Journal of Cultural Heritage 66 (2024) 204–214

Contents lists available at ScienceDirect

Journal of Cultural Heritage

journal homepage: www.elsevier.com/locate/culher

Original article

Undertaking the biological sex assessment of human remains: The applicability of minimally-invasive methods for proteomic sex estimation from enamel peptides

Jaroslav Brůžek[a,b,1], Ivan Mikšík[c,1], Anežka Pilmann Kotěrová[a,*], Marine Morvan[c,d], Sylva Drtikolová Kaupová[e], Fréderic Santos[b], Alžběta Danielisová[f], Eliška Zazvonilová[a,f], Bruno Maureille[b], Petr Velemínský[e]

[a] Department of Anthropology and Human Genetics, Faculty of Science, Charles University, Viničná 7, 128 00 Prague 2, Czech Republic
[b] Univ. de Bordeaux, CNRS, Ministère de la Culture, PACEA, UMR 5199, F-33600 Pessac, France
[c] Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Studentská 573, 532 10 Pardubice, Czech Republic
[d] Institute of Physiology of the Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague 4, Czech Republic
[e] Department of Anthropology, National Museum, Václavské náměstí 1700/68, 110 00 Prague 1, Czech Republic
[f] Institute of Archaeology of the Czech Academy of Sciences, Letenská 4, 118 00 Prague 1, Czech Republic

ARTICLE INFO

Article history:
Received 17 April 2023
Accepted 22 November 2023

Keywords:
Human skeletal collections
Cultural heritage
Proteomics
Sex estimation
Sampling

ABSTRACT

Being a part of the cultural heritage, skeletal human remains and grave objects are often the only evidence of people who lived many years, or even centuries or millennia, ago, and their preservation for future generations is thus of the utmost importance. The first task in analyzing skeletal remains is to build a biological profile of the individual, including in particular a sex estimation. Recently developed proteomic sex analysis, based on the detection of two sex-dependent forms of the amelogenin protein in tooth enamel, could offer a minimally-invasive and reliable approach applicable to both recent and past populations.

The aims of the present study are: 1) to validate the proteomic sex estimation approach with a delicate, minimally-destructive protocol using protein etching in recent and sub-recent identified samples of adult individuals; 2) for the first time, to evaluate the invasiveness of the extraction of amelogenin protein from teeth for proteomic analysis via scanning electron microscope (SEM) and microcomputed tomography (micro-CT); 3) to apply the method to an archaeological sample of unknown adult and juvenile individuals.

An assemblage of 60 teeth (32 males and 28 females) of recent and sub-recent origin was used to validate the approach. A sub-sample of 20 teeth (10 males and 10 females) was used to assess the invasiveness of the amelogenin extraction procedure. For the application of the method, samples of 15 adult and 32 juvenile teeth, both originating from medieval populations, were used.

Proteomic sex estimation achieved 100% accuracy in this sample. An SEM and micro-CT comparison of the dental surfaces before and after chemical treatment showed an approximately 10% loss of enamel and only 2% loss of dentine. The suitability and minimally-invasive character of the protocol for proteomic analysis in biological sex estimation was demonstrated, as was its applicability to archaeological samples.
© 2023 Consiglio Nazionale delle Ricerche (CNR). Published by Elsevier Masson SAS. All rights reserved.

Introduction

Human skeletal remains are an inseparable part of the cultural heritage, and are often the only information about people living in the past. They are preserved in museum and university collections, and are used for elaborating biological profiling methods [1]. Unfortunately, there are differences in legislation between countries [2–5] that affect the handling of skeletons, as well as differences in ethical standards e.g. [6,7]. The common denominators include some laws, such as the Valletta Treaty (Council of Europe, ETS No. 143, 1992), according to which all biological remains, artefacts, objects and any other remains of humankind from past epochs are subsumed under Cultural Heritage and are protected within the scope of a common European legacy. We must preserve for the future the status of archaeological human remains as part of

* Corresponding author at: Department of Anthropology and Human Genetics, Charles University, Viničná 7, Prague 2, 128 43, Czech Republic.
E-mail address: koterova@natur.cuni.cz (A. Pilmann Kotěrová).
[1] These authors contributed equally.

https://doi.org/10.1016/j.culher.2023.11.021
1296-2074/© 2023 Consiglio Nazionale delle Ricerche (CNR). Published by Elsevier Masson SAS. All rights reserved.

the cultural heritage, and as indispensable empirical sources for the reconstruction of human population history through time and space [8].

The uniqueness of skeletal remains from any period leads conservators to minimize any interference with their integrity. Knowledge of the methods used and the degree of damage they cause are of enormous importance, but such damage is not always fully known [9]. Within the field of human osteology, the debate on ethical issues regarding the sampling, research and display of archaeological human and animal remains has been ongoing for decades, and has become more vigorous with the recent increase in ancient biomolecular studies [10,11]. Although the amount of sample needed for analysis can be just a few milligrams of bone or dental tissue, museums too are being inundated with destructive sampling requests. With respect to anthropological collections, we must consider the need to reduce or completely eliminate the mechanical destruction of skeletal remains deemed useful for scientific evaluation.

The preservation of organic molecules in teeth and bones has proven crucial for understanding the human past [12]. In recent years, proteomics has become an attractive method for studying human, animal, and biological profiles and origins. It is an alternative to DNA analysis, which is limited by the DNA amplification that is present in ancient samples, contamination, its high cost, and the limited preservation of nuclear DNA [13–15]. Currently, three approaches are available for estimating biological sex: osteology, genomics, and proteomics [16], but little is known about the relative reliability of these methods in applied settings [17].

Correct and reliable biological sex estimation of skeletal remains is important in various areas, from bioarchaeology to forensic science. The varying degree of preservation of skeletal remains limits the methods that can be used [18]. Morphological methods are based on the existence of sexual dimorphism in the skeleton, and are subject to population specificity which causes various error rates [19,20]. The only part of the skeleton that allows a reliable estimate of sex is the pelvis [21]; in archaeological human skeletal assemblages, however, the pelvis is often heavily damaged or completely absent [22]. In addition, morphological methods have another major limiting factor: the inability to reliably or accurately estimate sex from immature elements with any degree of consistency [23,24]. According to Buonasera et al., biological sex estimation was possible for 100% of an archaeological sample by proteomics, for 91% by genomics, and for 51% by osteology; the agreement among the methods was high, but there were conflicts [17].

Proteomics provides a new, seemingly simple and cost-effective way to conduct sex estimation without the risk of contamination. It uses two sexually distinct forms of the amelogenin protein found in tooth enamel, detectable by liquid chromatography-tandem mass spectrometry (LC-MS/MS); the AMELY protein (amelogenin Y isoform) is present in enamel dental tissue only in males, while AMELX (isoform X) can be found in both sexes [13]. Enamel is the most mineralized part of the tooth, with mineral content making up about 97% of mature enamel; the rest is formed of proteins and other components, such as water. During enamel formation and maturation the matrix is removed almost completely through enzymatic degradation by proteases, resulting in its hardening and the extensive deposition of calcium-based minerals. Amelogenin is the predominant protein in the developing extracellular enamel matrix [25].

Although studies estimating biological sex by proteomic analysis of dental enamel use the same principle, they differ in their levels of sampling and pre-treatment. Some studies use more destructive sampling in the form of small enamel chunks extracted with a dentist's drill [26–30]; others offer a less invasive approach in the form of etching the enamel surface with hydrochloric acid [13,31,32].

Research aim

In this study, we aimed to (1) validate peptide-based enamel sex identification both in teeth from individuals of known sex from a recent population and in dissected individuals from the mid-20th century [33]; (2) assess the effect of protein extraction on the structural integrity of human dental enamel (damage to the enamel surface) via scanning electron microscope (SEM) and micro-computed tomography (micro-CT); and (3) to apply the method to an archaeological assemblage of unknown adult and immature individuals.

Material

To obtain and validate a predictor for biological sex based on enamel proteins, we used two different assemblages:

(1) a validation sample of teeth of individuals of known biological sex to verify the reliability of the method; a sub-sample of 20 teeth randomly selected from the validation sample served to verify the effect of protein etching on tooth enamel quality;
(2) an application sample of archaeological teeth from a museum collection, for which sex (in the case of adult individuals) was originally estimated by a morphological method.

Validation sample

This contains the identified teeth of adult individuals from two samples of the Czech population (Table 1). Group (1a) consists of 30 teeth of adult individuals of known age and sex (15 male and 15 female) from the recent Prague population, provided by dentists. Extractions were performed for medical reasons.

Group (1b) originates from the Pachner collection, housed in the Department of Anthropology and Human Genetics of the Faculty of Science, Charles University, Prague. It too comprises 30 identified teeth of individuals of known age and sex from the Prague population (17 male and 13 female), but who were autopsied in the 1940s. This collection dates to the 1930s and consists of individuals of known sex, age (date of birth and date of autopsy), and status (they represented the lower socio-economic classes of Bohemia) [33,34].

Table 1
Composition of validation sample, sub-sample subjected to micro-CT and SEM and application sample.

	Validation sample of identified individuals (n = 60)							Application sample (n = 47)	
	Extraction for medical reasons			Pachner's Identified Collection				Medieval population (9–11th century AD)	
	F (n)	M (n)	F and M (n)	F (n)	M (n)	F and M (n)	Total (n)	Adults	Non-adults
PSE	10	10	20	8	12	20	40	15	32
PSE + micro-CT and SEM	5	5	10	5	5	10	20	not performed	not performed

PSE = proteomic sex estimation; SEM = scanning electron microscope; micro-CT = microtomodensitometry.

Application sample

This contains 15 adult and 32 non-adult teeth from the burial grounds of an Early Medieval population (9–11th century AD) at the Mikulčice agglomeration in Moravia [35], stored in the Department of Anthropology of the National Museum, Prague (Table 1). We selected adult individuals based on the conflicting results of morphological sex estimation by osteological methods between those published in the 1960s [36–38] and the revised sex estimates published in 2020 [39]. We also included non-adult individuals whose biological sex cannot be assessed by morphological methods [32,40]; they were selected to study the differences in diet between boys and girls among the early Slavs, which is the subject of another study [41]. This research was approved by the Institutional Review Board of Charles University.

Methods

Biological sex estimation

Proteomic estimation of sex was done following the method of Stewart et al. [13], which is based on the analysis of two dimorphic peptides of amelogenin: AMELY-(58–64) peptide and AMELX-(44–52) peptide from tooth enamel. The teeth of males contain both peptides (AMELY and AMELX), while female teeth contain only one (AMELX).

1 step – Sample preparation

The tooth surface was cleaned with water to remove obvious surface contaminants. The tooth crown was then placed in the cap of a 2-mL Eppendorf tube, washed with 3% H_2O_2 (200 µL) for 30 s and then rinsed with ultrapure water. It was then treated with 200 µL of 5% (v/v) HCl. An initial etch was performed by lowering the tooth onto the HCl and maintaining contact for 2 min. This first etch was discarded. A second 2-minute etch was collected for the analysis.

2 step – Protein etching

For peptide extraction, a C18 resin-loaded ZipTip (ZTC18S096; EMD Millipore) was used. This had previously been conditioned three times with acetonitrile (100%), and three times with formic acid 0.1% (v/v) (each draw discarded). Peptide solution was then pipetted 10 times to maximize binding to the C18 resin of the ZipTip material. Finally, the ZipTip was washed six times with formic acid 0.1% (v/v) (each wash discarded). Bonded peptides were eluted by 4-µL of acetonitrile/formic acid (60%/0.1%, v/v) and the resulting solution was collected into small vials. This fraction was lyophilized and dissolved in 20 µL of formic acid (2%) in ultrapure water, centrifuged on a benchtop centrifuge for 5 min to remove any particulate matter, and transferred (18 µL) to autosampler vials. This sample was injected for analysis by reversed-phase nano-liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS).

3 step – Analysis method

The nanoLC apparatus was a Proxeon Easy-nLC (Proxeon, Odense, Denmark) coupled to a MaXis Q-TOF (quadrupole – time of flight) mass spectrometer with ultra-high resolution (Bruker Daltonics, Bremen, Germany) by nanoelectrosprayer. Five microliters of the peptide mixture were injected into a NS-AC-11 BioSphere C18 column (particle size: 5 µm, pore size: 12 nm, length: 152 mm, inner diameter: 75 µm), with an NS-MP-10 BioSphere C18 pre-column (particle size: 5 µm, pore size: 12 nm, length: 20 mm, inner diameter: 100 µm), both obtained from NanoSeparations (Nieuwkoop, Netherlands).

Peptide separation was achieved via a linear gradient between mobile phase A (ultrapure water) and B (acetonitrile), both containing formic acid 0.1% (v/v). Separation was started with a gradient elution from 5% to 30% mobile phase B at 70 min. The next step was gradient elution to 50% B in 10 min, followed by a gradient to 100% B in 10 min. Finally, the column was eluted with 100% B for 30 min. Equilibration before the subsequent run was achieved by washing the column with 3 µl of 5% mobile phase B for 5 min. The flow rate was 0.25 $\mu L.min^{-1}$, and the column was held at ambient temperature (25 °C).

Online nano-electrospray ionization (easy nano-ESI) in the positive mode was used. The ESI voltage was set at +4.5 kV, spectra rate 3 Hz. Operating conditions: drying gas (N_2), 4 $L.min^{-1}$; drying gas temperature, 180 °C; nebulizer pressure, 100 kPa. Experiments were performed by scanning from 420 to 550 *m/z*.

Proteomic determination of biological sex was done according to the peak area of EIC (extracted ion chromatogram) at a retention time of 35 min for *m/z* 440.22 (AMELY-peptide, marked as Y in the tables) and at a retention time of 43 min for *m/z* 540.28 (AMELX-peptide, marked as X in the tables).

Evaluation of the effect of protein etching on dental enamel

The effect of etching on enamel was performed in a subset of 20 teeth of individuals of known sex.

Scanning electron microscope (SEM)

Samples were scanned on a Hitachi S-3700 N Scanning Electron Microscope (SEM) at the National Museum in Prague. The teeth were documented overall, and then two areas on each were selected and scanned at several resolutions without tooth surface treatment (mechanical or ultrasonic cleaning). After the protein etching of the teeth, the same samples and the same areas on them were scanned again so that the changes that had occurred could be evaluated.

Microcomputed tomography (micro-CT)

Each tooth was scanned twice: before and after the application of the protocol of extraction for proteomic sex estimation with the same scanning parameters. The first scan took place under the same conditions as the SEM analysis of the surface (without mechanical or ultrasonic cleaning). Microtomodensitometric (micro-CT) data for these teeth were acquired using the micro-CT platform in the PACEA (Bordeaux). They were obtained with a "v|tome|x s 240″ micro-CT scanner microfocus tube (GE Sensing and Inspection Technologies Phoenix X ray).

A total of 1750 radiographic projections (i.e. 1750 angular increments for 360° rotation) were acquired with the following scan parameters: voltage 100 kV, current 160 µA, exposure 500 ms, voxel size 27.5 µm. The micro-CT data were reconstructed using Phoenix datos|x reconstruction 2 software and then exported as a 16-bit TIFF image stack. VG studio max software (Volume Graphics, release 2.2, Heidelberg, Germany) was used for the virtual slice visualization and three-dimensional rendering.

Using Avizo 9.5 (Thermo Fisher Scientific), a semi-automatic threshold-based segmentation was performed on the reconstructed images with subsequent manual corrections. After segmentation, volumetric reconstruction and visualisation of the micro-CT slices were performed using Avizo v. 9.5 software. Enamel thickness mapping before and after protein etching was performed with the Surface Distance module on the Avizo 9.5 software.

Results

Biological sex estimation via peptides in dental enamel

A biological female determination of the sample is indicated by the detection of only one diagnostic peptide (AMELX); a biological male determination is indicated by the detection of both diagnostic peptides derived from each isoform (AMELX-(44–52), or

Table 2
Amelogenin-sex estimation in a validation sample of adult teeth of known age and sex from a Czech population (30 males and 30 females).

Sample number	Sub-sample	Sex declared	Tooth sampled	Age (yrs)	Peak area		Proteomic sex
					X	Y	
Proteomic sex estimation							
8	A	F	${}_3M$	23	946,208	Not present	F
13	A	F	${}_2M$	39	1896,641	Not present	F
15	A	F	${}^3M$	20	1154,743	Not present	F
16	A	F	${}_3M$	21	1527,914	Not present	F
17	A	F	${}_3M$	40	1805,977	Not present	F
18	A	F	${}_3M$	25	874,56	Not present	F
19	A	F	P^2	64	3008,422	Not present	F
20	A	F	M_3	21	1325,183	Not present	F
21	A	F	M_3	55	3032,252	Not present	F
22	A	F	M^3	24	673,982	Not present	F
1	A	M	${}_3M$	17	748,564	550,882	M
2	A	M	M^3	41	1371,502	2604,241	M
23	A	M	${}^3M$	28	973,924	1296,839	M
24	A	M	${}_3M$	29	1033,295	2386,597	M
25	A	M	${}^3M$	55	2330,862	3339,066	M
26	A	M	${}_3M$	21	734,265	820,057	M
27	A	M	M_3	36	1609,180	2396,535	M
28	A	M	${}^3M$	35	1295,788	2389,316	M
29	A	M	${}^3M$	61	1565,209	865,203	M
30	A	M	${}_3M$	30	1490,656	2013,183	M
37	P	F	P_2	34	2323,905	Not present	F
40	P	F	${}^2P$	adult	929,849	Not present	F
43	P	F	M_3	51	4721,920	Not present	F
50	P	F	${}_3M$	38	2488,777	Not present	F
51	P	F	I^1	adult	258,116	Not present	F
52	P	F	M^1	70	4448,210	Not present	F
55	P	F	${}^3M$	65	2227,581	Not present	F
58	P	F	C	adult	2259,169	Not present	F
33	P	M	C	adult	1157,578	1258,985	M
34	P	M	P^1	43	1140,553	2664,629	M
35	P	M	${}^2P$	38	1967,799	3615,260	M
36	P	M	${}^2P$	adult-	1254,277	2890,431	M
38	P	M	P^2	62	2984,328	4386,024	M
39	P	M	${}^2P$	32	2070,259	4178,386	M
41	P	M	M^1	39	2536,816	3926,950	M
44	P	M	${}^1P$	adult	903,339	1473,504	M
45	P	M	P^1	32	4197,536	7638,730	M
56	P	M	${}^1M$	78	1487,187	1250,741	M
57	P	M	P_2	61	3344,463	4761,168	M
60	P	M	${}^2M$	35	2141,434	2029,143	M
Proteomic sex estimation with micro-CT and SEM analysis							
9	A*	F	M^3	32	1384,671	Not present	F
10	A*	F	${}_3M$	25	1271,997	Not present	F
11	A*	F	M_3	33	1322,932	Not present	F
12	A*	F	M_3	26	1831,753	Not present	F
14	A*	F	M_3	26	1258,762	Not present	F
3	A*	M	${}_3M$	48	1366,483	3291,729	M
4	A*	M	M_3	20	627,076	550,347	M
5	A*	M	${}_3M$	22	863,334	1037,607	M
6	A*	M	${}^3M$	31	611,035	1317,945	M
7	A*	M	${}_3M$	24	551,819	1299,878	M
31	P*	F	${}^3M$	34	2347,440	Not present	F
32	P*	F	${}_3M$	28	3000,280	Not present	F
46	P*	F	M^3	adult	778,678	Not present	F
48	P*	F	${}^2M$	32	5039,565	Not present	F
54	P*	F	M^1	adult	1210,069	Not present	F
42	P*	M	M^1	73	1092,764	876,029	M
47	P*	M	M^1	33	2718,667	3445,208	M
49	P*	M	${}^2M$	adult	2905,233	1947,298	M
53	P*	M	${}^1M$	42	3697,825	2906,365	M
59	P*	M	${}^2M$	72	2000,080	1784,672	M

A = group 1a (teeth from extractions in dental clinics in 2019–2021), *P* = group 1b (teeth from Pachner's Identified collection from the first half of the 20th century), * = sub-sample for the effect of protein etching on tooth enamel quality.
SEM = scanning electron microscope; micro-CT = microtomodensitometry.
Tooth nomenclature: *I* = incisor, C = canine, *P* = premolar, *M* = molar, upper index = upper jaw, lower index = lower jaw, side of the index indicates side of the tooth (left or right).

AMELY-(58–64)) of amelogenin proteins. For the total 60 samples of individuals of known sex, the accuracy of sex estimation was absolute (Table 2).

After this validation of the proteomic sex estimation method, the method was applied to a sample of 15 teeth of adult individuals of archaeological provenance. For the needs of another study [41], we also estimated the sex of 32 permanent teeth of juvenile individuals from a medieval population (in which morphological sex estimation is impossible). Table 3 shows the results of the proteomic sex estimation of the application sample from the Early

Table 3
Amelogenin-sex estimation in an application sample of 15 adult teeth and a sample of 32 non-adult teeth, both from burial grounds of the Early Medieval (9–11th century AD) population from the Mikulčice agglomeration in Moravia.

Skeleton number	Age-at-death (yrs)	Tooth class	Peak area		Proteomic sex
	(Stloukal 1963, 1964, 1967)		X	Y	
H87	40–50	C	3496,055	3991,318	M
H170	50–60	${}_2P$	3745,990	4076,427	M
H0171	40–59	C	3209,222	Not present	F
H0187	50–60	P_2	693,47	1445,756	M
H0292	30–59	P_2	835,227	Not present	F
H0314(bis)	adult	P_2	4902,831	Not present	F
H0314	30–40	P^2	2952,606	3665,371	M
H0324	30–40	I_1	7071,775	3665,371	F
H0352	50–60	P^2	4283,238	Not present	F
H0363	30–40	P_1	1986,341	2610,787	M
H0406	40–50	${}^2M$	2089,384	Not present	F
H0457	50–60	P_1	4678,292	Not present	F
H0647	40–50	${}_1P$	2551,808	3039,339	M
H0718	adult	P^2	5232,203	Not present	F
H1088	adult	${}^1P$	2893,400	3510,927	M
H73-VI	5–6	M^1	404,29	408,317	M
H143	6–7	${}^1M$	985,473	1057,251	M
H160-VI	6–7	${}_1M$	806,03	Not present	F
H207	10–11	${}^1M$	713,327	1009,648	M
H247	5–6	M^1	799,429	Not present	F
H253	4	M_1	1254,321	Not present	F
H266	9–10	M^1	187,41	446,308	M
H296	2	${}^1M$	770,229	1102,988	M
H315	3–4	${}_1M$	1211,179	Not present	F
H343	2–3	M^1	1260,716	1923,015	M
H393	4–5	${}_1M$	1186,517	Not present	F
H444	15–17	${}^1M$	799,936	1221,249	M
H447	2–3	${}_1M$	776,379	644,6	M
H454	6–7	${}_1M$	949,155	Not present	F
H455	2–3	${}_1M$	533,605	Not present	F
H462	4–5	M_1	1498,764	Not present	F
H473	4–5	M_1	556,056	702,444	M
H489	9	${}_1M$	759,236	1023,086	M
H496	3–4	${}^1M$	560,303	Not present	F
H497	6–7	${}_1M$	999,491	694,996	M
H526	2–3	M^1	348,887	593,259	M
H538	9	${}_1M$	297,597	Not present	F
H550	4–5	M^1	566,669	Not present	F
H582	12–14	${}_1M$	733,286	1023,541	M
H751	5–6	${}_1M$	388,359	Not present	F
H792	5–6	${}_1M$	1006,139	1233,096	M
H878	15–16	${}_1M$	915,958	825,651	M
H881	6	${}_1M$	949,087	Not present	F
H1041	6	M_1	367,721	Not present	F
H1058	5	M_1	465,283	Not present	F
H1154	5	M_1	671,2	1374,627	M
H1171	5–6	${}_1M$	640,652	Not present	F

Tooth nomenclature: *I* = incisor, C = canine, *P* = premolar, *M* = molar, upper index = upper jaw, lower index = lower jaw, side of the index indicates side of the tooth (left or right).

Medieval period. In the case of adults, 7 teeth showed both peptide signals and corresponded to males, while 8 teeth with only AMELX-peptide belonged to females. In the case of immature individuals, the nanoLC-MS/MS analysis showed that 16 teeth with only an AMELX-peptide signal belonged to female individuals; 16 individuals with the presence of both AMELX-peptide and AMELY-peptide signals corresponded to males.

Tooth enamel changes due to protein etching

From a macroscopic point of view (visual evaluation), protein extraction does not affect the observed details of the tooth surface. It is only necessary to mention subtle colour changes and the reduction of the gloss of the enamel surface.

SEM analysis was used to examine and compare the surface morphology of each tooth from the validation group before protein etching and after etching. Figs. 1 and 2 show SEM images of the enamel surface of two examples: tooth 42 from the Pachner collection and tooth 6 from recent extractions in dental clinics. The collected SEM images show that all the samples showed variable and distinct surface changes. Because the teeth were not treated in any way before applying the etching protocol, after protein extraction from the enamel all traces of tartar and other substances present on the enamel surface disappeared.

Additionally, most of the images from the SEM showed slight alterations of the surface with the presence of microporosity, possibly with deepening grooves and defects. The method of sample preparation for proteomic sex estimation appears to weaken the sample structure, which is also evident from the micro-CT analysis. We observed microcracks in some teeth before the analysis, which after protein etching were slightly wider (Fig. 3).

Quantitative changes in enamel thickness using colour maps are shown in Fig. 4. In the given example, the enamel surface on the occlusal plane shows visible enlargement of a cavity caused by

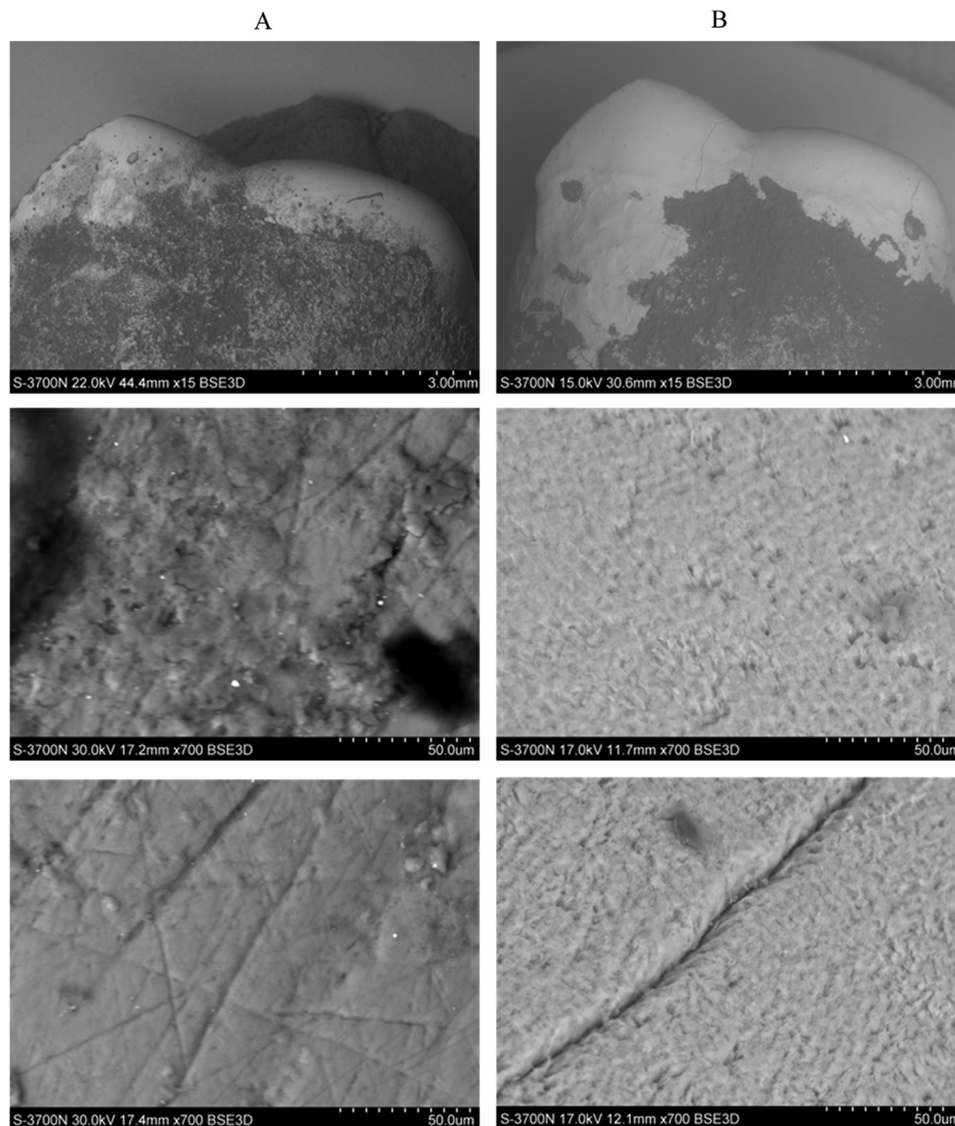

Fig. 1. Comparison of enamel surface alteration obtained by SEM before (A) and after (B) protein etching. Upper row – natural size; Middle row – area 1 at 500x magnification; Bottom row – area 2 at 500x magnification. Sample number 42 (from Pachner's Identified collection, from the first half of the 20th century).

tooth decay, which is caused by the action of the extraction solution. The enamel thickness is less, and the cavity after caries has increased due to the action of the extraction solution.

Another example of quantitative changes in enamel thickness using colour maps is shown in Fig. 5. After the application of the protein extraction protocol on the tooth enamel, there is a reduction in the thickness of the enamel, which is manifested by a change in colour from red to blue. This reduction in enamel thickness affects all of the teeth that have been treated. The colours are always less intense on the right colour map. Volume changes of both enamel and dentine, as well as of the entire tooth, are shown in Table 4. Due to the application of the extraction protocol, approximately 10% of the enamel is lost, but we also observed a 2% loss of dentine.

As can be seen in Fig. 6, the percentage of total volume loss depends on the proportion of enamel in the total volume of the tooth (this proportion is calculated as enamel volume before etching / total volume before etching). This relationship is of moderate intensity ($r = 0.46$) and weakly significant ($p = 0.042$).

Discussion

The validity of proteomic sex estimation

Proteomics is an approach with a wide range of applications. The main strength of tandem mass spectrometry (MS/MS) is its ability to analyse complex protein mixtures [42]. The use of the resulting MS/MS spectra to determine the sequence of peptides is increasingly common not only in biological sex estimation [27,29,30,32], but also in art history [43–45] and the material analysis of historical textiles [46,47]. The use of proteomic methods in palaeontology is also increasing rapidly, and it is expected that these methods will be helpful for many general applications, connecting molecular biology, palaeontology, archaeology, palaeoecology, and history [42].

The advantage of proteomics in sex estimation is its high reliability and, compared to DNA analysis, the low risk of contamination and relative cost-effectiveness [17]; it is also less destructive. The absolute accuracy of the method used for the extraction and

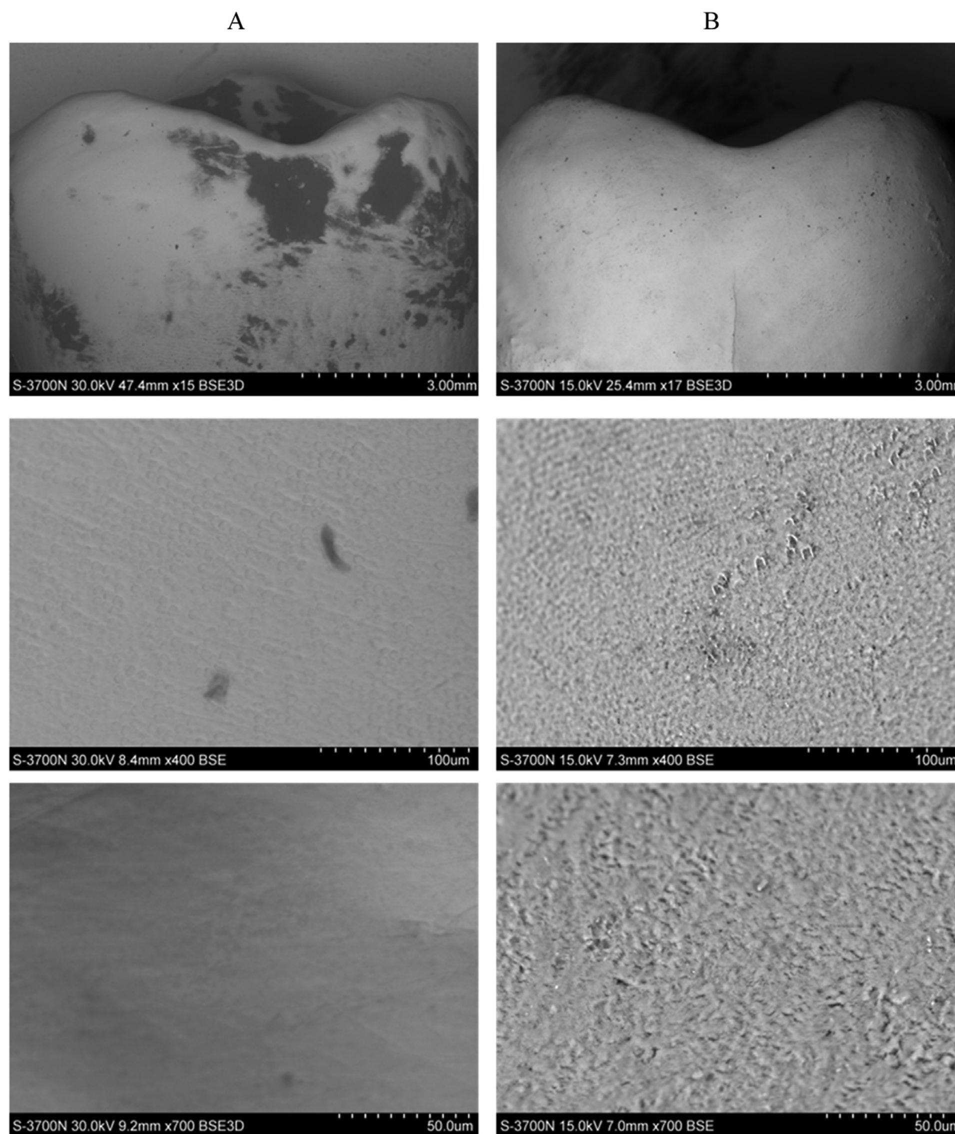

Fig. 2. Comparison of enamel surface alteration obtained by SEM before (A) and after (B) protein etching. Upper row – natural size; Middle row – area 1 at 400x magnification; Bottom row – area 1 at 700x magnification. Tooth sample number 6 (tooth from extraction in dental clinics in 2019–2021).

analysis of sex-specific proteins (Table 2) proved the suitability of the method for examining human skeletal remains, which, like cultural artefacts, form an integral part of the cultural heritage. Reliable estimation of sex in sub-adults (which is impossible to assess directly from skeletal remains due to the undevelopment of sexually dimorphic traits), so far uncommon in bioarchaeological practice, significantly increases the explanatory potential of sub-adult skeletons and helps to avoid interpretation bias resulting from unknown sex ratios in specific age classes or socioeconomic groups [41]. Therefore, if sex estimation is needed, it is advisable to use protein extraction methods [13,32] without the need for tooth destruction [26,30]. Other reasons for its use include the low impact of manipulation on the archaeological skeletal material, which manifests itself in minimal invasiveness, as also proven in our results.

In addition, the technique employed allows the first two steps (sample preparation and protein etching) to be carried out in a laboratory with minimal instrumentation and equipment. Only the third and last stage, the protein analysis itself, must take place at a workplace with the appropriate liquid chromatography-tandem mass spectrometry equipment.

Invasiveness of the amelogenin protein etching protocol

The issue of the invasiveness of protein etching is a major topic in terms of cultural heritage preservation efforts. The influence of the various chemicals to which the hard dental tissue, in the case of proteomics the enamel, is exposed should be known. However, we are not aware of any previous study evaluating the extent of enamel loss when applying proteomics for sex estimation; to the best of our knowledge, the effect of protein extraction on the structural integrity of enamel assessed here for the first time. The protocol used in the present study uses low concentrations of H_2O_2 (3%) for 30 s for cleaning and demineralization of the tooth surface to remove calcium phosphate salts (calculus), and HCl (5%) etching for only 2 min [13] with immersion of only the dental crown. Such a concentration of H_2O_2 is even lower than the maximum concentration (up to 6% H_2O_2) in tooth whitening products, which a 2011 European directive considered to be cosmetic products according to Dias et al. [48]. With regard to HCl concentration and time to its exposition, the only possibility for comparison is offered by studies that deal with the effect of acids on teeth dissolution in a forensic context. The destructive effect of highly

Table 4
Micro-CT of selected teeth before and after protein etching. Absolute and relative values of tooth volume, enamel and dentine volume.

Sample number*	Tooth class	Enamel volume (mm^3)			Dentin volume (mm^3)			Total volume (mm^3)		
		Before	After	Difference (%)	Before	After	Difference (%)	Before	After	Difference (%)
3	${}_{3}M$	248.03	224.25	9.6	475.14	463.93	2.4	723.2	688.18	4.8
4	M_3	285.38	264.53	7.3	632.08	623.11	1.4	917.5	887.64	3.3
5	${}_{3}M$	311.76	287.41	7.8	706.53	697.15	1.3	1018.3	984.56	3.3
6	${}^{3}M$	191.38	167.6	12.4	499.1	485.5	2.7	690.5	653.1	5.4
7	${}_{3}M$	290.16	270.04	6.9	738.75	728.22	1.4	1028.9	998.26	3.0
9	M^3	151.3	131.57	13.0	403.34	393.32	2.5	554.6	524.89	5.4
10	${}_{3}M$	205.22	178.91	12.8	390.81	383.48	1.9	596.0	562.39	5.6
11	M_3	191.6	166.52	13.1	620.17	603.85	2.6	811.8	770.37	5.1
12	M_3	182.37	157.88	13.4	465.99	453.56	2.7	648.4	611.44	5.7
14	M_3	162.68	139.6	14.2	389.2	372.79	4.2	551.9	512.39	7.2
31	${}^{3}M$	155.17	139.73	10.0	480.56	473.3	1.5	635.7	613.03	3.6
32	${}_{3}M$	177.24	160	9.7	476.13	471.85	0.9	653.4	631.85	3.3
42	M^1	196.09	180.22	8.1	728.44	720.29	1.1	924.5	900.51	2.6
46	M^3	155.55	141.07	9.3	531.76	516.31	2.9	687.3	657.38	4.4
47	M^1	277.28	260.76	6.0	936.66	928.53	0.9	1213.9	1189.29	2.0
48	${}^{2}M$	237.48	220.53	7.1	553.83	547.77	1.1	791.3	768.3	2.9
49	${}^{2}M$	185.94	166.71	10.3	688.07	675.21	1.9	874.0	841.92	3.7
53	${}^{1}M$	171.39	148.34	13.4	798.43	786.98	1.4	969.8	935.32	3.6
54	M^1	100.7	91.35	9.3.	1005.0	984.73	2.0	1105.7	1076.08	2.7
59	${}^{2}M$	237.44	218.2	8.1	1197.51	1178.42	1.6	1435.0	1396.62	2.7
Mean				10.10			1.92			4.02
Standard deviation				2.59			0.84			1.36
95% confidence interval for the mean				[8.88, 11.30]			[1.53, 2.31]			[3.38, 4.65]

*Numbers correspond to the sample number in Table 2.
Tooth nomenclature: *I* = incisor, C = canine, *P* = premolar, *M* = molar, upper index = upper jaw, lower index = lower jaw, side of the index indicates side of the tooth (left or right).

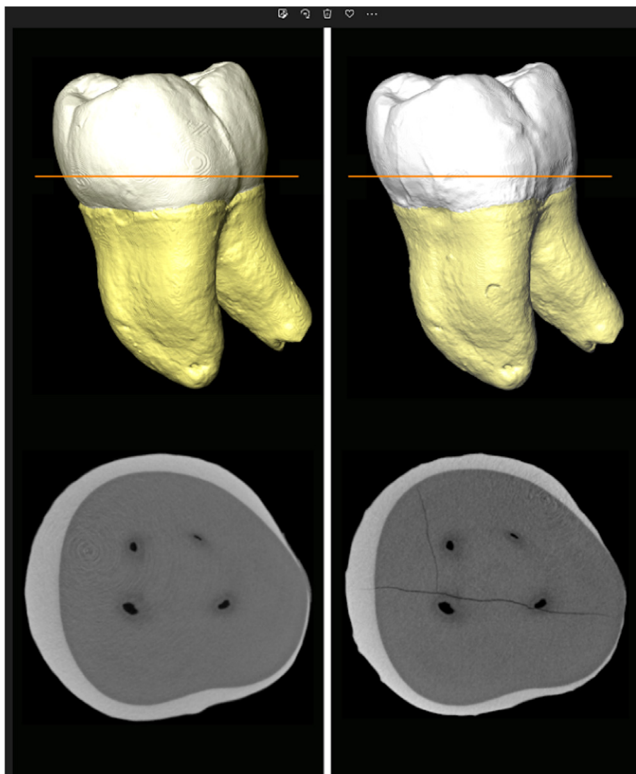

Fig. 3. Structural changes in tooth due to protein etching (Tooth sample number 3). Top left: 3D reconstruction of Tooth 3 before protocol application. Top right: after enamel etching according to the protocol to obtain protein for sex estimation. The horizontal line shows the plane of the section.

concentrated HCl (37%) on human dentition is indisputable. Several studies have established that highly concentrated HCl (depending, among other things, on the type of tooth) cause teeth to completely dissolve after a few hours [49–51]. Concerning the exposure

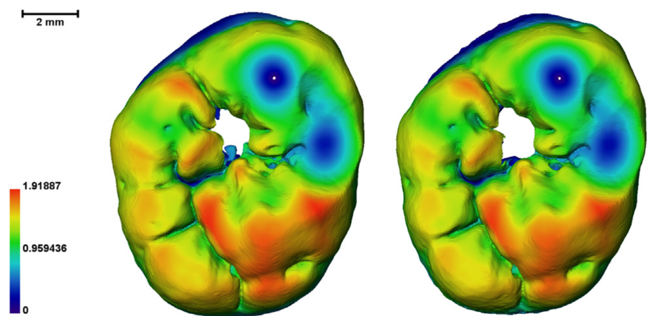

Fig. 4. Colour map of the enamel thickness on the molar occlusal surface. Red indicates areas of thicker enamel surface. The white area in the middle corresponds to an opening caused by dental caries. As a result of the extraction and the action of the acid, the opening became larger (left before etching, right after etching). Tooth sample number 46.

Table 5
Contradictory morphological sex estimation in an application sample of 15 adult teeth performed by Stloukal (1963, 1964, 1967) and by Zazvonilová et al. (2020), in comparison with amelogenin-sex estimation. Concordance of the anthropological estimation with the proteomic sex estimation is indicated by an asterisk.

Skeleton number	Anthropological sex estimation		Proteomic sex estimation
	Stloukal (1963, 1964, 1967)	Zazvonilová et.al. (2020)	Present study
H87	F?	M*	M
H170	M*	F	M
H0171	M	F*	F
H0187	F?	M?*	M
H0292	M	F*	F
H0314(bis)	Not determined	M	F
H0314	Not determined	M*	M
H0324	M	F*	F
H0352	F*	M	F
H0363	M*	F	M
H0406	F*	M	F
H0457	F?*	M	F
H0647	M*	F	M
H0718	M?	F*	F
H1088	F?	M*	M

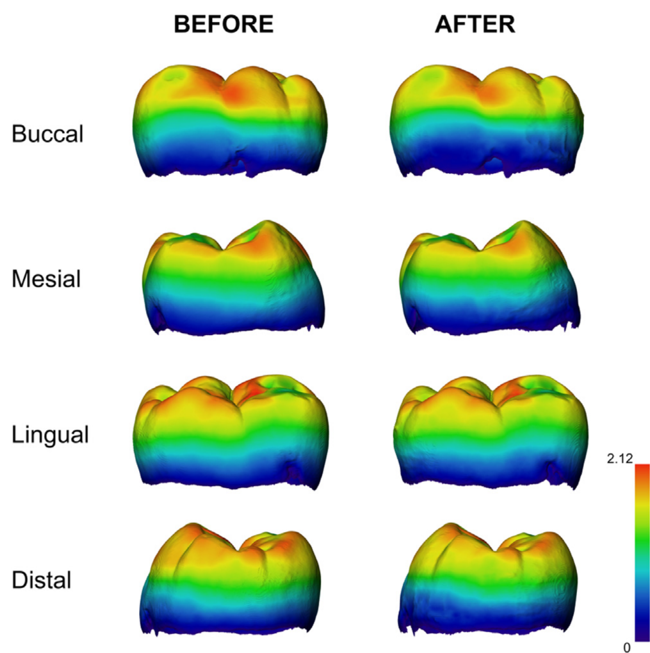

Fig. 5. Colour map of the tooth showing the enamel thickness before and after protein etching. Red indicates areas of thicker enamel surface. Tooth sample number 7.

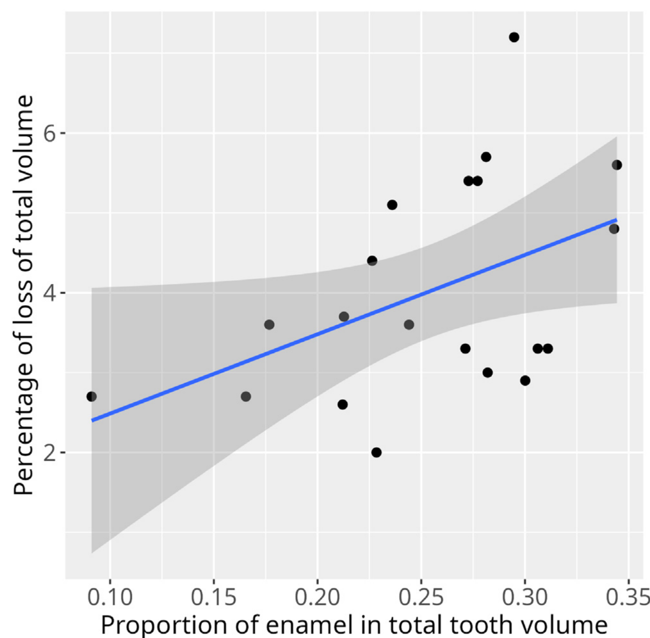

Fig. 6. Relationship between percentage of loss of total volume and the proportion of enamel in total tooth volume.

time, Mazza et al. have reported no visible effect after 5 min of immersion in 37% HCl [49]. Gupta and Johnson observed morphological and radiographic changes to enamel after 30mins – 1 hour [52]. Jones et al. 2020 reported almost complete disintegration of enamel after 4 h and no enamel present after 12 h in HCl (37%) [51]. It has been confirmed that the acid concentrations as well as the length of exposure is important for the final morphological and chemical impact [49,50], and left no traces behind. In our case, a much lower concentration and a much shorter exposure time were used, which leave no visible traces. We observed enamel loss of 10% and dentine loss of only 2% using micro-computed tomography (micro-CT) (see Table 4). However, it should be noted that this also involves the removal of surface dirt and dental calculus, which are included in the volume of dental tissue in the first scanning and are removed only before the protein etching itself.

Importance for sexing in archaeological assemblages

The application of proteomic sex estimation in an archaeological collection to an individual with questionable or contradictory results of biological sex estimation by anthropological methods allows the obtaining of correct information about biological sex, which can be used to study a whole range of issues such as demography, diet and burial rites [15,53]. In the present study, adult individuals, for whom the morphological sex assessments performed by two teams half a century apart showed differences, were selected for the application sample. Table 5 shows the results of proteomic sex estimation in 15 adults from the Early Medieval period with biological sex estimate discrepancy. The proteomic analysis agrees with the first morphological sex estimate by Stloukal in approximately half of the cases [36–38], while the same ratio of morphological sex agreement occurs in the case of the second morphological sex estimate by Zazvonilová et al. [39]. Sexing by proteomic analysis was successful for all individuals, contrary to the morphological sex estimates where some individuals were not determined or where sex was estimated with uncertainty (in Table 5 indicated by question marks). Unlike morphological sex estimation, which provides a probability of the estimated sex, in proteomic analysis sex can be assigned.

Regarding the non-adult individuals described in detail elsewhere [41], the current sample of sexed non-adults with known dietary history helped to reveal that there were no dietary differences between Great Moravian boys and girls during the first decades of their lives. Proteomics provides a new, relatively simple, and quite inexpensive method of sex estimation without the risk of contamination.

Conclusion

Our results demonstrated the suitability of a protocol that uses protein etching from an intact tooth and does not require its mechanical destruction, or that of parts of the crown. Results show the absolute accuracy of biological sex estimation in a sample of 60 individuals of known sex. Based on micro-CT analysis ($n = 20$), the etching protein process is minimally invasive and does not cause visible changes to the dental enamel surface. Application to an archaeological sample of non-adult ($n = 32$) and adult ($n = 15$) individuals proved the suitability of proteomic biological sex estimation. Proteomics provides a convenient way to estimate sex in juveniles where anthropological methods cannot be applied. The status of such a procedure allows for wider dissemination of the method, which can thus become a routine technique in the analysis of archaeological skeletal material.

Author contributions

Conceptualization JB, IM, PV, BM
Wrote the paper JB, IM, SDK, BM
Contributed to writing the paper APK, EZ
Provided tooth samples and specific expertise APK, PV
Contributed new analytical tools IM, MM, APK, PV
Performed the statistical analysis FS
Provided critical comments on the paper AD

Acknowledgements

The research was carried out with the financial support of the Laboratory of 3D Imaging and Analytical Methods, Department of

Anthropology and Human Genetics, Faculty of Science of Charles University in Prague, Ministry of Culture of the Czech Republic (DKRVO 2019-2023/7.I.e, 00023272). This research also benefited from the scientific framework of the University of Bordeaux's Idex "Investments for the Future" program / GPR "Human Past". The authors would like to thank Jiřina Dašková of the Palaeontology Department of the National Museum, Prague for obtaining the scanning electron microscope (SEM) images. We also thank Nicolas Vanderesse and Adrien Thibaut (UMR 5199 PACEA) for help with micro-CT, tooth tissue segmentation and the generation of colour maps. Our thanks also go to Mrs. Armenuhi Kirakosjan of the Institute of Physiology, Czech Academy of Sciences in Prague for her help with the proteomic analysis, and Alastair Millar for linguistic revision of the text.

References

[1] M.G. Belcastro, A. Pietrobelli, T. Nicolosi, M. Milella, V. Mariotti, Scientific and ethical aspects of identified skeletal series: the case of the documented human osteological collections of the University of Bologna (Northern Italy), Forensic Sci. 2 (2022) 349–361, doi:10.3390/forensicsci2020025.

[2] C. Knüsel, B. Maureille, Archaeological approaches to human remains: France, in: B. O'Donnabhain, B. Lozada (Eds.), Archaeol. Hum. Remain, Springer, Cham, 2018, pp. 57–80, doi:10.1007/978-3-319-89984-8_5.

[3] A. Santos, Skulls and skeletons from documented, overseas and archaeological excavations: Portuguese trajectories, in: B. O'Donnabhain, B. Lozada (Eds.), Archaeol. Hum. Remain, Springer, Cham, 2018, pp. 111–125.

[4] M. Licata, A. Bonsignore, R. Boano, F. Monza, E. Fulcheri, R. Ciliberti, Study, conservation and exhibition of human remains: the need of a bioethical perspective, Acta Bio Medica Atenei Parm. (2020) 91.

[5] M.G. Belcastro, V. Mariotti, The place of human remains in the frame of cultural heritage: the restitution of medieval skeletons from a Jewish cemetery, J. Cult. Herit. 49 (2021) 229–238.

[6] K. Squires, D. Piombino-Mascali, Ethical considerations associated with the display and analysis of juvenile mummies from the capuchin catacombs of Palermo, Sicily, Public Archaeol. 20 (2021) 66–84, doi:10.1080/14655187.2021.2024742.

[7] D.G. Jones, Anatomists' uses of human skeletons: ethical issues associated with the India bone trade and anonymized archival collections, Anat. Sci. Educ. 16 (2023) 610–617, doi:10.1002/ase.2280.

[8] G. Grupe, J. Wahl, Changing perceptions of archaeological human remains in Germany, in: B. O'Donnabhain, B. Lozada (Eds.), Archaeol. Hum. Remain, Springer, Cham, 2018, pp. 81–92.

[9] M. Sponheimer, C.M. Ryder, H. Fewlass, E.K. Smith, W.J. Pestle, S. Talamo, Saving old bones: a non-destructive method for bone collagen prescreening, Sci. Rep. 9 (2019) 13928, doi:10.1038/s41598-019-50443-2.

[10] R.M. Austin, S.B. Sholts, L. Williams, L. Kistler, C. Hofman, To curate the molecular past, museums need a carefully considered set of best practices, PNAS 116 (2019) 1471–1474, doi:10.1073/pnas.1822038116.

[11] A.H. Pálsdóttir, A. Bläuer, E. Rannamäe, S. Boessenkool, J.H. Hallsson, Not a limitless resource: ethics and guidelines for destructive sampling of archaeofaunal remains, R. Soc. Open Sci. 6 (2019) 191059, doi:10.1098/rsos.191059.

[12] M. Buckley, Proteomics in the analysis of forensic, archaeological, and paleontological bone, in: Appl. Forensic Proteomics Protein Identif. Profiling, American Chemical Society, 2019, pp. 125–141. SE–810.1021/bk-2019-1339.ch008.

[13] N.A. Stewart, R.F. Gerlach, R.L. Gowland, K.J. Gron, J. Montgomery, Sex determination of human remains from peptides in tooth enamel, Proc. Natl. Acad. Sci. 114 (2017) 13649–13654.

[14] F. Bray, S. Flament, G. Abrams, D. Bonjean, K.D. Modica, C. Rolando, C. Tokarski, P. Auguste, Extinct species identification from Upper Pleistocene bone fragments not identifiable from their osteomorphological studies by proteomics analysis, BioRxiv. (2020). https://doi.org/10.1101/2020.10.06.328021.

[15] K. Rebay-Salisbury, P. Bortel, L. Janker, M. Bas, D. Pany-Kucera, R.B. Salisbury, C. Gerner, F. Kanz, Gendered burial practices of early Bronze Age children align with peptide-based sex identification: a case study from Franzhausen I, Austria, J. Archaeol. Sci. 139 (2022) 105549, doi:10.1016/j.jas.2022.105549.

[16] I. Mikšík, M. Morvan, J. Brůžek, Peptide analysis of tooth enamel – a sex estimation tool for archaeological, anthropological, or forensic research, J. Sep. Sci. 46 (2023) 2300183, doi:10.1002/jssc.202300183.

[17] T. Buonasera, J. Eerkens, A. de Flamingh, L. Engbring, J. Yip, H. Li, R. Haas, D. DiGiuseppe, D. Grant, M. Salemi, C. Nijmeh, M. Arellano, A. Leventhal, B. Phinney, B.F. Byrd, R.S. Malhi, G. Parker, A comparison of proteomic, genomic, and osteological methods of archaeological sex estimation, Sci. Rep. 10 (2020) 1–15, doi:10.1038/s41598-020-68550-w.

[18] A.R. Klales, Sex Estimation of the Human Skeleton, Academic Press, 2020.

[19] A. Kotěrová, J. Velemínská, J. Dupej, H. Brzobohatá, A. Pilný, J. Brůžek, Disregarding population specificity: its influence on the sex assessment methods from the tibia, Int. J. Legal Med. 131 (2016) 251–261.

[20] E.K. Oikonomopoulou, E. Valakos, E. Nikita, Population-specificity of sexual dimorphism in cranial and pelvic traits: evaluation of existing and proposal of new functions for sex assessment in a Greek assemblage, Int. J. Legal Med. 131 (2017) 1731–1738.

[21] J. Brůžek, P. Murail, Methodology and reliability of sex determination from the skeleton, in: A. Schmitt, E. Cunha, J. Pinheiro (Eds.), Forensic Anthropol. Med. Complement. Sci. From Recover. to Cause Death, Humana Press Inc., Totowa, 2006, pp. 225–242.

[22] T. Waldron, The relative survival of the human skeleton: implications for palaeopathology, in: A. Boddington, A. Garland, R. Janaway (Eds.), Death, Decay Recon-Struction Approaches to Archaeol. Forensic Sci, Manchester University Press, 1987, pp. 55–64.

[23] L.K. Corron, F. Santos, P. Adalian, K. Chaumoitre, P. Guyomarc'h, F. Marchal, J. Brůžek, How low can we go? A skeletal maturity threshold for probabilistic visual sex estimation from immature human os coxae, Forensic Sci. Int. 325 (2021) 110854, doi:10.1016/j.forsciint.2021.110854.

[24] M.E. Lewis, The Bioarchaeology of Children: Perspectives from Biological and Forensic Anthropology, Cambridge University Press, Cambridge, 2006, doi:10.1017/CBO9780511542473.

[25] M. Jágr, A. Eckhardt, S. Pataridis, Z. Broukal, J. Duskova, I. Miksik, Proteomics of human teeth and saliva, Physiol. Res. 63 (2014) S141.

[26] F. Lugli, G. Di Rocco, A. Vazzana, F. Genovese, D. Pinetti, E. Cilli, M.C. Carile, S. Silvestrini, G. Gabanini, S. Arrighi, L. Buti, E. Bortolini, A. Cipriani, C. Figus, G. Marciani, G. Oxilia, M. Romandini, R. Sorrentino, M. Sola, S. Benazzi, Enamel peptides reveal the sex of the Late Antique 'Lovers of Modena, Sci. Rep. 9 (2019) 13130, doi:10.1038/s41598-019-49562-7.

[27] F. Lugli, C. Figus, S. Silvestrini, V. Costa, E. Bortolini, S. Conti, B. Peripoli, A. Nava, A. Sperduti, L. Lamanna, L. Bondioli, S. Benazzi, Sex-related morbidity and mortality in non-adult individuals from the Early Medieval site of Valdaro (Italy): the contribution of dental enamel peptide analysis, J. Archaeol. Sci. Rep. 34 (2020) 102625, doi:10.1016/j.jasrep.2020.102625.

[28] G.J. Parker, J.M. Yip, J.W. Eerkens, M. Salemi, B. Durbin-Johnson, C. Kiesow, R. Haas, J.E. Buikstra, H. Klaus, L.A. Regan, D.M. Rocke, B.S. Phinney, Sex estimation using sexually dimorphic amelogenin protein fragments in human enamel, J. Archaeol. Sci. 101 (2019) 169–180, doi:10.1016/j.jas.2018.08.011.

[29] C. Froment, M. Hourset, N. Sáenz-Oyhéréguy, E. Mouton-Barbosa, C. Willmann, C. Zanolli, R. Esclassan, R. Donat, C. Thèves, O. Burlet-Schiltz, C. Mollereau, Analysis of 5000 year-old human teeth using optimized large-scale and targeted proteomics approaches for detection of sex-specific peptides, J. Proteomics 211 (2020) 103548, doi:10.1016/j.jprot.2019.103548.

[30] A. Gasparini, F. Lugli, S. Silvestrini, A. Pietrobelli, I. Marchetta, S. Benazzi, M.G. Belcastro, Biological sex vs. archaeological gender: enamel peptide analysis of the horsemen of the Early Middle age necropolises of Campochiaro (Molise, Italy), J. Archaeol. Sci. Rep. 41 (2022) 103337, doi:10.1016/j.jasrep.2021.103337.

[31] M. Fonović, T. Leskovar, I. Štamfelj, Determination of sex based on sexually dimorphic amelogenin peptides in human tooth enamel, J. Crim. Investig. Criminol. 72 (2021) 2.

[32] R. Gowland, N.A. Stewart, K.D. Crowder, C. Hodson, H. Shaw, K.J. Gron, J. Montgomery, Sex estimation of teeth at different developmental stages using dimorphic enamel peptide analysis, Am. J. Phys. Anthropol. 174 (2021) 859–869, doi:10.1002/ajpa.24231.

[33] P. Pachner, Pohlavní Rozdíly Na Lidské Pánvi (Sexual Differences in Human Pelvis), Česká akademie věd a umění, Praha, 1937.

[34] L. Borovanský, Pohlavní Rozdíly Na Lebce člověka (Sexual differences in Human Skulls), Česká akademie věd a umění, Praha, 1936.

[35] L. Poláček, Great Moravian Elites From Mikulčice, Czech Academy of Sciences, Institute of Archaeology, Brno, 2021.

[36] M. Stloukal, První pohřebiště na hradišti „Valy "u Mikulčic, Památky Archeol. 54 (1963) 114–140.

[37] M. Stloukal, Čtvrté pohřebiště na hradišti „Valy "u Mikulčic, Památky Archeol. 55 (1964) 479–505.

[38] M. Stloukal, Druhé pohřebiště na hradišti „Valy "u Mikulčic, Památky Archeol. 58 (1967) 272–319.

[39] E. Zazvonilová, P. Velemínský, J. Brůžek, Paleodemografická interpretace kosterních souborů minulých populací: nové hodnocení raně středověkých pohřebišť u 3. a 6. kostela v Mikulčicích (Palaeodemographic interpretation of skeletal assemblages of past populations: a new evaluation of early mediev, Archeol. Rozhl. 72 (2020) 67–101.

[40] D. Ferembach, I. Schwidetzky, M. Stloukal, Recommendations for age and sex diagnoses of skeletons, J. Hum. Evol. 9 (1980) 517–549.

[41] S.D. Drtikolová Kaupová, J. Brůžek, J. Hadrava, I. Mikšík, M. Morvan, L. Poláček, L. Půtová, P. Velemínský, Early life histories of Great Moravian children – carbon and nitrogen isotopic analysis of dentine serial sections from the Early Medieval population of Mikulčice (9th-10th centuries AD, Czechia), Archaeol. Anthropol. Sci. (2022) Preprint.

[42] C. Warinner, K. Korzow Richter, M.J. Collins, Paleoproteomics, Chem. Rev 122 (2022) 13401–13446, doi:10.1021/acs.chemrev.1c00703.

[43] A. Lluveras-Tenorio, R. Vinciguerra, E. Galano, C. Blaensdorf, E. Emmerling, M.Perla Colombini, L. Birolo, I. Bonaduce, GC/MS and proteomics to unravel the painting history of the lost Giant Buddhas of Bāmiyān (Afghanistan), PLoS One 12 (2017) e0172990, doi:10.1371/journal.pone.0172990.

[44] W. Fremout, S. Kuckova, M. Crhova, J. Sanyova, S. Saverwyns, R. Hynek, M. Kodicek, P. Vandenabeele, L. Moens, Classification of protein binders in artist's paints by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry: an evaluation of principal component analysis (PCA) and soft independent modelling of class analogy (SIMCA), Rapid Commun. Mass Spectrom. 25 (2011) 1631–1640, doi:10.1002/rcm.5027.

[45] I.K. Levy, R. Neme Tauil, A. Rosso, M.P. Valacco, S. Moreno, F. Guzmán, G. Siracusano, M.S. Maier, Finding of muscle proteins in art samples from mid-18th

century murals by LC–MSMS, J. Cult. Herit. 48 (2021) 227–235, doi:10.1016/j.culher.2020.11.005.
[46] A.K. Popowich, T.P. Cleland, C. Solazzo, Characterization of membrane metal threads by proteomics and analysis of a 14th c. thread from an Italian textile, J. Cult. Herit. 33 (2018) 10–17, doi:10.1016/j.culher.2018.03.007.
[47] C. Solazzo, S. Clerens, J.E. Plowman, J. Wilson, E.E. Peacock, J.M. Dyer, Application of redox proteomics to the study of oxidative degradation products in archaeological wool, J. Cult. Herit. 16 (2015) 896–903, doi:10.1016/j.culher.2015.02.006.
[48] S. Dias, A. Mata, J. Silveira, R. Pereira, A. Putignano, G. Orsini, R. Monterubbianesi, D. Marques, Hydrogen peroxide release kinetics of four tooth whitening products—in vitro study, Materials 14 (2021), doi:10.3390/ma14247597.
[49] A. Mazza, G. Merlati, C. Savio, G. Fassina, P. Menghini, P. Danesino, Observations on dental structures when placed in contact with acids: experimental studies to aid identification processes, J. Forensic Sci. 50 (2005) JFS2004292–JFS2004295.
[50] M. Raj, K. Boaz, N. Srikant, Are teeth evidence in acid environment, Forensic Dent. Sci. 5 (2013) 7.
[51] C. Jones, T. Bracewell, A. Torabi, C.C. Beck, T.B. Harvey, The effect of hydrochloric acid (HCl) on permanent molars: a scanning electron microscope (SEM) and energy dispersive X-ray spectroscopy (EDS) study, Med. Sci. Law. 60 (2020) 172–181, doi:10.1177/0025802420905981.
[52] K.K. Gupta, A. Johnson, Morphologic and radiographic effects of acids on the teeth: an in-vitro forensic study, Indian J. Forensic Med. Toxicol. 14 (2020) 28–33.
[53] C. McFadden, The past, present and future of skeletal analysis in palaeodemography, Philos. Trans. R. Soc. B Biol. Sci. 376 (2020) 20190709, doi:10.1098/rstb.2019.0709.