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Protein sequence comparison of human and non-human primate tooth proteomes

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Conflict of interest

The authors declare that they have no conflict of interest.

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Abstract

In the context of human evolution, the study of proteins may overcome the limitation of the high degradation of ancient DNA over time to provide biomolecular information useful for the phylogenetic reconstruction of hominid taxa. In this study, we used a shotgun proteomics approach to compare the tooth proteomes of extant human and non-human primates (gorilla, chimpanzee, orangutan and baboon) in order to search for a panel of peptides able to discriminate between taxa and further help reconstructing the evolutionary relationships of fossil primates. Among the 25 proteins shared by the five genera datasets, we found a combination of peptides with sequence variations allowing to differentiate the hominid taxa in the proteins AHSG, AMBN, APOA1, BGN, C9, COL11A2, COL22A1, COL3A1, DSPP, F2, LUM, OMD, PCOLCE and SERPINA1. The phylogenetic tree confirms the placement of the samples in the appropriate genus branches. Altogether, the results provide experimental evidence that a shotgun proteomics approach on dental tissue has the potential to detect taxonomic variation, which is promising for future investigations of uncharacterized and/or fossil hominid/hominin specimens.

1 1. Introduction

2 In the last ten years, major advances have been achieved in the field of human
3 evolution with the increasing recovery of Late Pleistocene human genomes. These advances
4 have unveiled the existence of a new human group, the Denisovan hominins, an extinct
5 relative of Neanderthals [1, 2], in addition to recurrent interbreeding between archaic and
6 modern humans during their period of cohabitation in Eurasia [3, 4]. However, despite the
7 exceptional retrieval of 0.4 million-year (My) old hominin genomic data from Sima de los
8 Huesos in Spain [5, 6], the use of a DNA-based approach for older specimens, beyond the
9 Middle Pleistocene [7], remains limited due to a high degree of fragmentation of ancient DNA
10 over time [8, 9]. Therefore, the next challenging step is the biomolecular characterization of
11 fossils further back in time, in particular for the Early Pleistocene-Late Pliocene key period (3-
12 2 My ago) corresponding to the emergence of the genus *Homo*. Such invaluable missing
13 data are essential for a more accurate reconstruction of the phylogenetic relationships
14 between the different hominin taxa [10].

15 As an alternative to DNA, proteins appear to be more resistant to post-mortem
16 damage, potentially allowing biomolecular investigation of the deeper time [11, 12]. Even in
17 unfavorable conditions proteins survive longer than DNA, as demonstrated by the recovery of
18 > 3 My old peptides entrapped in ostrich egg shell in the warm African environment [13] and
19 more recently, of dental proteins from a subtropical Early Pleistocene specimen (1.9 My ago)
20 representative of the extinct Asian hominid *Gigantopithecus blacki* [14]. Given the ever
21 increasing performance of mass spectrometers, in terms of sensitivity, sequencing speed
22 and resolution, it is now possible to study the protein content of ancient samples, using small
23 amount of precious paleontological and paleoanthropological material. This opens the way to
24 a new field of research: Palaeoproteomics. Proteins have been sequenced from Late to Early
25 Pleistocene faunal and human fossils [7, 14-17], providing invaluable functional information
26 on past human (patho)physiology [18-20] and ancient diets [21, 22]. In addition, phylogenetic
27 reconstruction and taxonomic placement based on protein identification have also been
28 possible for extinct species and/or when DNA was no longer available [16, 23-27]. The two
29 main examples below on debated hominin fossils, have recently shed light on the
30 relationships between human lineages during evolution. On the basis of a collagen variant,
31 Chen et al. [24] were able to assign the mandible of Xiahe from the Tibetan plateau to a
32 Denisovan individual. For the first time, the presence of this hominin outside the Siberian
33 Altaï area was therefore demonstrated together with its anatomical relationship with other
34 archaic hominin fossils in China. By analyzing the enamel proteome of a representative of
35 *Homo antecessor* from Atapuerca (Spain) dated to 0.8-0.9 My ago, Welker et al. [27] have

36 suggested a very close affinity between this species and the last common ancestor of
37 modern humans, Neanderthals and Denisovans.

38 Collagen sequences are widely used to identify or characterize specimens [9, 28, 29],
39 but they are sometimes not enough informative because of a high conservation between
40 species limiting accurate phylogenetic reconstruction [30]. It is therefore necessary to identify
41 other proteins with sufficient taxonomic variability. In this context, the tooth proteome is
42 expected to be more informative than the bone proteome [9, 14, 16, 27]. Indeed, in addition
43 to collagens, it also contains a variety of specific non-collagenous proteins with taxonomic
44 interest, including the X and Y forms of amelogenin able to provide information about the sex
45 of ancient individuals [27, 31-34].

46 In the present study we used a shotgun proteomics approach to compare the tooth
47 proteomes of modern humans from the 21th century and non-human primates (gorilla,
48 chimpanzee, orangutan, baboon) from the 19th-mid 20th century, in order to search for a
49 panel of peptides able to discriminate between taxa. We focused on the analysis of the
50 proteins shared by the five genera datasets to allow for comparison. We found in 14 proteins
51 a combination of peptides with taxonomic variation potentially useful for future studies on
52 uncharacterized and/or older hominid/hominin specimens.

53

54 2. Materials and Methods

55 2.1 Samples

56 Non-human primate teeth (2 individuals per genus) from 19th-mid 20th century
57 collections were obtained from the University of Poitiers with the agreement of Pr R.
58 Macchiarelli (samples T1: *Gorilla*, T2: *Papio*, T5: *Pan.*, T6: *Pan*), from the University of
59 Toulouse with the agreement of Pr J. Braga (samples T3: *Papio*, T4: *Gorilla*), and from the
60 University of Montpellier with the agreement of S.Jiquel (samples T7: *Pongo*, T8: *Pongo*).
61 Except for the two chimpanzee specimens that are attributed to *Pan troglodytes*, the species
62 of the other non-human primate samples is not known. Human teeth were obtained from two
63 colleagues who kindly donated their own M3 that had been surgically extracted three (LOS2)
64 and fifteen (LKS2) years ago. Description of the teeth is given in Table S1. Teeth were
65 handled under a laminar flow hood and the operator was equipped with disposable clothes.
66 The surface of the teeth was carefully cleaned with a solution of 1% SDS and abundantly
67 rinsed with sterile pure water. The tooth area to be sampled, (preferably chosen on the
68 cervical part of the crown so that the damaged area is minimally visible after repositioning the
69 tooth on the jaw) was first manually drilled with a micromotor STRONG 207-106 (Pouget-
70 Pellerin, France) to abrade the surface. Then a spherical carbide drill (1-1.2 mm in diameter)
71 was used to pierce the enamel through the dentine tissue, over a sterile microtube to collect
72 the powder. Sample amounts ranged from 2 to 26 mg for non-human primates and 60 mg for

73 humans, respectively (Table S1). Collected powder was immediately processed for protein
74 extraction.

75 2.2. Protein extraction and Trypsin digestion

76 Samples were prepared as five independent series, each including an extraction blank
77 with no material (Blk) that was processed in the same way as the tooth samples. Protein
78 extraction was performed by using the filter-aided sample preparation (FASP) protocol
79 described in [31]. It was slightly modified to include an additional step for recovering the flow-
80 through content of the first Amicon™ Ultra-4 (10kDa) filter unit (Merck Millipore) after
81 centrifugation which may contain material of interest (Fig. 1). After a demineralization step in
82 0.5 M EDTA pH 8 for 18 h at room temperature under rotation, the pellet was extracted in 0.1
83 M Tris pH 8, 0.1 M DTT, 4% SDS for 2h at 60 °C. The supernatants from the
84 demineralization and extraction steps were mixed with 8 M urea in 0.1 M Tris pH 8 and ultra-
85 filtered through an Amicon™ Ultra-4 (10kDa) centrifugal filter unit (4000g, swinging rotor,
86 room temperature). The flow-through (except for LOS2) was collected and ultra-filtered
87 through an Amicon™ Ultra-4 (3kDa) centrifugal filter unit to recover smaller protein fragments
88 excluded from the 10kDa filtration. The two filtration units (giving at end samples referred as
89 T and Tpep, respectively) were then similarly processed. After a wash with 2 ml of 8 M urea
90 in 0.1M Tris pH 8, protein alkylation (50 mM 2-Chloroacetamide in 8M urea, 0.1 M Tris pH8)
91 was performed on the filter units for 20-30 min at room temperature in the dark. The units
92 were then washed (2 x 1 ml) with 8M urea in 0.1 M Tris pH 8, followed by 50 mM ammonium
93 bicarbonate washes (1 x 1 ml, 1 x 0.5 ml). Proteins (T samples) and peptides (Tpep
94 samples) retained on the filter were dissolved in 50 mM ammonium bicarbonate and an
95 aliquot was harvested for quantification using the Qubit protein assay kit (Thermo Fisher
96 Scientific). They were digested by overnight incubation at 37 °C with 2 µg sequencing grade
97 modified porcine trypsin (Promega). The digestion was prolonged the next day for 4-6 h with
98 2 µg additional trypsin. The tryptic peptide mixtures were recovered by centrifugation over a
99 new tube. The centrifugates were then transferred to microtubes, dried by using a centrifugal
100 vacuum concentrator and kept at -20°C until mass spectrometry analysis.

101 EDTA, Tris, and SDS were purchased from Invitrogen, urea and ammonium bicarbonate
102 from Acros Organics, chloroacetamide from Sigma-Aldrich.

103 2.3. nanoLC-MS/MS analysis

104 The dried peptides were resuspended with 0.05% trifluoroacetic acid in 2% acetonitrile
105 at an estimated concentration of 1µg/µl based on protein quantification, and then analyzed
106 by online nanoLC using an UltiMate® 3000 RSLCnano LC system (Thermo Scientific,
107 Dionex) coupled to an Orbitrap Fusion Tribrid™ mass spectrometer (Thermo Scientific,
108 Bremen, Germany). 1µl of the samples were loaded on a 300 µm ID x 5 mm PepMap C18

109 pre-column (Thermo Scientific, Dionex) at 20 μ l/min in 2% acetonitrile, 0.05% trifluoroacetic
110 acid. After 5 minutes of desalting, peptides were on-line separated on a 75 μ m ID x 50 cm
111 C18 column (in-house packed with Reprosil C18-AQ Pur 3 μ m resin, Dr. Maisch, and
112 equilibrated in 95% of buffer A (0.2% formic acid)) with a gradient of 5 to 25% of buffer B
113 (80% acetonitrile, 0.2% formic acid) for 80 min then 25% to 50% for 30 min at a flow rate of
114 300 nL/min.

115 The instrument was operated in the data-dependent acquisition (DDA) mode using a
116 top-speed approach (cycle time of 3s). The survey scans MS were performed in the Orbitrap
117 over m/z 350-1550 with a resolution of 120,000 (at 200 m/z), an automatic gain control
118 (AGC) target value of 4e5, and a maximum injection time of 50 ms. Most intense ions per
119 survey scan were selected at 1.6 m/z with the quadrupole and fragmented by Higher Energy
120 Collisional Dissociation (HCD). The monoisotopic precursor selection was turned on, the
121 intensity threshold for fragmentation was set to 50,000 and the normalized collision energy
122 was set to 35%. The resulting fragments were analyzed in the Orbitrap with a resolution of
123 30,000 (at 200 m/z), an automatic gain control (AGC) target value of 5e4, and a maximum
124 injection time of 60 ms. The dynamic exclusion duration was set to 30 s with a 10 ppm
125 tolerance around the selected precursor and its isotopes. For internal calibration the
126 445.120025 ion was used as lock mass.

127 Each sample was subjected to two independent LC-MS/MS runs (TR1, TR2) for
128 assessing the identification reproducibility. To control for carry-over contamination, the MS
129 workflow process included a washing step followed by two blank MS runs using gradient
130 conditions similar to those of the samples and performed before and after each sample MS
131 run (including the blank samples).

132 2.4. Bioinformatics analysis of nanoLC-MS/MS data

133 All raw mass spectrometry files were processed in parallel using two different protein
134 identification softwares: Proteome DiscovererTM software 2.3.0.523 (Thermo Fischer
135 Scientific) with Mascot 2.6.2 (Matrix Science, London, UK) combined with the Percolator
136 algorithm (version 2.05) for PSM search optimization, and PEAKSTM Studio 10.0 software
137 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) using the full set of available
138 processes PEAKS de novo > PEAKS DB > PEAKS PTM> PEAKS SPIDER [35, 36]. For both
139 softwares, data obtained from T and Tpep samples were searched against the UniProtKB
140 Swiss-Prot and TrEMBL protein databases (including canonical and isoform sequences, and
141 supplemented with frequently observed contaminants) corresponding to their own taxon:
142 Uniprot_isoF_Human database released 2019_09 with *Homo sapiens* taxonomy (195349
143 sequences), Uniprot_isoF_Gorilla database released 2019_07 with *Gorilla* taxonomy (46070
144 sequences), Uniprot_isoF_Pan database released 2019_06 with *Pan* (chimpanzees)

145 taxonomy (154055 sequences), Uniprot_isoF_Pongo released 2019_02 with *Pongo*
146 (orangutan) taxonomy (96058 sequences), Uniprot_isoF_Papio released 2019_02 with *Papio*
147 (baboons) taxonomy (46692 sequences). Data obtained from the blank samples were
148 searched against the five databases.

149 For Proteome Discoverer analysis, Mascot database searches were performed
150 individually for each raw file using a processing workflow consisting of the following
151 parameters: mass tolerances in MS and MS/MS were set to 10 ppm and 0.02 Da,
152 respectively. Carbamidomethylation of cysteine was set as a fixed modification. The enzyme
153 specificity was selected as semi-tryptic, with a maximum of three missed cleavages. The
154 main protein modifications commonly observed in damaged and ancient proteins were set as
155 variable modifications: deamidation (N, Q), oxidation (M, P), carbamylation (K, N-terminal
156 protein), and conversion to pyro-glutamic acid (N-terminal Q). The Percolator algorithm was
157 used to validate PSMs and peptides based on Posterior Error Probability (PEP) values at a
158 $FDR \leq 1\%$ [37, 38]. FDR was estimated by a target-decoy approach using the reversed
159 database. Afterwards, the processing workflow results (.msf files) were combined into
160 sample (technical replicates TR1 + TR2), genus (technical replicates TR1 + TR2, T and Tpep
161 samples) or extraction blank multiconsensus reports. Each resulting dataset was then filtered
162 using a consensus workflow consisting of the following parameters: Only PSMs with rank 1
163 and Mascot ion score ≥ 20 were considered. Peptide identifications were grouped into
164 proteins according to the law of parsimony and filtered to 5% FDR.

165 For PEAKS analysis, all the raw files belonging to the same genus (technical replicates
166 TR1 + TR2, T and Tpep samples) or to the extraction blanks were loaded into a single
167 identification workflow per genus or per extraction blank and processed using the following
168 parameters: mass tolerances in MS and MS/MS were set to 10 ppm and 0.02 Da,
169 respectively. Carbamidomethylation of cysteine was set as a fixed modification, deamidation
170 (N, Q) and oxidation (M, P) as variable modifications. A maximum of 3 modifications per
171 peptide was allowed. In the PEAKS PTM module, all the 313 modifications and also a
172 maximum of 3 modifications per peptide were considered, and the validation was based on
173 an average local confidence (ALC) score $\geq 15\%$. Trypsin with semi-specific digest mode and
174 a maximum of three missed cleavages were selected. Finally, each resulting dataset per
175 genus and per extraction blank obtained from the PEAKS SPIDER module, was filtered and
176 exported using the following threshold values: Peptide score of $-10\lg P \geq 20-22$ adjusted to
177 obtain a $FDR \leq 1\%$ for PSMs and peptides, Protein score of $-10\lg P \geq 25-49$ adjusted to
178 obtain a $FDR \leq 5\%$ without taking into account the criterion of unique peptide and only
179 considering significant peptides, and *de novo* only $ALC (\%) \geq 50$. FDR was estimated by the
180 PEAKS “decoy-fusion” approach.

181 For each analysis, the proteins marked as contaminant or found in the extraction
182 blanks were excluded from the datasets analyzed. All the MS/MS spectra of the taxon-
183 specific peptides were inspected manually. The species specificity of peptides with
184 taxonomic interest were checked by a protein Blast (BlastP) search in Uniprot
185 (<https://www.uniprot.org/>) and NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

186 All the RAW data files, the output files corresponding to Proteome Discoverer™ (.msf
187 files) and PEAKS (.csv files and HTML reports) analyses, and all the fasta files used for the
188 database searches have been deposited on the ProteomeXchange Consortium [PMID
189 24727771] via the PRIDE partner repository and can be accessed with the dataset identifier
190 PXD018933.

191 2.5. Proteomic data analysis

192 To be able to compare proteomes between genera, the accession numbers of the
193 proteins identified in the different taxa were converted to human gene names by using the
194 Retrieve ID functions of Uniprot (<https://www.uniprot.org/>), or the db2db conversion tool of
195 bioDBnet (<https://biodbnet-abcc.ncifcrf.gov/>), and/or by BLAST alignment and manual
196 inspection. Classification of the identified proteins into functional categories according to GO
197 terms was performed by using the GSEA software at [https://www.gsea-
198 msigdb.org/gsea/msigdb/annotate.jsp](https://www.gsea-
198 msigdb.org/gsea/msigdb/annotate.jsp) [39]. Graphic representations and statistical analysis of
199 the data were performed by using Prism 7 (GraphPad Software Inc., USA). Online tool found
200 at <http://bioinformatics.psb.ugent.be/webtools/Venn/>
201 diagrams.

202 2.6 Phylogenetic analysis

203 The phylogenetic tree was based on the alignment of 14 proteins (AHSG, AMBN,
204 APOA1, BGN, C9, COL11A2, COL22A1, COL3A1, DSPP, F2, LUM, OMD, PCOLCE,
205 SERPINA1). The concatenated protein sequences identified in each genus sample were
206 aligned to the concatenated corresponding protein sequences from 8 hominoids (*Homo*
207 *sapiens*, *Gorilla gorilla*, *G.gorilla gorilla*, *Pan troglodytes*, *Pan paniscus*, *Pongo abelii*, *Pongo*
208 *pygmaeus*, *Nomascus leucogenys*) and 3 Papionini (*Papio anubis*, *Papio hamadryas*,
209 *Mandrillus leucophaeus*) by using the Catfasta2phymI tool available at
210 <https://github.com/nylander/catfasta2phymI>. The concatenated alignment is given in the
211 supplementary Phylotreealign.fasta file. The best-fit model for generating the phylogenetic
212 tree was selected based on the Akaike's information criterion corrected (AICc) implemented
213 in jModelTest 2 [40]. The tree was built using RAXML-NG [41] with a bootstrapping procedure
214 (X1000) as statistical test for branch support.

215

216 3. Results

217 3.1. Comparative analysis of human, gorilla, chimpanzee, orangutan and baboon tooth 218 proteomes.

219 For each genus, two types of samples (T and Tpep) were prepared (Fig. 1). The T
220 samples correspond to proteins retained on the filter unit of the FASP protocol and usually
221 analyzed in proteomic experiments. The Tpep samples were recovered from the elution of
222 the filter units that is usually discarded but might also contain material of interest such as
223 peptides or protein fragments present in the demineralized/lysed extracts. The samples from
224 2 individuals per genus (*Homo*, *Gorilla*, *Pan*, *Pongo* and *Papio*) were analyzed in duplicate
225 by using a nanoLC-MS/MS data-dependent analysis of the tryptic digestions. The Proteome
226 Discoverer software was used with the Mascot search engine for protein identification in
227 genus-specific protein databases. This led to identification of 32 to 172 proteins per T
228 samples, with only a few additional ones identified in Tpep samples (Fig. S1A and S1B).

229 The total number of proteins identified per taxon by Proteome Discoverer ranged from
230 33 in chimpanzee (*Pan*) to 228 in human (Fig. S1C and Table S2 for the raw list of proteins).
231 To allow for the comparison between the five proteomes, the protein accessions were
232 converted into the corresponding human gene names. This resulted in a reduced number of
233 identification (Fig.S1C) because of redundant proteins corresponding to multiple accession
234 numbers (isoforms, incomplete sequence, etc.) for a same gene. The analysis of the
235 distribution of proteins between the five proteomes (Fig. 2A) indicates that 25 proteins are
236 common to the five genera (Table 1). Less than 15% of proteins are unique to each taxon,
237 with the exception of *Homo* (45%) and *Papio* (25%). A gene set overlap analysis according
238 to GO annotation terms indicates that the Top 20 most significant gene sets are different
239 between the common and the exclusive pooled proteins (Fig.2B). While shared proteins are
240 annotated with terms related to extracellular matrix organization/ossification and
241 blood/wound healing, 20% to 46% of the proteins unique in the *Homo*, *Gorilla*, *Pongo* and
242 *Papio* samples are significantly associated with immunity, in addition to extracellular matrix
243 organization and coagulation (Fig.2B, Table S3). Identifying proteins involved in the immune
244 system in the variable proteome is not unexpected since it may reflect the different sanitary
245 status between taxa and/or individuals, in addition to a possible genus-specific expression of
246 proteins [42].

247 3.2. Analysis of the proteins of interest for taxonomic discrimination

248 A comparative analysis of the peptide lists from the proteins common to the five
249 primate proteomes was performed to search for the presence of taxon-specific peptides that
250 could be useful markers for specimen identification. Compared to the only use of protein
251 database search engines, *de novo* peptide sequencing algorithms that do not require a

252 protein sequence database [35], or error-tolerant search algorithms that utilize protein
253 sequence databases while allowing sequence deviation [43], represent powerful approaches
254 in palaeoproteomics to allow for the identification of novel amino acid substitutions.
255 Therefore, to potentially improve the identification of taxon-specific peptides, an additional
256 round of bioinformatic data analysis was performed using PEAKS software which uses a *de*
257 *novo*-assisted database search algorithm to maximize the peptide identification efficiency
258 [35, 36]. As shown in Fig.S3A, PEAKS yielded slightly more protein identifications than
259 Proteome Discoverer (Fig. S1C), with a 40-72% overlap of the identifications between the
260 two datasets (Fig. S3B) if considering gene name correspondence rather than protein
261 accessions to avoid protein redundancy.

262 The 25 proteins found in the five primate samples, especially the collagens, were
263 mainly identified by peptides covering regions of highly conserved sequences. However, a
264 number of peptides showing amino acid variations between taxa were identified in 14
265 proteins (AHSG, AMBN, APOA1, BGN, C9, COL11A2, COL22A1, COL3A1, DSPP, F2, LUM,
266 OMD, PCOLCE, SERPINA1). The lists of all the peptides identified for each protein in each
267 dataset per genus with Proteome Discoverer or PEAKS softwares are given in Tables S4 to
268 S17. The phylogenetic tree based on the concatenated alignment of the 14 proteins confirms
269 the placement of the five samples in the appropriate genus branches (Fig. 3). Because of
270 missing protein sequences in the database for some species, in particular *G. gorilla*, *P.*
271 *pygmaeus*, *P. hamadryas*, and as no peptide strictly specific to a species was identified in
272 the dataset, with the exception of *H. sapiens* (Table 2), the taxonomic discrimination at the
273 level of the species is less guaranteed. The main representative peptides showing amino
274 acid substitutions able to discriminate between the hominids taxa are presented in Table 2,
275 after the validation of their specificity by a BlastP search. The HCD MS/MS spectra are
276 shown in Fig. 4 and 5 and Fig. S4. Peptides specific to the taxa *Homo*, *Gorilla* and *Pongo*
277 were identified. No *Pan*-specific peptides were detected, probably because of the lower
278 protein coverage in this sample (see discussion). Despite the phylogenetic distance and a
279 high number of amino acid variations with respect to the four other primates, no *Papio*-
280 specific peptide was found (Tables S4-S17).

281 The protein displaying the most diversity is alpha-2-HS-glycoprotein/Fetuin A (AHSG)
282 which is well covered in the five genera (Table 1). A number of discriminating peptides were
283 identified in this protein (Tables 2 and S4). Peptides covering AHSG-[72-99] with a lysine
284 residue at the position 99 are specific to *Gorilla* (Fig. 4A) while the peptides covering AHSG-
285 [104-117] with a lysine residue at the position 117 and AHSG-[328-337] with a leucine
286 residue at the position 329 are specific to *Pongo* (Fig. 4B and 4C). Interestingly, a particular
287 combination of amino acids in the peptides covering AHSG-[318-337] specifically
288 differentiates *Homo* (P329/V333) and *Pongo* (L329/V333) from the two other taxa

289 (P329/A333) (Fig. 4C, and Table 2). In addition, the position 45 and 52 in the peptide AHSG-
290 [29-57] allows to distinguish *Homo* and *Gorilla* (I45/L52) from *Pan* and *Pongo* (I45/H52) and
291 from *Papio* (V45/L52) (Fig.4D and Tables 2 and S4). Among the other proteins, *Homo*-
292 specific peptides are identified in the F2 protein (F2-[199-217]), *Gorilla*-specific peptides are
293 identified in the proteins COL3A1 (COL3A1-[351-368] and -[902-923]), DSPP (DSPP-[403-
294 411]) and SERPINA1 (SERPINA1-[126-149]), and *Pongo*-specific peptides are identified in
295 the proteins APOA1 (APOA1-[185-195]) and BGN (BGN-[220-239]) (Table 2 and Fig. S4).
296 Beside the taxon-specific peptides, peptides with a variable signature or a combination of
297 variable amino acids among taxa, may be also informative. For example, peptides covering
298 PCOLCE-[305-320] distinguish *Homo* and *Pan* (S309) from *Gorilla* and *Pongo* (T308) (Table
299 2 and Fig. 5). Peptides C9-[232-242] bearing A238, C9-[546-558] bearing I546/E554/N557
300 and DSPP-[362-370] bearing T365/A367 are specific to the Homininae taxon (Table 2 and
301 Fig. S4). The peptide DSPP-[56-66] with a leucine residue at the position 60 is specific to
302 *Homo* and *Pan*. Taken together, the data demonstrate therefore that it is possible by using a
303 MS-based proteomics approach on dental tissue to identify a combination of peptides
304 enabling the distinction between members of the four hominid genera (*Homo*, *Gorilla*, *Pan*
305 and *Pongo*) in accordance with the phylogenetic tree (Fig. 3).

306

307 4. Discussion

308 The present study is, to our knowledge, the first comparative analysis of tooth
309 proteomes from five living primate genera, including one cercopithecidae (*Papio*) and the
310 four extant hominids (*Homo*, *Pan*, *Gorilla*, *Pongo*). It is noteworthy that we had to deal with
311 the incomplete genomic annotation of non-human primates to convert protein accessions
312 from each species into the corresponding canonical (human) gene names, a prerequisite for
313 allowing comparison between taxa. However, even if a few mis-conversions or
314 unrecognitions might still remain, a total of 312 proteins corresponding to the pooled proteins
315 from all samples across all genera after removing of duplicates, were identified (Fig. S5A).
316 67% belong to dentine tissue, compared to the recently reported comprehensive human
317 dentine proteome [44]. The other 30% proteins include the amelogenin protein specific to the
318 enamel tissue, diverse collagens, in addition to constituents of the extracellular matrix and
319 immune system proteins (Fig S5B). The later components may reflect the dynamic and
320 heterogeneous part of dentine, a tissue rich in diverse bioactive peptides involved in host
321 defense, regenerative process, angiogenesis, growth and differentiation [44-47]. These are
322 expected to vary between species or individuals depending on health status and/or traumatic
323 injury [42, 47, 48]. The number of proteins identified was lower in chimpanzee compared to
324 the other specimens, and this was not due to a smaller tooth sampling (same amount as in

325 orangutan, Table S1). Without excluding a possible poor preservation of proteins in the
326 chimpanzee specimens, or a less efficient protein extraction, the low number of proteins
327 could also reflect a different level of protein expression in the dental tissue in this taxon [49].
328 Indeed, the chimpanzee has a particularly thin enamel which is more prone to tooth damage
329 than in the other great apes [50]. Another explanation could be related to the tooth types that
330 were sampled. While for all the other specimens, enamel and dentine powder were collected
331 from permanent teeth, for the two chimpanzees we could only sample their deciduous teeth.
332 The nature and degree of protein expression might differ between permanent and deciduous
333 teeth, although this does not appear to be the case in humans [51] and this will need to be
334 investigated further.

335 All the proteins common to the five proteomes, with the exception of COL10A1 and
336 COL23 A1, have already been identified in human dentine extracts [44, 46, 52], although
337 AMBN may also be derived from the enamel [45, 53]. COL22A1 and F2 have been detected
338 at the enamel-dentine junction (EDJ), an interface between the mineralized tissues involved
339 in mechanical load [54]. The analysis of the peptides in the proteins common to the five
340 proteomes allowed for the identification of sequence variations in AHSG, AMBN, APOA1,
341 BGN, C9, COL11A2, COL22A1, COL3A1, DSPP, F2, LUM, OMD, PCOLCE and SERPINA1
342 enabling a taxonomic placement at the genus level (Fig.3 and Table 2). However, no species
343 marker was detected, excepted for the species *H.sapiens*. Interestingly, nine of the proteins
344 have been detected in 5000-year-old bovine dentin samples [55]. These include AHSG also
345 identified in the 1.9 My old enamel of the extinct primate *Gigantopithecus blacki* [14] and
346 COL3A1 identified in the dentine from the Xiahe specimen attributed to Denisova [24].
347 Therefore, some of the proteins described here and showing a potential taxonomic interest
348 survive in time and to fossilization processes.

349 AHSG has already been reported to be resistant to degradation and to display
350 enough sequence variation to be of interest for phylogenetic studies [9, 12]. Here, although
351 the protein was identified by 3 peptides in only 2 out of 20 blank replicates (Table S2), it was
352 identified by a larger number of peptides in the samples (Table 1) suggesting a probable
353 endogenous origin of the protein in the samples. AHSG was therefore kept in the analysis to
354 make a comparison with the data from the literature on ancient specimens. Among the
355 peptides identified in AHSG, those bearing amino acids K-99 or L-329 are specific to *Gorilla*
356 and *Pongo*, respectively. In addition, the combination of P-329 and V-333 is specific to the
357 *Homo* taxon. In *Gigantopithecus blacki* [14] only one peptide was identified in a highly
358 conserved region of the protein (AHSG-[133-145]) susceptible to contamination in our
359 analysis (Table S4). AMBN was poorly covered in our samples (Tables 1 and S5) and not
360 detected in the dentine of the Xiahe Denisovan [24], in contrast to the other paleontological
361 specimens sampled from dental enamel [14, 27]. This suggests that using enamel tissue to

362 extract this protein is more appropriate than using dentine alone, or both dentine and
363 enamel. Similarly to ancient samples (*Homo antecessor*, *Homo erectus*, *Gigantopithecus*
364 *blacki*), the N-terminal part of the AMBN including the substitutions S34T and R55G that
365 distinguish *Papio* from the other hominid taxa were covered in our samples (Table S5).
366 However, no peptides were identified in the region overlapping a combination of amino acid
367 substitutions that differentiates *Pongo* (V264/G269) and *Papio* (M265/G170) from the others
368 taxa (V265/E270), and which indeed helps to affiliate *Gigantopithecus* to the pongine clade.
369 The dentine proteome of the Denisovan specimen from Xiahe exclusively contains collagens.
370 Interestingly, all the peptides identified in the ancient COL3A1 were also identified in our
371 samples (Table S11). In particular, they include the peptides covering the substitution A364V
372 specific to *Gorilla*, and the substitution S796G that differentiates *Homo/Gorilla/Pan* (S) from
373 *Pongo/Papio* (G).

374 A number of taxon-specific peptides were identified in the other dentine proteins
375 (Table 2), with the exception of *Pan* due to the low protein coverage of the chimpanzee
376 samples, and probably also because of the high sequence homology with *Homo* (lower
377 bootstrap at the hominin node, Fig.3). Peptides with taxonomic specificity were generally
378 identified together with the corresponding peptides in the other taxa proteins (Fig. 4, Fig. 5,
379 Fig. S4 and Tables S4-S17). As some positions were also covered in ancient samples, the
380 results support the potential of a MS-based proteomics approaches for protein identification
381 and taxonomic discrimination of extant and fossil primates from tooth samples.

382 In conclusion, the present comparison between human and non-human primates
383 tooth proteomes shows that a shotgun proteomics approach on dental tissue has the
384 potential to discriminate between the hominid taxa *Homo*, *Gorilla*, *Pan* and *Pongo*, despite a
385 high protein sequence homology (Fig.3). The results also suggest that dentine proteins offer
386 informative variability. However the data highlight the limitation of the method to differentiate
387 individual species. A targeted MS-based approach using a combination of the peptides
388 identified in this study, especially in AHSG, APOA1, BGN, COL3A1, DSPP, F2 and
389 PCOLCE, could be applied for further in-depth taxonomic investigations of ancient samples,
390 as previously done with amelogenin peptides for sex estimation [31]. In light of the recent
391 papers on Pleistocene specimens [14, 27] a promising way is open to characterize the
392 taxonomic attribution and phylogenetic relationships of fossil hominid remains, notably for
393 those older than the Middle Pleistocene for which DNA information may not be preserved or
394 not retrievable with the currently available methods.

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Table 1: List of the proteins common to the five proteomes.

The list of proteins was retrieved from the comparison of the protein lists obtained from each dataset per genus with Proteome Discoverer (PD) or PEAKS (PX) softwares. For each protein, Uniprot accession numbers correspond to the master protein and the first protein of the protein group identified by PD and by PX, respectively. The number of total identified peptides (and unique peptides) that allowed for protein identification is indicated. * In the case of proteins identified with only 1 unique peptide, the HCD MS/MS spectra of unique peptides are presented in Fig. S2. nd (not detected)

Gene name	Protein name	Ident Software	Homo		Gorilla		Pan		Pongo		Papio	
			Uniprot Acession	# Peptides (unique)	Uniprot Acession	# Peptides (unique)	Uniprot Acession	# Peptides (unique)	Uniprot Acession	# Peptides (unique)	Uniprot Acession	# Peptides (unique)
AHSG	Alpha 2-HS glycoprotein	PD	P02765	27 (27)	E1U7Q5	47 (44)	A0A2R9ADR9	5 (5)	H2PC98 E1U7Q6	24 (2)	A0A096NPS0	36 (36)
		PX	B7Z8Q2	51 (19)	E1U7Q5	89 (85)	A0A2R9ADR9	29 (29)	H2PC98 E1U7Q6	59 (4)	B9MSS3	71 (71)
AMBN	Ameloblastin	PD	Q9NP70	6 (6)	G3RCU1	2 (2)	A0A2R9CKL8	2 (2)	A0A2J8UTQ2	2 (2)	A0A096NFU6	3 (3)
		PX	Q9NP70	8 (8)	nd	nd	A0A2J8PF83	2 (2)	nd	nd	A0A096NFU6	1(1)
APOA1	Apolipoprotein A1	PD	A0A024R3E3	12 (12)	G3QY98	7 (7)	K7D1U8	5 (5)	P0DJG1	9 (9)	P68293	12 (12)
		PX	P02647	17 (15)	G3QY98	11 (11)	P0DJG0	8 (8)	A0A2J8X1C8	12 (12)	P68293	17 (16)
BGN	Biglycan	PD	B4DDQ2	11 (11)	A0A2I2YJ91	15 (15)	H2R1R5	10 (10)	H2PX51	14 (14)	A0A096NEE7	27 (27)
		PX	A6NLG9	17 (17)	A0A2I2YJ91	38 (37)	H2R1R5	20 (19)	H2PX51	17 (17)	A0A096NEE7	61 (59)
C3	Complement C3	PD	P01024	6 (6)	G3RBJ0	7 (7)	A0A2R9B9K1	4 (4)	A0A2J8R6I7	3 (3)	A0A0A0MUD9	3 (3)
		PX	V9HWA9	18 (17)	G3RBJ0	17 (17)	K7CUE1	11 (11)	A0A2J8R6I7	4 (4)	A0A0A0MUD9	13 (13)
C9	Complement C9	PD	A0A024R035	3 (3)	G3RIM1	12 (12)	A0A2R9BNI9	2 (2)	H2PFE3	4 (4)	A0A096N4A4	12 (12)
		PX	P02748	6 (6)	G3RIM1	26 (26)	A0A2R9BNI9	10 (10)	H2PFE3	6 (6)	A0A096N4A4	23 (23)
CLEC11A	C-type lectin domain family 11A	PD	M0R081	3 (3)	G3S6C9	2 (2)	A0A2J8J1C9	2 (2)	A0A2J8U8Y9	1 (1)*	A0A2I3M1B2	5 (5)
		PX	Q5U0B9	4 (2)	G3S6C9	4 (4)	H2QGY4	3 (0)	nd	nd	A0A2I3M1B2	8 (8)
COL10A1	Collagen alpha-1(X) chain	PD	Q5QPC7	1 (1)*	G3S3J2	3 (2)	A0A2R9A6P1	2 (1)*	A0A2J8U152	1 (1)*	A0A096NA96	2 (2)
		PX	nd	nd	A0A2I2YVF5	4 (3)	nd	nd	A0A2J8U152	1 (1)	A0A096NA96	5 (4)
COL11A1	Collagen alpha-1(XI) chain	PD	D3DT71	9 (6)	A0A2I2Z7V8	20 (18)	A0A2I3TI23	6 (5)	A0A2J8VCH4	7 (7)	A0A2I3LKH0	19 (17)
		PX	P12107-4	12 (9)	G3QG18	24 (23)	A0A2I3RPT1	10 (9)	A0A2J8VCG6	8 (8)	A0A096MQI3	27 (24)
COL11A2	Collagen alpha-2(XI) chain	PD	A0A1U9X7I9	30 (27)	G3R2X9	34 (32)	H2R4E0	8 (7)	A0A2J8Y2T4	7 (7)	A0A096NHF5	33 (31)
		PX	P13942	34 (30)	G3R2X9	52 (50)	A0A2J8NYG5	15 (0)	A0A2J8Y2T2	11 (11)	A0A2I3NDC2	48 (47)
COL22A1	Collagen alpha-1(XXII) chain	PD	Q8NFW1	7 (7)	G3R3K3	19 (19)	H2QWR6	5 (5)	A0A2J8SAY6	9 (9)	A0A096NYZ3	9 (9)
		PX	Q8NFW1-2	10 (1)	G3R3K3	27 (27)	H2QWR6	9 (2)	A0A2J8SAY6	7 (1)	A0A2I3NDC7	16 (16)
COL23A1	Collagen alpha-	PD	Q86Y22	1 (1)*	G3QVJ2	2 (2)	A0A2J8JTY4	4 (3)	A0A2J8SD64	1 (1)*	A0A096NDQ8	2 (2)

	1(XXIII) chain	PX		nd		nd	A0A2R9C687	2 (1)		nd		nd
COL3A1	Collagen alpha-1(III) chain	PD	P02461	90 (31)	G3RK87	38 (26)	K7D718	45 (15)	A0A2J8WW41	50 (33)	A0A096N2I8	59 (41)
		PX	P02461	92 (69)	G3RK87	46 (28)	K7D718	53 (6)	A0A2J8WW41	48 (10)	A0A096N2I8	84 (58)
COL5A1	Collagen alpha-1(V) chain	PD	B2ZZ86	17 (14)	G3R760	15 (13)	K7CMZ9	11 (10)	A0A2J8UIU7	13 (13)	A0A096NWJ0	18 (17)
		PX	Q59EE7	17 (13)	G3R760	25 (24)	K7D718	22 (3)	A0A2J8UIT7	18 (18)	A0A096NWJ0	35 (32)
COL5A2	Collagen alpha-2(V) chain	PD	P05997	39 (37)	G3RDT1	37 (33)	H2R6B8	18 (14)	H2P838	34 (31)	A0A096MVP2	42 (41)
		PX	P05997	39 (37)	G3RDT1	60 (14)	A0A2R9AJC2	32 (28)	H2P838	32 (29)	A0A096MVP2	65 (62)
DSPP	Dentin sialophosphoprotein	PD	Q9NZW4	7 (7)	G3SE58	11 (11)	A0A2J8MA47	2 (2)	A0A2J8V572	4 (4)	A0A2I3M6W8	4 (4)
		PX	Q9NZW4	11 (11)	G3SE58	21 (21)	A0A2J8MA47	6 (6)	A0A2J8V572	9 (9)	A0A2I3M6W8	9 (9)
F2	Prothrombin	PD	P00734	20 (3)	G3QVP5	25 (25)	A0A2R9C6X1	6 (6)	Q5R537	8 (8)	A0A096N4Z1	11 (11)
		PX	P00734	31 (4)	G3QVP5	35 (7)	H2Q3I2	13 (13)	Q5R537	16 (16)	A0A096N4Z1	24 (24)
HSPG2	Heparan sulfate proteoglycan	PD	P98160	2 (2)	A0A2I2YAC3	8 (8)	H2PY96	3 (3)	H2N8P5	6 (6)	A0A096N531	3 (3)
		PX	A0A024RAB6	5 (5)	A0A2I2YAC3	10 (10)	A0A2R9C5E1	4 (4)	H2N8P5	6 (6)	A0A096N531	5 (5)
LUM	Lumican	PD	P51884	10 (10)	G3S376	9 (9)	A0A2R8ZXH3	5 (5)	Q5RFG1	3 (3)	A0A096MQ49	9 (9)
		PX	Q53FV4	10 (10)	G3S376	13 (13)	H2Q6L3	7 (7)	H2NI87	6 (6)	A0A096MQ49	15 (15)
OMD	Osteomodulin	PD	B2R7N9	7 (7)	A0A2I2YQC5	7 (7)	H2QXG5	2 (2)	H2PSP3	2 (2)	A0A096P237	6 (6)
		PX	B2R7N9	11 (11)	A0A2I2YQC5	11 (11)	H2QXG5	6 (6)	H2PSP3	4 (4)	A0A096P237	11 (11)
PCOLCE	Procollagen C-endopeptidase enhancer1	PD	A4D2D2	8 (8)	G3R5A8	11 (11)	A0A2R9C060	3 (3)	A0A2J8XUJ5	3 (3)	A0A096NNW4	11 (11)
		PX	Q15113	10 (10)	G3R5A8	16 (16)	H2QV35	5 (5)	A0A2J8XUJ5	4 (4)	A0A096NNW4	14 (14)
SERPINA1	Alpha-1-antitrypsin	PD	A0A384MDQ7	9 (9)	S4UFD6	4 (4)	A0A2J8QMJ5	2 (2)	Q5RCW5	5 (5)	P01010	3 (3)
		PX	E9KL23	24 (24)	G3QXZ8	16 (16)	A0A2J8QMJ5	4 (4)	Q5RCW5	8 (8)	P01010	5 (5)
SERPINC1	Antithrombin-III	PD	P01008	9 (9)	G3S9Q7	6 (5)	A0A2R9CFX9	5 (5)	Q5R5A3	4 (4)	A0A096N0R9	7 (7)
		PX	A0A0K0Q2Z1	13 (10)	G3S9Q7	9 (8)	A0A2R9CFX9	13 (9)	Q5R5A3	5 (5)	A0A096N0R9	19 (11)
SPARC	Secreted protein acidic rich in C/Osteonectin	PD	P09486	5 (5)	G3RJ76	13 (13)	A0A2R9BZI6	6 (6)	Q5R767	10 (10)	A0A096MNJ1	15 (15)
		PX	D3DQH8	7 (1)	G3RJ76	27 (27)	H2QRU3	17 (17)	Q5R767	11 (11)	A0A096MNJ1	48 (48)
VTN	Vitronectin	PD	P04004	8 (8)	G3R679	7 (7)	H2QCH3	2 (2)	H2NT31	2 (2)	A0A096P388	6 (6)
		PX	D9ZGG2	18 (18)	G3R679	11 (11)	A0A2R9BDP7	6 (6)	A0A2J8TMB3	3 (3)	A0A096P388	9 (9)

Table 2: Proteins with the main representative peptides showing a taxonomic variation among the hominids.

The list of peptides was retrieved from each dataset obtained per genus with Proteome Discoverer (PD) or PEAKS (PX) softwares (the complete lists of peptides is given in Tables S4-S17, with more detailed information). For each peptide, a Blast search on protein (BlastP) was performed in Uniprot and NCBI to check the species specificity (100% identity and Query cover).

Red bold high size character indicates a position showing an amino acid variation specific to the taxon. *The ancestral/derived amino substitution is provided with respect to the position in the human Uniprot protein accession indicated in brackets. Black bold high size character indicates a discriminative variation between groups of hominids. The spectra of the peptides are shown in Fig. 4, Fig. 5 and Fig. S4.

Homo sample				
Gene name	Uniprot accession	Position	Ancestral /derived*	BlastP result
AHSG	P02765			
	QPNCCDDPETEEAALVA IDYINQNL PWGYK	29-57		<i>H. sapiens, G. gorilla gorilla, G. gorilla</i>
	HTFMGVVSLG SPSGE VSHPR	318-337		<i>H. sapiens</i>
	LGS SPSGE VSHPR	326-337		<i>H. sapiens</i>
DSPP	Q9NZW4			
	ESGV L VHEGDR	56-66		<i>H. sapiens, P. troglodytes, P. paniscus</i>
	ESE THA VGK	362-370		<i>H. sapiens, G. gorilla gorilla (na G. gorilla), P. troglodytes, P. paniscus</i>
F2	P00734			
	SEGSSVNLSP P LEQCVDR	199-217	S210L	<i>H. sapiens</i>
	N.LSP P LEQCVDR.G	206-217		<i>H. sapiens</i>
PCOLCE	A4D2D2/Q15113			
	TEE S PSAPDAPTCPK	306-320		<i>H. sapiens, P. troglodytes, P. paniscus</i>
Gorilla sample				
Gene name	Uniprot accession	Position	Ancestral /derived*	BlastP result
AHSG	E1U7Q5/A0A2I2ZQ06		(P02765)	
	QPNCCDDPETEEAALVA IDYINQNL PWGYK	29-57		<i>H. sapiens, G. gorilla gorilla, G. gorilla</i>
	QPSGELFEIEIDTLETTCHVLDPT PVA K	72-99	R99K	<i>G. gorilla gorilla, G. gorilla</i>
	VLDPT PVA K	91-99		<i>G. gorilla gorilla, G. gorilla</i>
	HTFMGVV SLGSPSGE ASHPR	318-337		<i>G. gorilla gorilla, G. gorilla, P. troglodytes, P. paniscus</i>
	LGS SPSGE ASHPR	326-337		<i>G. gorilla gorilla, G. gorilla, P. troglodytes, P. paniscus</i>
C9	G3RIM1			
	TSNFNA A ISLK	232-242		<i>H. sapiens, G. gorilla gorilla (na G. gorilla), P. troglodytes, P. paniscus</i>
	ISEGLPAL E FPNE	546-558		<i>H. sapiens, G. gorilla gorilla (na G. gorilla), P. troglodytes, P. paniscus</i>
COL3A1	G3RK87		(P02461)	
	GEVGPAGSPGSNG V PGQR	351-368	A364V	<i>G. gorilla gorilla (na G. gorilla)</i>

	DGP A GPAGNTGAPGSPGVSGPK	902-923	P405A	G. gorilla gorilla (na G. gorilla)
DSPP	G3SE58		(Q9NZW4)	
	ESE T H A VGK	362-370		<i>H.sapiens, gorilla gorilla, P. troglodytes, P. paniscus</i>
	GQHGMIL S K	403-411	G410S	G. gorilla gorilla (na G. gorilla)
F2	G3QVP5			
	K. G QPSVLQVVNLPIVER.P	518-533		<i>G.gorilla gorilla (na G. gorilla), H. sapiens, P. troglodytes, P. paniscus</i>
PCOLCE	G3R5A8			
	TEE T PSAPDAPTCPK	306-320		<i>G. gorilla gorilla (na G. gorilla), P. abelii (na P. hamadryas)</i>
SERPINA1	S4UFD6/G3QXZ8		(P01009)	
	TLNQPDSQLQLTTG S GFLFSEGLK	126-149	N140S	G. gorilla gorilla, G. gorilla
Pongo sample				
Gene name	Uniprot accession	Position	Ancestral /derived*	BlastP result
AHSG	H2PC98/ E1U7Q6		(P02765)	
	QPNCCDPETEEAALVA I DYINQ N HPWGYK	29-57		<i>P abelii, P. pygmaeus, P. troglodytes, P. paniscus</i>
	QLKEHAVEGDCDF K	104-117	Q117K	P. abelii, P. pygmaeus
	S LSGE V SHPR	328-337	P329L	P. abelii, P. pygmaeus
APOA1	P0DJG1/A0A2J8X1C8		(P02647)	
	THLAPY T DEL R	185-195	S191T	P. abelii, P.pygmaeus
BGN	H2PX51		(P21810)	
	ELHLDNNK L A G VPSGLPDLK	220-239	R291G	P. abelii (na P. pygmaeus)
	L A G VPSGLPDLK	228-239		P. abelii (na P. pygmaeus)
PCOLCE	A0A2J8XUJ5			
	TEE T PSAPDAPTCPK	305-319		<i>G. gorilla gorilla (na G. gorilla), P. abelii (na P. pygmaeus)</i>

Legend to figures

Figure 1: Schematic of the FASP protocol describing the steps for the preparation of the T samples (tryptic peptides issued from protein digestion) and the Tpep samples (tryptic peptides issued from the digestion of peptides and/or fragmented proteins already present in the demineralized/lysed extract). See Materials and Methods for details.

Figure 2: Comparison of human (*Homo*); gorilla (*Gorilla*) chimpanzee (*Pan*), orangutan (*Pongo*) and baboon (*Papio*) tooth proteomes.

(A) Venn diagram (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) showing the distribution between genera of the proteins identified in each resulting dataset per genus using Proteome Discoverer, and converted into the corresponding human gene names for allowing comparison. (B) Gene set overlap analysis according to GO annotation terms of the proteins common to the five taxa (Common), or uniquely identified in each genus, by using the GSEA tool at <https://www.gsea-msigdb.org/gsea/msigdb/annotate.jspdb>. The bar graph shows the pattern of distribution of the proteins sorted with significant FDR q-values <0.05 in the Top 20 GO Gene Sets. The distribution was calculated as the ratio of the protein counts in each category (blood/wound-healing, extracellular matrix/ossification, immune response and others the rest of the less represented GO terms) to the total protein counts in the Top 20 gene sets, and was represented as stacked bars for each sample.

Figure 3: Phylogenetic tree based on the concatenated alignment of 14 proteins (AHSG, AMBN, APOA1, BGN, C9, COL11A2, COL22A1, COL3A1, DSPP, F2, LUM, OMD, PCOLCE, SERPINA1).

The position of the sample datasets is visualized in bold characters.

The tree was built using RAxML-NG. Node values (%) correspond to 1000 bootstraps and branch length indicates the rate of amino acid substitution.

Figure 4: HCD MS/MS spectra of AHSG peptides with taxonomic interest.

(A) AHSG-[91-99] *Gorilla*-specific peptide VLDPTPVAK (doubly charged precursor ion, MH2+, at m/z 470.2787; scan 17775; OFCCF180623_18_SP01_CCF01530_T4_TR1_Gorilla.raw). (B) AHSG-[104-117] *Pongo*-specific peptide QLKEHAVEGDCDFK (triply charged precursor ion, MH3+, at m/z 559.5901; scan 12157; OFCCF200122_13_SP03_CCF01646_T8_TR1_Pongo.raw). (C) AHSG-[318-337] *Homo*-specific peptide HTFMGVVSLGSPSGEVSHPR (triply charged precursor ion, MH3+, at m/z 694.3470; scan 57611;

OFCCF180912_14_S3_CCF01452_LOS2_TR1_Human.raw), AHS-[318-337] *Gorilla* peptide HTFMGVVSLGSPSGEASHPR (triply charged precursor ion, MH₃⁺, at m/z 685.0026; scan 57284; OFCCF180623_18_SP01_CCF01530_T4_TR1_Gorilla.raw) and AHS-[328-337] *Pongo*-specific peptide SLSGEVSHPR (doubly charged precursor ion, MH₂⁺, at m/z 534.7749; scan 6900; OFCCF200122_31_SP03_CCF01646_T8_TR2_Pongo.raw). (D) AHS-[29-57] *Homo*- and *Gorilla*- specific peptide QPNcDDPETEEAALVAIDYINQNLPGWK (triply charged precursor ion, MH₃⁺, at m/z 1121.8558; scan 65473; OFCCF180623_24_SP01_CCF01530_T4_TR2_Gorilla.raw) and the corresponding AHS-[29-57] *Pongo*-specific peptide QPNcDDPETEEAALVAIDYINQNHPPWK (triply charged precursor ion, MH₃⁺, at m/z 1129.8445; scan 54151; OFCCF200122_31_SP03_CCF01646_T8_TR2_Pongo.raw). Series of y- and b-ions are highlighted in blue and red, respectively. c: carbamidomethylated cysteine residue.

Figure 5: HCD MS/MS spectra of PCOLCE peptides with taxonomic interest.

PCOLCE-[306-320] *Homo* peptide TEESPSAPDAPTcPK (doubly charged precursor ion, MH₂⁺, at m/z 793.8555; scan 14888; OFCCF180912_22_S3_CCF01452_LOS2_TR2_Human.raw) and, the corresponding *Gorilla* PCOLCE-[306-320] and *Pongo* PCOLCE-[305-319] peptides TEETPSAPDAPTcPK (doubly charged precursor ion, MH₂⁺, at m/z 800.8607; scan 19435; OFCCF180623_24_SP01_CCF01530_T4_TR2_Gorilla.raw, and doubly charged precursor ion, MH₂⁺, at m/z 800.8613; scan 14388; OFCCF200122_13_SP03_CCF01646_T8_TR1_Pongo.raw, respectively).

Series of y- and b-ions are highlighted in blue and red, respectively. c: carbamidomethylated cysteine residue.

Figure 1: Schematic of the FASP protocol describing the steps for the preparation of the T samples (tryptic peptides issued from protein digestion) and the Tpep samples (tryptic peptides issued from the digestion of peptides and/or fragmented proteins already present in the demineralized/lysed extract). See Materials and Methods for details.

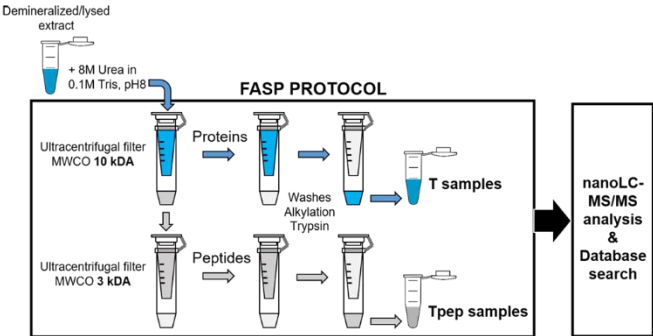


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(A) Venn diagram (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) showing the distribution between genera of the proteins identified in each resulting dataset per genus using Proteome Discoverer, and converted into the corresponding human gene names for allowing comparison. (B) Gene set overlap analysis according to GO annotation terms of the proteins common to the five taxa (Common), or uniquely identified in each genus, by using the GSEA tool at <https://www.gsea-msigdb.org/gsea/msigdb/annotate.jspdb>. The bar graph shows the pattern of distribution of the proteins sorted with significant FDR q-values <0.05 in the Top 20 GO Gene Sets. The distribution was calculated as the ratio of the protein counts in each category (blood/wound-healing, extracellular matrix/ossification, immune response and others the rest of the less represented GO terms) to the total protein counts in the Top 20 gene sets, and was represented as stacked bars for each sample.

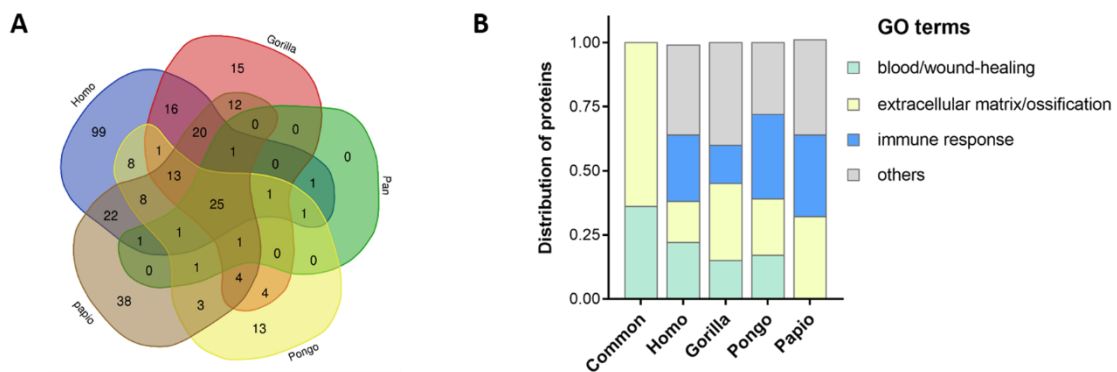


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The position of the sample datasets is visualized in bold characters.

The tree was built using RAxML-NG. Node values (%) correspond to 1000 bootstraps and branch length indicates the rate of amino acid substitution.

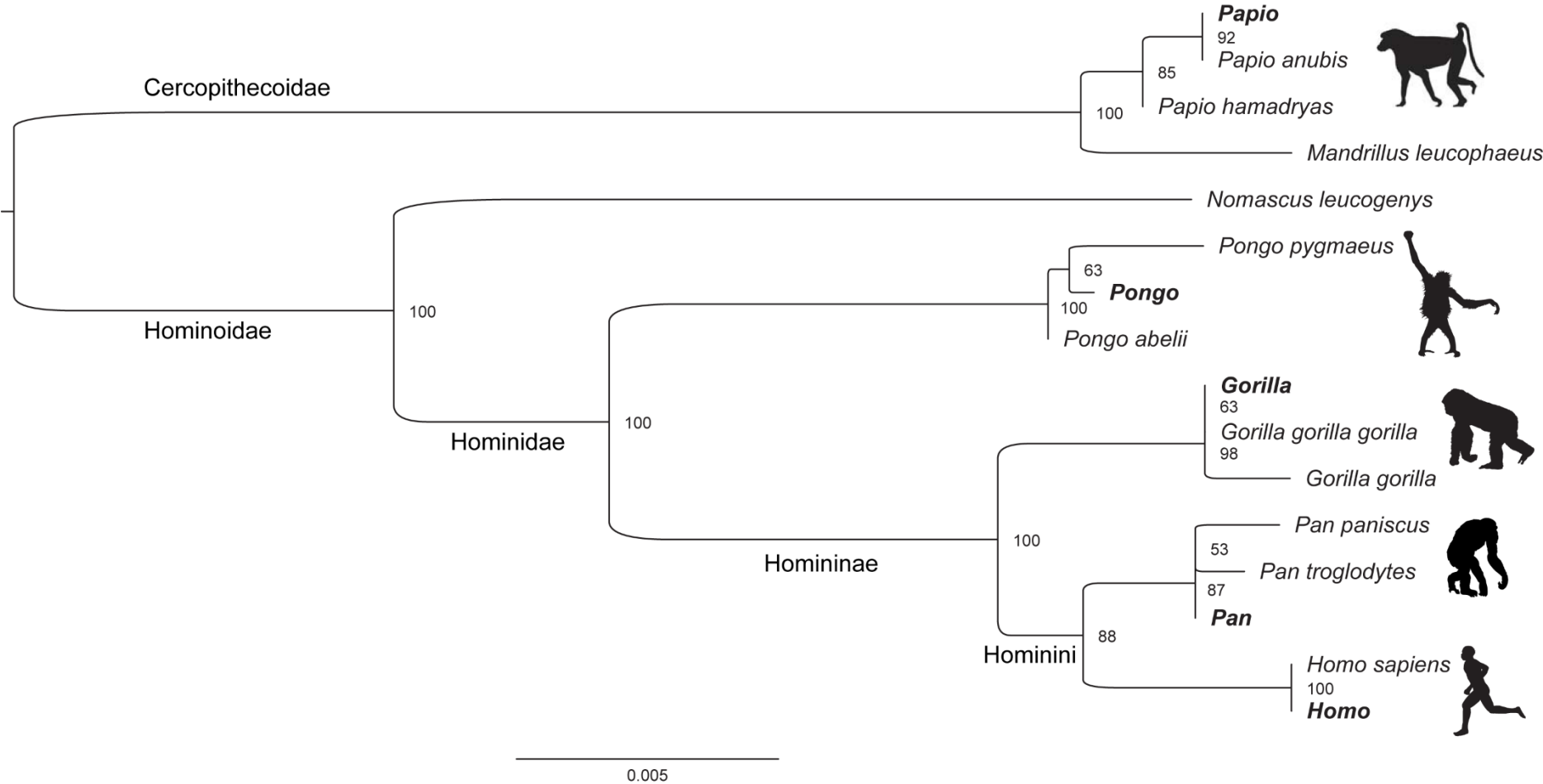


Figure 4: HCD MS/MS spectra of AHSB peptides with taxonomic interest.

(A) AHSB-[91-99] *Gorilla*-specific peptide VLDPTPVAK (doubly charged precursor ion, MH₂⁺, at m/z 470.2787; scan 17775; OFCCF180623_18_SP01_CCF01530_T4_TR1_Gorilla.raw). (B) AHSB-[104-117] *Pongo*-specific peptide QLKEHAVEGDCDFK (triply charged precursor ion, MH₃⁺, at m/z 559.5901; scan 12157; OFCCF200122_13_SP03_CCF01646_T8_TR1_Pongo.raw). (C) AHSB-[318-337] *Homo*-specific peptide HTFMGVVSLGSPSGEVSHPR (triply charged precursor ion, MH₃⁺, at m/z 694.3470; scan 57611; OFCCF180912_14_S3_CCF01452_LOS2_TR1_Human.raw), AHSB-[318-337] *Gorilla* peptide HTFMGVVSLGSPSGEASHPR (triply charged precursor ion, MH₃⁺, at m/z 685.0026; scan 57284; OFCCF180623_18_SP01_CCF01530_T4_TR1_Gorilla.raw) and AHSB-[328-337] *Pongo*-specific peptide SLSGEVSHPR (doubly charged precursor ion, MH₂⁺, at m/z 534.7749; scan 6900; OFCCF200122_31_SP03_CCF01646_T8_TR2_Pongo.raw). (D) AHSB-[29-57] *Homo*- and *Gorilla*- specific peptide QPNcDDPETEEAALVAIDYINQNLPGWK (triply charged precursor ion, MH₃⁺, at m/z 1121.8558; scan 65473; OFCCF180623_24_SP01_CCF01530_T4_TR2_Gorilla.raw) and the corresponding AHSB-[29-57] *Pongo*-specific peptide QPNcDDPETEEAALVAIDYINQNHPGWK (triply charged precursor ion, MH₃⁺, at m/z 1129.8445; scan 54151; OFCCF200122_31_SP03_CCF01646_T8_TR2_Pongo.raw). Series of y- and b-ions are highlighted in blue and red, respectively. c: carbamidomethylated cysteine residue.

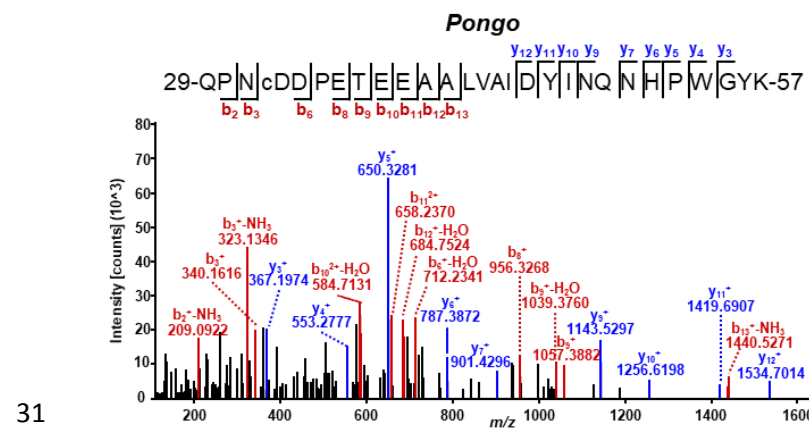
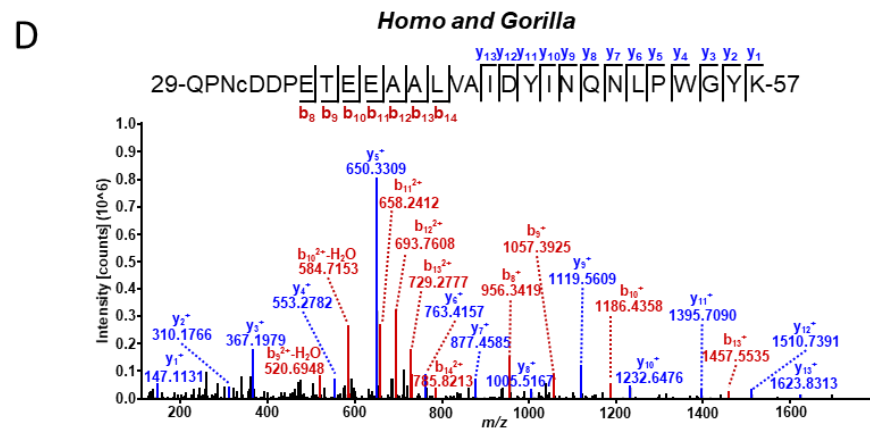
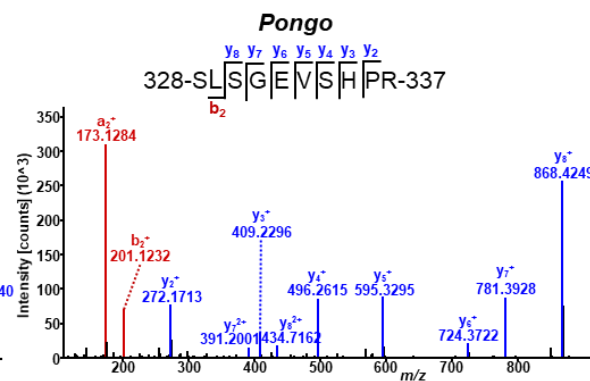
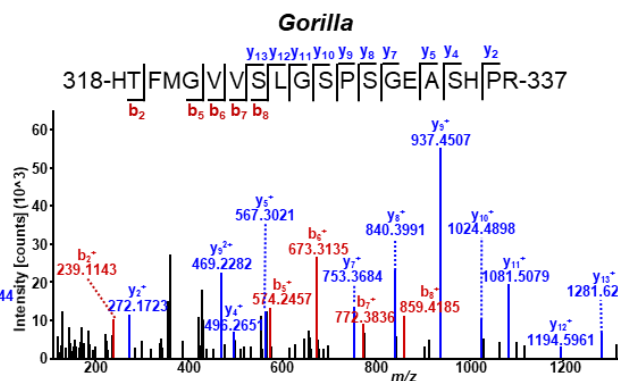
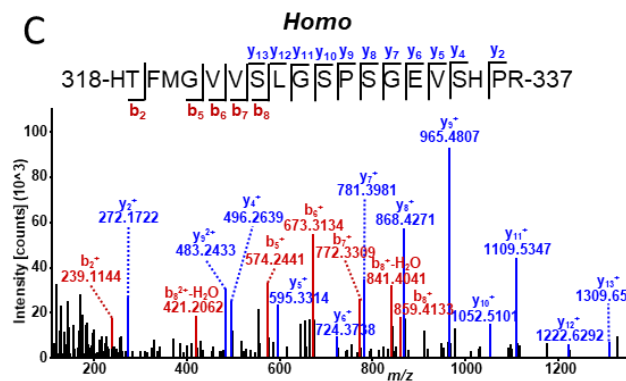
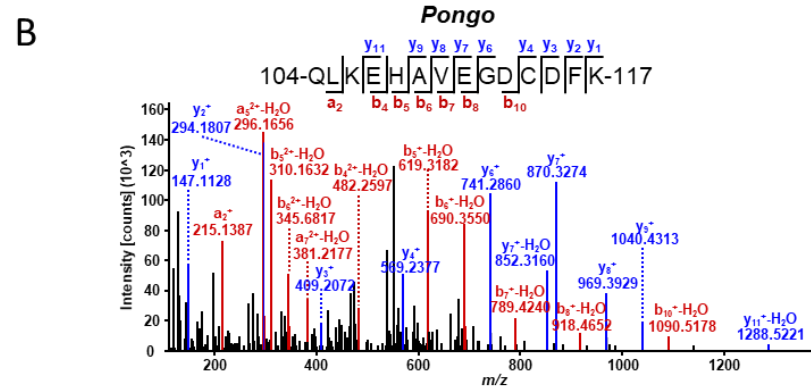
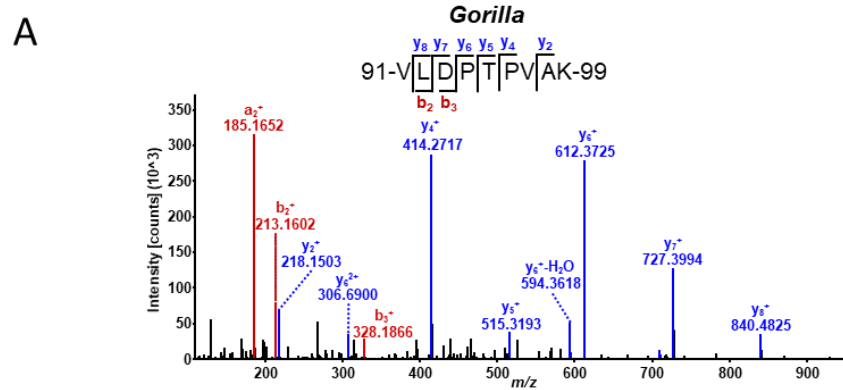


Figure 5: HCD MS/MS spectra of PCOLCE peptides with taxonomic interest.

PCOLCE-[306-320] *Homo* peptide TEESPSAPDAPTcPK (doubly charged precursor ion, MH₂⁺, at m/z 793.8555; scan 14888; OFCCF180912_22_S3_CCF01452_LOS2_TR2_Human.raw) and, the corresponding *Gorilla* PCOLCE-[306-320] and *Pongo* PCOLCE-[305-319] peptides TEETPSAPDAPTcPK (doubly charged precursor ion, MH₂⁺, at m/z 800.8607; scan 19435; OFCCF180623_24_SP01_CCF01530_T4_TR2_Gorilla.raw, and doubly charged precursor ion, MH₂⁺, at m/z 800.8613; scan 14388; OFCCF200122_13_SP03_CCF01646_T8_TR1_Pongo.raw, respectively).

Series of y- and b-ions are highlighted in blue and red, respectively. c: carbamidomethylated cysteine residue.

