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Sex assessment in a hominin upper second molar (RV’23-350) from the Ruidera (Spain) Middle Pleistocene paleoanthropological site: a proteome-based analysis of the amelogenin

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ABSTRACT

Sex determination in fossil hominin remains is often challenging due to the fragmentary nature of the record and the limitations of morphological assessments. In this study, we apply a proteomic approach to assess the sex of an upper second molar (RV’23-350) from the Middle Pleistocene site of Ruidera (Spain). By analyzing the presence of amelogenin, a key enamel protein encoded by genes on both the X and Y chromosomes, we provide molecular evidence supporting the classification of this fossil as belonging to a male individual. To the best of our knowledge, this represents the first application of amelogenin-based sex estimation in European Middle Pleistocene hominin teeth. Our findings have significant implications for understanding hominin demographic structures and population dynamics in the Iberian Peninsula during this period. The ability to integrate proteomic analyses with traditional morphological approaches offers a novel and reliable methodology for paleoanthropological investigations, particularly in cases where skeletal preservation is insufficient for standard osteological assessments. This study highlights the potential of ancient protein analysis in refining taxonomic and biological reconstructions, further enhancing our knowledge of human evolution and variability in the Middle Pleistocene.

1. Introduction

Estimating sex in fossil human remains is crucial for understanding ancient populations’ evolutionary anatomy, demographic structure, social behaviors, and evolutionary dynamics (Smith, 1980; Leutenegger and Shell, 1987; McHenry, 1991; Wood et al., 1991; Plavcan, 1994; Rehg and Leigh, 1999; Plavcan et al., 2005). Traditionally, this estimation combined morphological and metric analyses, leveraging features such as pelvic and skull characteristics for assessing sexual dimorphism. The pelvis, with its distinct differences in shape and size between males and females, has been a primary focus due to its role in childbirth (Gardner, 1936; Coleman, 1969; Tague, 1992). Similarly, cranial features such as the robustness of the brow ridge, mastoid processes, and nuchal crest have provided valuable insights into sex differentiation (Uytterschaut, 1986; Anemone and Swindler, 1999; Graw et al., 1999; Boucherie et al., 2022). These methods, while effective,

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often require well-preserved and relatively complete skeletal remains, which are not always available in paleoanthropological contexts. Consequently, researchers have sought alternative approaches to sex estimation, including the analysis of dental remains, which are more likely to be preserved and can offer additional data points for understanding the biological and social aspects of ancient human populations (García-Campos et al., 2018a; García-Campos et al., 2018b, 2020, 2024).

In addition to those methods, the molecular analyses, particularly proteomic analysis focusing on the amelogenin protein in dental enamel, have become essential in sex estimation for fossil specimens (Nielsen-Marsh et al., 2009; Zanolli et al., 2017; Masuyama et al., 2017; Hendy et al., 2018; Parker et al., 2019; Froment et al., 2020; Gowland et al., 2021; Demeter et al., 2022; Shaw et al., 2024; Gamble et al., 2024; Madupe et al., 2025a, 2025b). Amelogenin, a sexually dimorphic protein encoded by genes on the X and Y chromosomes in AMELX and AMELY proteins, can be analyzed using mass spectrometry and proteomic techniques on preserved enamel peptides (Gowland et al., 2021; Demeter et al., 2022; Shaw et al., 2024; Madupe et al., 2025a, 2025b). This method offers significant advantages in cases where traditional methods are inconclusive or unfeasible due to fragmentary remains, providing direct molecular evidence of biological sex without significantly damaging samples (Buonasera et al., 2020). Due to its presence in both male and female individuals, but with distinct sequences, the amelogenin protein allows for precise sex determination. Proteomic analysis involves extracting and identifying these protein fragments, which can then be compared to known sequences to determine the individual's sex. This approach has been successfully used in various paleoanthropological studies, providing a reliable alternative to morphological assessments. By integrating proteomic data with traditional methods, researchers can achieve a more comprehensive understanding of the sex composition of ancient populations, thereby enhancing our knowledge of their social structures, reproductive strategies, and evolutionary dynamics.

Moreover, proteomic analyses offer several significant advantages over genetic or genomic analyses. Firstly, proteomic analyses are generally more cost-effective. The technology and reagents required for proteomics are typically less expensive than those needed for genetic or genomic sequencing, making this methodology more accessible to many laboratories and research projects. This cost reduction allows for larger-scale studies and more frequent analyses, facilitating faster advancements in paleoanthropology. Secondly, proteomic analyses provide reliable results even when the DNA in fossils is poorly preserved. Fossil remains are often found in conditions that degrade DNA, such as exposure to heat, moisture, and microbial activity over extended periods. Furthermore, peptide diagenetic modifications can be used to confirm the antiquity of the extracted peptides (Taurozzi et al., 2024; Madupe et al., 2025a, 2025b), allowing us to rule out modern external contamination as a source of amelogenin.

Proteins, particularly those in dental enamel, are more resilient to these degrading factors and can remain intact for extended periods. This resilience makes proteomic analysis a valuable tool for extracting biological information from ancient specimens where DNA analysis might fail. By leveraging the stability of proteins like amelogenin, researchers can obtain direct molecular evidence of biological sex and other biological traits, enhancing our understanding of ancient populations' sex composition and complementing traditional morphological methods.

1.1. *Regional setting: Ruidera paleoanthropological site*

In this study, we aimed to employ proteomic methods to estimate the sex of a second upper molar, labelled RV'23-350 (also known as RV-16), which was discovered during the 2023 excavation campaign at the Middle Pleistocene paleoanthropological site of Ruidera (formerly known as "Ruidera-Los Villares" site), located in Castilla-La Mancha, Spain, on the Southern Iberian Plateau (García-Martínez et al., 2022). This site, currently dated around 300-400 Kya through a combined geochronological approach, has yielded numerous significant fossil findings, contributing valuable insights into the hominin populations that once inhabited the region. Currently, the hominin fossils from this site have not been attributed to any specific species and are classified as Homo sp., pending further analysis. By focusing on the proteomic analysis of the amelogenin protein in the dental enamel of this molar, we seek to provide a reliable sex estimation that complements traditional morphological assessments.

The Ruidera archaeological site is located in the Ruidera Lakes Natural Park, along the Upper Guadiana River Valley. This Natural Park consists of fifteen lakes confined by tufa dams, the oldest dated by U-Th age at 190-250 Kya (González Martín et al., 1987, 2004; Ordóñez et al., 2005). The Upper Guadiana River valley develops along the interface between Upper Triassic gypsiferous clays and Jurassic dolostones. The Triassic sequence is characterized by the presence of Keuper facies, defined by the occurrence of reddish sandstones and siltstones, as well as by gypsiferous marls and dolostones at the top. The Jurassic carbonate rocks are mainly composed of well-stratified dolostones, massive dolostones, and breccia dolostones, with minor intercalations of marls (F. Leyva Cabello, J. Matas González, P. Cabra Gil, J.C. Gutiérrez Marco, R. M^{a}. Rodríguez González Martín et al., 1987). These carbonate beds have suffered significant karstic processes, resulting in depressions and dry and steep-headed valleys that can be found all over the region (Ordóñez et al., 2005).

This region constitutes the high plateau of "Campo de Montiel," which was shaped by several stepped erosion surfaces (900-1100 m.a.s.l.) during the Pliocene (González Martín et al., 1987). A progressive drainage network incision and tufa terrace deposit formation characterize the Quaternary. Concave profile ramps or "glacis" are also interpreted as sedimentary deposits formed under cool conditions in the Middle Pleistocene (Ordóñez et al., 2005). Four sedimentary levels have been identified at the site to date, named from top to bottom RV-1 to RV-4 (Fig. 1 from Supplementary Material). The fossil comes from level RV-1, an approximately 1-m-thick heterogeneous layer composed of clastic-supported dolostones and red breccia clasts with an open fabric and detrital matrix. The top of the formation is characterized by a very dark brown edaphic sub-layer formed by detrital sediments with organic matter. The size of the clasts is variable, some reaching more than 30 cm in diameter. This layer is interpreted as the debris resulting from the construction of a road in the 20th century. The source of the sediment appears to be a karstic filling of reddish breccia situated approximately 5 m up the slope; the dental fossil RV'23-350 belongs to this level.

1.2. *Aims of this study*

This study aims to utilize sex assessment based on the amelogenin protein in the RV'23-350 M, thereby contributing to the paleodemographic knowledge of the fossil record from the Middle Pleistocene in the Iberian Peninsula and Europe. By applying proteomic techniques to analyze the amelogenin protein, we aim to provide a reliable determination of the sex of this specimen. This information is crucial for reconstructing the demographic structure of ancient populations, offering insights into their social organization, reproductive strategies, and evolutionary dynamics. The findings from this study will not only enhance our understanding of the specific population at the Ruidera site but also contribute to broader discussions on the variability and adaptation of hominins across Europe during the Middle Pleistocene. By integrating molecular data with traditional archaeological and morphological analyses, we can better understand the biological and behavioral aspects of human evolution during this critical period. The size or morphological assessment of the human tooth is not addressed in this study, as it is currently under study elsewhere.

2. Materials and methods

The RV tooth RV'23-350 is an upper second molar recovered in the first excavation campaign at the real estate "Los Villares," where the Ruidera site is located (Ruidera, Ciudad Real, Iberian Peninsula) (Fig. 1). The tooth was recovered during sediment sieving on June 12, 2023. Although it was not found in situ during initial exposure, its contextual origin is well established. The specimen derives from Level RV-I, a well-defined Middle Pleistocene unit, and was recovered while excavating a small access stair to the site. Its find spot is shown in Fig. 1 from the Supplementary Material. All sediment removed from this area was systematically processed, and the stratigraphic integrity of Level RV-I is well documented. Consequently, despite its recovery through sieving rather than direct exposure, the contextual antiquity and stratigraphic position of the tooth are secure.

The Ruidera RV'23-350 specimen corresponds to a permanent maxillary second molar (UM2) based on its crown morphology. The tooth shows fully developed enamel with no resorption surfaces, and its crown size and proportions clearly exclude attribution to the deciduous dentition. The identification as *Homo* is supported by several traits that fall within the range of European Middle Pleistocene hominins, including a subrectangular to trapezoidal occlusal outline, a four-cusp configuration with well-developed protocone and metacone, and cusp relief and enamel thickness patterns consistent with published Middle Pleistocene *Homo* specimens (Martinón-Torres et al., 2012; Bermúdez de Castro et al., 2019, 2024). The assignment to UM2 is based exclusively on crown morphology, as the roots are not preserved. The intermediate crown dimensions between UM1 and UM3, the moderately reduced hypocone compared to UM1, the configuration of the occlusal polygon,

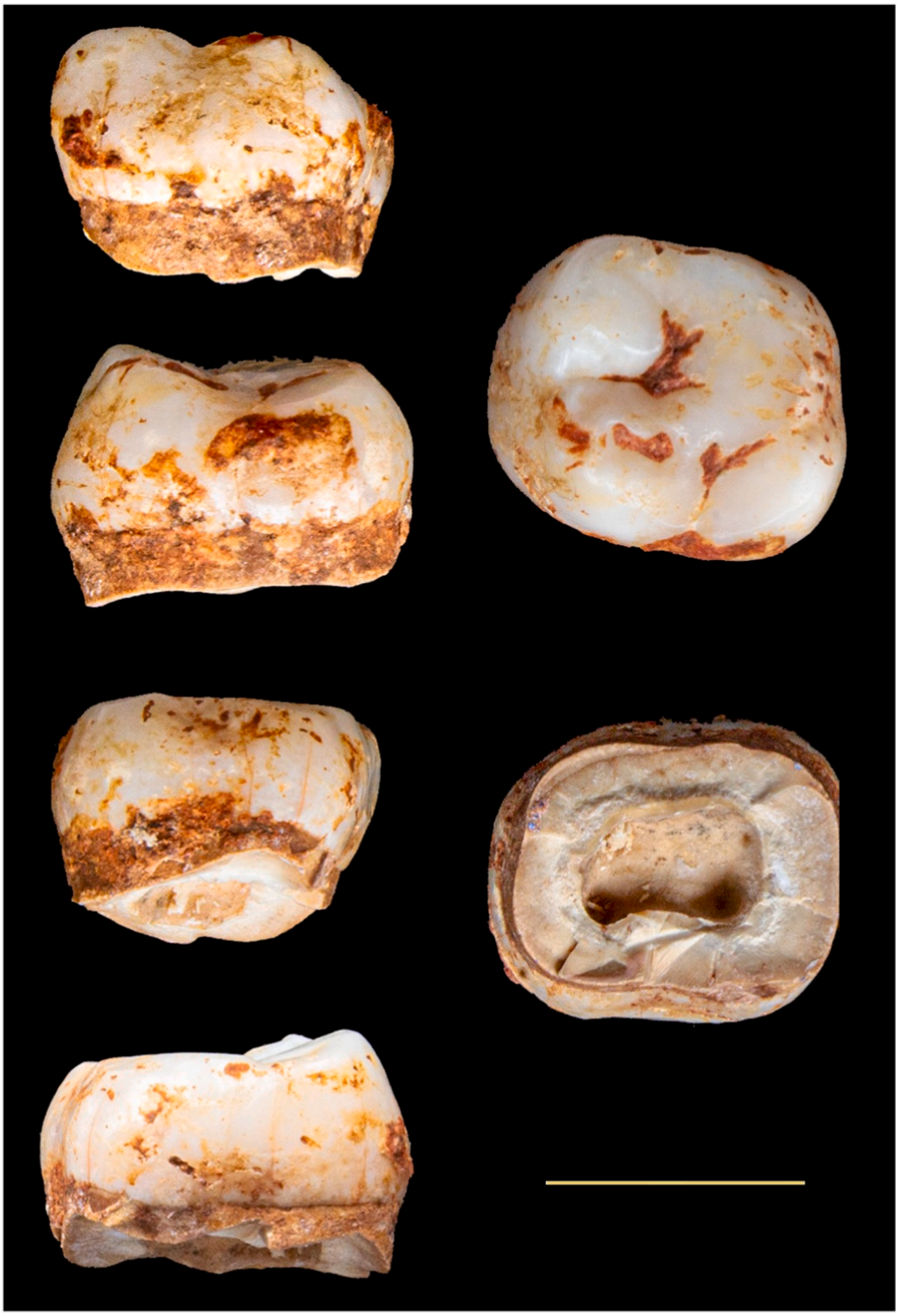

Fig. 1. Multi-view photographs of the Ruidera permanent maxillary second molar (UM2). The specimen is shown in occlusal (top right), mesial, distal, buccal, lingual, and basal views. The crown displays the characteristic four-cusp configuration and occlusal outline typical of *Homo* Middle Pleistocene maxillary molars. Enamel preservation is excellent, with limited adhering sediment. The basal view reveals the absence of roots, which are not preserved. Scale bar = 1 cm.

and the relative proportions of the buccal and lingual aspects align well with diagnostic criteria for UM2 in the genus *Homo* (Bermúdez de Castro et al., 2024).

Before studying the RV tooth, we tested the amelogenin extraction protocol using a double-blinded approach to ensure its accuracy and reliability. The test involved 19 teeth from individuals of known biological sex (self-identified) kindly donated by local dental clinics. As we do not record any demographic, personal, or medical data on the donors but the self-identified biological sex, and do not perform any medical analysis on the samples, ethical review and approval were not required for the study, in accordance with the local legislation and institutional requirements. This sample treatment complies with the Helsinki declaration (Goodyear et al., 2007). The results demonstrated a 100% success rate in correctly identifying the sex of all samples, validating the effectiveness of the amelogenin extraction protocol. This preliminary testing provided a robust foundation for applying the same protocol to the RV tooth, ensuring confidence in the accuracy of the sex determination for this Middle Pleistocene specimen. Once this protocol was carried out and the double-blinded samples of known sex were accurately measured and tested, we analyzed the RV tooth.

Each of the samples previously mentioned was subjected to the peptide extraction process, adjusting the protocol described by previous researchers (Stewart et al., 2017; Hendy et al., 2018). The tooth enamel was etched with 5% hydrochloric acid (HCl) for 2 min followed by a sequential washing process with 3% hydrogen peroxide (H_2O_2), double-distilled water (ddH_2O) twice, and 5% HCl; all steps were performed under a CLASS II Biosafety cabinet following usual GLP instructions. This meticulous cleaning ensured the removal of any contaminants that could interfere with the proteomic analysis; no further precautions were needed as only one sample was processed. The peptides were then subjected to SPE (Solid Phase Extraction) purification on ZipTip C18 tips (Millipore) following manufacturer instructions and analyzed by liquid mass spectrometry at the Faculty of Pharmacy Proteome Unit of the Complutense University of Madrid. In brief, peptides were loaded on EVOSEP tips following the manufacturer's instructions and loaded into the EVOSEP ONE instrument for reverse phase liquid chromatography in a 15 cm column (PepSec C18 15 cm × 150 μm, 1,5 μm, Bruker Daltonics) on a 15 SPD (samples per day) gradient (88 min). A mass spectrometer tims TOF Pro II, coupled in line with an EVOSEP ONE chromatograph, was operated in positive mode using a standard DDA method in a range of 100 to 1700 m/z MS scans using 0.6 to 1.6 1/K0 mobility ramp. Up to 10 precursors were selected per mobility ramp using a ramp and accumulation time of 100 ms each.

Raw data files were analyzed using fragpipe, a LC-MS analysis pipeline that integrates identification and validation tools (Hsiao et al., 2024). Cleavage of proteins was set to nonspecific, as were the load rules, peptide length to 7-20 amino acids, and modification to oxidized methionine and tryptophan, deamidation of asparagine and glutamine, proline hydroxylation, and arginine to ornithine conversion. As the reference database, we used the UniProt entries that include all annotated isoforms of the AMELX and AMELY genes, together with a curated list of proteins previously detected by our group in the dental enamel of modern individuals. Decoy sequences required for FDR estimation were automatically generated and appended by the FragPipe software. PSM validation was performed by the Percolator algorithm set at 0,5% FDR, and protein inference was performed by ProteinProphet (Nesvizhskii et al., 2003; Kong et al., 2017; Li et al., 2019; da Veiga Leprevost et al., 2020; Teo et al., 2020; Yu et al., 2020; Kong et al., 2017; Teo et al., 2020; Yu et al., 2020; Nesvizhskii et al., 2003; da Veiga Leprevost et al., 2020; Li et al., 2019). A brief description and a schematic workflow of proteomic analysis can be found in Fig. 2 from the Supplementary Material.

3. Results

In the double-blinded approach, we assessed the sex of male and female teeth with a 100% success rate. This validation step was crucial before applying the same protocol to the RV'23-350 molar, ensuring that our methods were robust and could provide accurate sex determinations even in ancient and potentially degraded samples. The detailed results obtained with these samples can be found in Table 1 from the Supplementary Material. Proteomics sex assignment was done by the presence of AMELY unique PSMs in the sample. The minor presence of AMELY unique PSMs in a particular sample (like DI5, DI7, DI9) was fixed manually using the spectra visualized in PDV-viewer, since most of them can be attributed to mis-assignment of the software (Ziganshin et al., 2020). Results were then compared with the self-identified biological sex of each sample.

In the RV'23-350 s molar, 241 PSMs identified peptides unique for the AMELY protein (Table 3 from Supplementary Material). Consequently, we identify the individual as a male. Comparison of modifications between RV'23-350 and modern samples suggests the ancient origin of the identified proteins, proving, at the same time, there is no contamination with modern material (Fig. 3 from Supplementary Material). In Figs. 4 and 5 from the Supplementary Material, we provide representative MS/MS spectra of the most abundant AMELX- and AMELY-specific unique peptides identified in the RV'23-350 sample. As shown, the fragmentation patterns unambiguously confirm the sequence assignments for peptides from both amelogenin proteins. As no enzymatic cleavage was performed, the resulting peptides show substantial overlap with only slight sequence differences. The distribution of peptides along the AMEL sequences in the RV'23-350 sample is presented in Fig. 6 from the Supplementary Material, while the corresponding distribution in modern samples is shown in Fig. 7 from the Supplementary Material. These figures highlight the differences in peptide location between the RV'23-350 specimen and modern controls, which are particularly informative for assessing diagenetic effects and sequence preservation. We hypothesize that the differences in unique peptide locations are attributable to the age of the RV'23-350 sample. In AMEL proteins, certain sequence regions—particularly those located at both terminal ends—are naturally more abundant, but these peptides are expected to be preferentially lost during diagenesis. Additional proteins were also identified during the analysis. Supplementary Tables 2 and 3 summarize the proteins detected in the modern ("Current") samples and in RV'23-350, respectively. As expected, many more proteins were identified in the modern teeth (38 proteins across 19 samples) than in the RV'23-350 specimen (15 proteins), reflecting degradation associated with time, environmental conditions, or both.

4. Discussion

4.1. Relevance of sex estimation, its limitations, and new methodologies

Estimating the sex of fossil remains is crucial for understanding the demographic structure, social behaviors, and evolutionary dynamics of ancient populations (Smith, 1980; Leutenegger and Shell, 1987; McHenry, 1991; Wood et al., 1991; Rehg and Leigh, 1999; Plavcan et al., 2005). Accurate sex determination allows researchers to reconstruct population profiles, including the ratio of males to females, which can provide insights into reproductive strategies, social organization, and survival patterns. However, this task becomes particularly complex when the remains are neither pelvic nor cranial, as these bones typically exhibit the most pronounced sexual dimorphism (Gardner, 1936; Coleman, 1969; Uytterschaut, 1986; Tague, 1992; Anemone and Swindler, 1999; Graw et al., 1999). Researchers must rely on other skeletal elements in such cases, which may show less obvious differences between sexes. This complexity necessitates the use of advanced techniques, such as proteomic analysis of dental enamel proteins like amelogenin (Nielsen-Marsh et al., 2009; Masuyama et al., 2017; Zanolli et al., 2017; Parker et al., 2019; Froment et al., 2020; Gowland et al., 2021; Gamble et al., 2024; Shaw et al., 2024; Madupe et al., 2025a, 2025b), which can provide molecular evidence of sex even from fragmentary remains. By overcoming the limitations of traditional morphological assessments,

Table 1
Summary of all known Pleistocene hominin individuals for which sex has been estimated using enamel palaeoproteomics. The table includes Early, Middle, and Late Pleistocene specimens, ordered chronologically, and reports the presence/absence of AMELX and AMELY peptides when available. Sex assignments follow the criteria established in recent proteomic studies.

Taxon/Specimen	Site/Locality	Approx. Age (ordered)	Reference	AMELX PSMs	AMELY PSMs	Sex Assigned
***Paranthropus robustus* (SK 850)**	Swartkrans (South Africa)	2.2–1.8 Ma	Madupe et al., 2025a	High	**Present**	**Male**
***Paranthropus robustus* (SK 835)**	Swartkrans	2.2–1.8 Ma	Madupe et al., 2025a	High	**Present**	**Male**
***Paranthropus robustus* (SK 830)**	Swartkrans	2.2–1.8 Ma	Madupe et al., 2025a	High	**Absent**	**Female**
***Paranthropus robustus* (SK 14132)**	Swartkrans	2.2–1.8 Ma	Madupe et al., 2025a	High	**Absent**	**Female**
***Homo erectus* (D4163)**	Dmanisi (Georgia)	1.77 Ma	Welker et al. (2020)	Low–moderate	**Absent**	**Undetermined**
***Homo antecessor* (ATD6–92)**	Atapuerca (Spain)	0.9–0.8 Ma	Welker et al. (2020)	High	**Present**	**Male**
Ruidera (RV'23–350)	Ruidera (Spain)	350–300 Kya	*This study*	High	**Present**	**Male**
Denisovan (Penghu 1)	Penghu Channel, Taiwan	190–130 Kya	Tsutaya et al. (2025)	Present	**Present**	**Male**
Denisovan (TNH2-1)	Tam Ngu Hao 2 (Laos)	164–131 Kya	Demeter et al. (2022)	Present	**Absent**	**Female (probable)**
Neandertal (Scladina)	Belgium	~100 Kya	Nielsen-Marsh et al. (2009)	Present (TRAP-X only)	**Absent**	**Undetermined**
Neandertal (Oliveira 9)	Portugal	92–90 Kya	Shaw et al. (2024)	Present	**Present**	**Male**
Neandertal (Lakonis)	Greece	~40 Kya	Nielsen-Marsh et al. (2009)	Very low signal	**Absent**	**Undetermined**

these molecular methods enhance our ability to accurately determine sex and, consequently, deepen our understanding of the biological and social aspects of ancient human populations.

4.2. Sex estimation through the amelogenin study in the paleoanthropological fossil record

Amelogenin as a molecular marker provides a novel and direct approach to sex determination in fossil remains, overcoming potential challenges associated with morphological assessments alone. Traditional morphological methods, while valuable, often require well-preserved and relatively complete skeletal remains, which are not always available. In contrast, the analysis of amelogenin, a protein found in dental enamel, offers a robust alternative that can yield accurate results even from fragmentary specimens.

The application of proteomics in this context not only enhances the accuracy of our sex determinations but also highlights the utility of molecular techniques in paleoanthropological research, particularly when dealing with fragmentary or poorly preserved specimens. Recent studies have applied similar approaches to several ancient fossil species from different chronological periods. Regarding the Late Pleistocene fossils, early attempts to extract and sequence enamel proteins from Neanderthal teeth demonstrated the technical feasibility of recovering ancient amelogenin peptides but did not succeed in determining sex. In their pioneering study, Nielsen-Marsh et al. (2009) analyzed two Neanderthal molars—one from Lakonis, Greece (~40 Kya), and another from Scladina Cave, Belgium (~100 Kya)—and succeeded in sequencing fragments of the tyrosine-rich amelogenin peptide (TRAP). However, all recovered peptides corresponded exclusively to the AMELX isoform. As the authors emphasised, the AMELY isoform was never detected, even in modern male controls, making chromosomal sex determination impossible for these fossils. Rather than providing sex estimates, this study revealed the limitations of MALDI-TOF/TOF for detecting Y-linked enamel peptides and highlighted the need for more sensitive proteomic approaches to achieve reliable sex identification in ancient hominins. A very recent study (Shaw et al., 2024) applied the minimally destructive acid-etching protocol to a Neanderthal mandibular premolar (P3) from Gruta da Oliveira (Portugal), dated to ~90 Kya. In contrast to earlier MALDI-based attempts, this analysis successfully recovered both AMELX and AMELY peptide isoforms via nanoLC-MS/MS, enabling an unambiguous determination that the Oliveira 9 individual was male. This work demonstrates that enamel proteomics can reliably identify chromosomal sex in Neanderthals even when only very small enamel fragments remain, and it confirms the applicability of the acid-etch approach to Middle Pleistocene hominins.

A recent palaeoproteomic study on a Middle Pleistocene Denisovan molar from Laos (Demeter et al., 2022) illustrates the limitations of enamel peptide preservation in tropical environments. Although amelogenin peptides were recovered, no AMELY-specific peptides could be detected. As the authors note, the absence of AMELY may indicate that the tooth belonged to a female individual, but it may equally reflect extensive diagenetic loss of Y-linked peptides beyond the limits of detection, preventing a definitive molecular sex determination. The "female" attribution proposed in the study is therefore based on dental developmental morphology rather than proteomic evidence. In contrast, a recent analysis of a Denisovan mandible from Penghu (Taiwan), dated to the Middle Pleistocene, successfully recovered multiple AMELY-specific peptides using LC–MS/MS, allowing for an unambiguous male sex determination (Tsutaya et al., 2025). To the best of our knowledge, aside from these Denisovan specimens, no Middle Pleistocene hominin—such as *Homo heidelbergensis* or other contemporaneous forms from Europe, Asia, or Africa—has yet been securely sexed using enamel proteomics.

Finally, recent palaeoproteomic advances now allow direct biological sex determination and the detection of intra- and intertaxon variability across the Early Pleistocene record. In addition to the enamel-protein analyses performed on *Homo antecessor* from Gran Dolina—-where AMELY-specific peptides identified ATD6-92 as a male—and the Dmanisi *Homo erectus* specimen D4163 (Welker et al., 2020), the new proteomic study from Swartkrans provides the first robust molecular data for Early Pleistocene *Paranthropus*. Four *P. robustus* teeth dated to ~1.8–2.2 Ma yielded authentic ancient enamel proteomes, with two specimens (SK 850, SK 835) sexed as males based on multiple AMELY-specific peptides, while the remaining two (SK 830, SK 14132) were attributed to females using a quantitative AMELX/AMELY intensity threshold (Madupe et al., 2025a). Beyond sex estimation, the study also revealed protein-sequence variability— including a polymorphism at ENAM-137—suggesting potential subgroup structure within southern African *Paranthropus*. When viewed together, proteomic evidence from *Paranthropus, H. antecessor, and H. erectus* demonstrates that enamel proteins now provide the a reliable biomolecular avenue for assessing sex and genetic diversity across the Lower Pleistocene, far beyond the temporal limits of ancient DNA preservation.

The current proteomic record of Pleistocene hominins reveals an uneven sex distribution, with male individuals clearly dominating the dataset. Of the eleven specimens for which sex can be confidently assessed, six are male, compared with only two females, while the remaining cases (n = 3) remain undetermined due to low protein preservation or lack of AMELY detection in early-generation studies. Male individuals appear across all chronological intervals—Early, Middle, and Late Pleistocene—and encompass a wide taxonomic range (*Paranthropus, Homo antecessor*, Denisovans, and Neandertals). Female individuals, in contrast, are represented only in the Early Pleistocene (Paranthropus) and in the Middle Pleistocene Denisovan record. This pattern may reflect a combination of taphonomic factors, sampling biases, and differential biochemical preservation, but it is nonetheless striking that male individuals are substantially more frequent in the proteomic dataset. As the field expands and more specimens are analyzed with high-sensitivity LC-MS/MS workflows, it will become possible to evaluate whether this male overrepresentation is a genuine demographic signal or a product of preservation and sampling history. Table 1 presents, to the best of our knowledge, the most comprehensive compilation of fossil hominins whose sex has been determined using enamel-derived palaeoproteomic data.

4.3. *Relevance of the Ruidera results for hominin evolution during the Middle Pleistocene*

Within this emerging framework, the new proteomic data obtained from the Middle Pleistocene site of Ruidera (García-Martínez et al., 2022) represent a crucial contribution to the demographic and biological characterization of European hominins. While enamel-protein studies on Early Pleistocene taxa—such as southern African *Paranthropus, Homo erectus* from Dmanisi, and *Homo antecessor* from Atapuerca—have demonstrated the power of palaeoproteomics to determine sex and detect population-level variability deep in time, the Ruidera results extend this approach into the Middle Pleistocene of western Europe. The recovery of AMELX/AMELY peptides from the Ruidera molar provides the first proteomic sex determination for a hominin individual from this region and period, thereby filling a critical temporal and geographic gap between the Early Pleistocene record and later Neanderthal populations.

As previously mentioned, the Ruidera hominins are still classified as *Homo* sp. because their taxonomic species identification is pending. This is due to the issue commonly known as “the muddle in the middle” (Rosas and Bermúdez de Castro, 1998; Bermúdez de Castro et al., 2001; Roksandic et al., 2022; Bae et al., 2023). The Middle Pleistocene ages stand out as one of the most perplexing stages in studying human evolution. Lasting from approximately 774 Kya to 129 Kya, it was marked by the appearance of modern humans and closely related species like Neanderthals and Denisovans. Fossil evidence from Africa, Europe, and Asia provides valuable insights into the origins of our species and our connection to these relatives. However, the significant variability and the unusual characteristics of many fossils from this time make them particularly challenging to interpret, earning this period the informal nickname of “the muddle in the middle” among anthropologists (Roksandic et al., 2022; Bae et al., 2023). In this context, solving the problem of not knowing the sex of the fossil specimens could help to remove from the equation the variability added by sexual dimorphism. As size and robustness are sexually dimorphic traits that influence the morphology and tissue proportions of fossils (both dental and skeletal) (García-Campos et al., 2020), accurately determining the sex is crucial to avoid misidentifying taxa (Plavcan, 2012). The identification of the Ruidera tooth as belonging to a male individual has direct implications for ongoing debates surrounding Middle Pleistocene human diversity (Roksandic et al., 2022; Bae et al., 2023). Part of the morphological variation and disparity reported for European Middle Pleistocene hominins—including Iberian assemblages (Martinón-Torres et al., 2012; Bermúdez de Castro et al., 2019)—may reflect sexual dimorphism rather than taxonomic differentiation. Establishing the sex of the Ruidera specimen, therefore, provides a crucial baseline for assessing intra-site morphological variability, helping to distinguish differences attributable to sex from those that may represent genuine phylogenetic or population-level variation. This integrative perspective contributes to refining interpretations within the broader context of the Middle Pleistocene “muddle in the middle” (Bae et al., 2023).

4.4. *Limitations of the use of paleoprotemics in this work*

Although the power of enamel proteomics for sex determination is well established, several methodological limitations must be considered when interpreting the results obtained in this study. Proteomic sex estimation relies on the detection of X- and Y-specific amelogenin peptides preserved in the enamel matrix. When AMELY-specific peptides are present, sex attribution is unequivocally male; however, the absence of AMELY peptides is inherently ambiguous. As highlighted recently by Štamfelj (2021), a small proportion of phenotypically normal males carry structural deletions of the AMELY locus on the Y chromosome, which would lead to false female assignments in both genomic and proteomic assays. Although this possibility exists, the reply by Parker and colleagues (Parker et al., 2021) demonstrates that such deletions are exceedingly rare and have not been detected in large, systematically sampled populations such as the 1000 Genomes Project. Consequently, while AMELY deletion represents a theoretical limitation, its practical impact on routine palaeoproteomic sex estimation—particularly in non–South Asian contexts—is likely negligible. Nevertheless, two caveats remain relevant. First, proteomics cannot independently rule out Y-chromosome microdeletions, chromosomal anomalies, or sex-chromosome mosaicism, all of which can be evaluated more comprehensively through genomic methods when ancient DNA is preserved. Second, proteomic sex estimation is constrained by protein preservation and abundance: very low-signal samples may fail to yield detectable AMELY peptides even in biologically male individuals. For this reason, recent analytical frameworks incorporate thresholds based on AMELX peptide abundance to distinguish genuine female profiles from low-signal male false negatives. In our case study, the enamel of specimen RV’23-350 proved exceptionally well preserved, and amelogenin PSMs fall almost within the range of modern controls (Table 1 from Supplementary Material). Following the interpretative criteria proposed by previous researchers (Wasinger et al., 2019), the robust AMELX signal and the consistent presence/absence of AMELY peptides in fossils support a confident sex determination. However, as with all proteomic studies, this attribution should be interpreted within the broader evolutionary, taphonomic, and demographic context, acknowledging the intrinsic limitations of the method and the low-probability alternative hypotheses outlined above.

Finally, proteomics-based results are not exempt from potential sources of contamination, including exogenous modern proteins introduced through human handling, cross-sample contamination during laboratory processing, or methodological artefacts. However, in the specific case of amelogenin-based sex determination, the risk of external contamination is exceedingly low due to the highly restricted tissue distribution of AMELX and AMELY gene products. Public proteomic and transcriptomic repositories (e.g., PeptideAtlas; Human Protein Atlas) show that amelogenin expression is essentially confined to dental enamel–related tissues, making the introduction of modern AMELX/AMELY peptides from skin contact, saliva aerosols, or other environmental sources extremely improbable. Additional resources—such as the Salivary Proteome Database and skin mass-spectrometry datasets—confirm the negligible likelihood of these tissues contributing to false amelogenin signals. Moreover, all molecular indicators of protein antiquity in our dataset—including peptide-length distributions, N/Q deamidation profiles, and R-to-ornithine conversion—display patterns fully consistent with ancient proteins and clearly distinct from modern contaminants (Taurozzi et al., 2024; Madupe et al., 2025a, 2025b), as illustrated in Fig. 3 from the Supplementary Material. To further minimise laboratory contamination, all sampling and extractions were performed inside a P2 laminar-flow cabinet following strict Good

Laboratory Practice (GLP) standards. No plastic consumable or solution was used on more than one specimen, and all materials were discarded after each extraction (see proteomics workflow, Fig. 2 from the Supplementary Material). Additionally, we processed a batch of 16 samples alongside control injections of 40 ng of digested HeLa cells after each tooth sample. These controls were analyzed with identical parameters to verify the absence of AMELX or AMELY peptides (Table 4 from the Supplementary Material). The EVOSEP chromatography system coupled to a Bruker timsTOF Pro 2 instrument demonstrated complete prevention of carry-over, confirming that sample-to-sample contamination did not occur.

5. Conclusions

To the best of our knowledge, this study presents the first proteomics-based sex estimation for a hominin tooth from the Middle Pleistocene of western Europe. This finding carries important implications for the study of anatomical variability within the Ruidera hominin assemblage, provisionally attributed to *Homo sp.* The use of amelogenin peptides as molecular markers offers a direct and independent line of evidence for determining sex in fossil individuals, overcoming the limitations inherent to morphological assessments—particularly in fragmentary or developmentally ambiguous samples. In the case of Ruidera, the recovered enamel proteome enables the tentative attribution of the RV’23-350 molar to a male individual, with peptide abundance and degradation patterns fully consistent with ancient biomolecules.

The Ruidera proteomic data contribute meaningfully to broader efforts aimed at reconstructing the demographic structure and population dynamics of hominins inhabiting the Iberian Peninsula during the Middle Pleistocene. By adding a new sex-identified individual to the European fossil record, this study helps bridge the gap between Early Pleistocene proteomic datasets—such as those from Paranthropus, *Homo erectus* (Dmanisi), and *Homo antecessor*—and the more abundant Late Pleistocene records of Neanderthals. Moreover, the present results integrate seamlessly with ongoing methodological discussions regarding the reliability of enamel-based sex determination. While the debate surrounding rare AMELY deletions underscores the need for cautious interpretation, current genomic evidence indicates that such deletions are exceedingly uncommon and do not compromise the validity of the molecular diagnosis in this case. This molecular approach also provides an ideal complement to existing dental-tissue–based protocols for morphological sex discrimination, such as those developed by previous researchers. In subsequent phases of the project, we plan to combine amelogenin-derived sex determinations with quantitative analyses of enamel and dentine proportions. This integrative framework will allow us to refine and recalibrate non-invasive morphological methods for sex estimation—an important step for expanding their applicability to Middle Pleistocene hominins, where comparative samples remain limited.

Finally, establishing the sex of individuals from Ruidera provides a solid foundation for future morphometric and taxonomic work. Accurate sex attribution minimizes the risk of conflating sexual dimorphism with phylogenetic signal when evaluating dental morphology. As such, this study strengthens both the taxonomic and demographic interpretations of the Ruidera assemblage, contributing to a more nuanced understanding of hominin variability and population structure in the Middle Pleistocene of southwestern Europe.

CRediT authorship contribution statement

Daniel García-Martínez: Writing – original draft, Investigation, Funding acquisition, Data curation, Conceptualization. **Paula Sanz-Henche:** Conceptualization, Writing – original draft, Writing – review & editing. **Inés Zapico:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Luis Felipe Clemente:** Writing – original draft, Methodology, Investigation, Formal analysis. **Maria Luisa Hernáez:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Luis Ríos:** Writing – review & editing, Data curation, Conceptualization. **Mar Casquero:** Writing – review & editing, Data curation. **Candelas Buenestado Ruíz:** Writing – review & editing. **Darío Fidalgo:** Writing – review & editing. **Sara Díaz-Pérez:** Writing – review & editing, Data curation. **Isidoro Campaña Lozano:** Writing – review & editing. **Lucía Bermejo:** Writing – review & editing. **Tomás Torres-Medina:** Writing – review & editing. **Carlos A. Palancar:** Writing – review & editing, Data curation.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the authors used ***OpenAI's ChatGPT*** to assist with language editing, stylistic refinement, and improvement of clarity in some sections of the manuscript. After using this tool, the authors thoroughly reviewed, verified, and edited all generated content, and take full responsibility for the final version of the manuscript and all scientific interpretations presented therein.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.quaint.2026.110182.

Data availability

All the proteomic data is uploaded to PRIDE website under a password to protect privacy, but all data will be available upon reasonable request to the corresponding author.

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