monuments. All of these identified individuals date to the late 19th century. Etches were performed on teeth from seven adult individuals of known identity and sex. Sex was assigned by coffin plates and confirmed by osteological analysis (13) (Table 1). All samples were anonymized after removal of peptides and analyzed blind.

In addition, male and female pairs from three archaeological sites ranging in date from the early Neolithic (ca. 5,700 BP) to the Medieval period were tested to determine the survival and recovery of sufficient proteins over archaeological timescales and a variety of burial contexts. For each pair, sex had been previously assigned using standard osteological methods, as described in Table 1. These pairs were not analyzed blind.

Samples from Whitwell, Seaham, and St. Guthlac’s were prepared and analyzed as described previously (9). All reagents used were of analytical grade, and solvents used for nanoLC-MS/MS were MS grade. The Fewston samples analyzed as described previously (9). All reagents used were of analytical grade, and thus sex can be established for adults as well as juvenile skeletons in the absence of key skeletal identifiers. In addition, the dimorphic peptide sequence is identical in apes (Fig. S1) and so should be present in all hominins. Finally, this technique will also have a transformative effect on human identification in medicolegal contexts, such as mass disasters and war graves, allowing sex to be established both reliably and cost-effectively.

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