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Ana Arzelier, Harmony de Belvalet, Marie-Hélène Pemonge, Pauline Garberi, Didier Binder, et al.. Ancient DNA sheds light on the funerary practices of late Neolithic collective burial in southern France. *Proceedings of the Royal Society B: Biological Sciences*, 2024, 291 (2029), rspb.2024.1215. 10.1098/rspb.2024.1215 . hal-04679849

HAL Id: hal-04679849

<https://hal.science/hal-04679849>

Submitted on 30 Aug 2024

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1 **Ancient DNA sheds light on the funerary practices of late Neolithic collective**
2 **burial in southern France**

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11

12 **Abstract**

13 The Aven de la Boucle (Corconne, Gard, Southern-France) is a karst shaft used as a collective
14 burial between 3,600 and 2,800 cal BCE. The site encompasses the skeletal remains of
15 approximately 75 individuals comprising a large majority of adult individuals, represented by
16 scattered and commingled remains. To date, few studies have explored the potential of ancient
17 DNA to tackle the documentation of Neolithic collective burials and the funerary selection rules
18 within such structures remain largely debated.

19 In this study, we combine genomic analysis of 37 individuals with archaeo-anthropological data
20 and bayesian modelling of radiocarbon dates. Through this multidisciplinary approach, we aim
21 to characterize the identity of the deceased and their relationships, as well as untangle the
22 genetic diversity and funerary dynamics of this community.

23 Genomic results identify 76% of male Neolithic individuals, suggesting a marked sex-biased
24 selection. Available data emphasize the importance of biological relatedness and a male-
25 mediated transmission of social status, as the affiliation to a specific male-lineage appears as
26 a preponderant selection factor. The genomic results argue in favor of “continuous” deposits
27 between 3,600 and 2,800 BCE, carried out by the same community, despite cultural changes
28 reflected by the ceramic material.

29

30 **Introduction**

31 The use of collective burial, i.e., the successive gathering of several individuals within the same
32 funerary structure over time, is an emblematic phenomenon of the second half of the Neolithic
33 period throughout Western Europe. In southern France, it became widespread during the
34 second half of the 4th millennium BCE, which corresponds to the transition from the broad
35 Chasséen culture to one of the various cultures that emerged at the regional level at the end

36 of the Neolithic, i.e., the Ferrières culture. Identifying the social motivation underlying the use
37 of collective burial practices remains challenging for several reasons. For example, the
38 dichotomy between megalithic monuments and cavities gave rise to debates surrounding the
39 reasons for these distinct choices in funerary locations. Since the natural settings of certain
40 regions within southern France permit both types of structures to coexist, the choice of location
41 calls for arguments other than environmental opportunism and could be connected to cultural
42 preferences and traditions (1–4). Frequently used over several generations, collective burials
43 are generally seen to include individuals from the same community, symbolizing collective
44 ancestry without emphasizing individual identities. However, it is also suggested that such
45 burials could have included individuals from various families, thus serving to strengthen
46 sociopolitical ties between distinct local groups (5,6). Here, we propose a multidisciplinary
47 approach combining archaeological, radiocarbon and genomic data from the Aven de la Boucle
48 natural cavity to document for the first time the dynamics of the use of a Neolithic collective
49 burial in France.

50 The Aven de la Boucle is located near Corconne (Gard) in southern France and was used as
51 a collective burial from the second half of the 4th millennium BCE (Figure 1, SI, Figure C1). The
52 group of the deceased is represented by scattered remains across the funerary space, with
53 few anatomical connections (7–10). The archaeo-anthropological study of the Aven de la
54 Boucle assemblages demonstrated the primary deposition of the dead inside this cavity, with
55 marked selection according to the age at death of the individuals, displayed by the massive
56 exclusion of children.

57 The quantitative analysis of skeletal parts identified a minimum number of individuals
58 approximating 75, based on calcaneus bones (MNI by exclusions, SI, Figure C2), with
59 evidence of the retrieval of some skeletal parts by the living (7–11). Indeed, voluminous pieces
60 such as coxal, cranial remains and long bones account for approximately 30 to 45 individuals
61 (SI, Figure C2).

62 Two stratigraphic horizons are superposed and distinguished through archeological material,
63 notably ceramic elements. At the base, the first corresponds to a transitional stage from the
64 Final Chasséen to the Ferrières cultures, also referred to as the “Recent Neolithic” or Pre-
65 Ferrières. The second belongs to the Ferrières culture *stricto sensu* (10). These funerary
66 occupations, whether continuous or not, took place as the cavity was accessible through the
67 rearranged joint. This passage was condemned after its use for funerary purposes, and the
68 cavity remained closed until a vertical shaft opened accidentally. Therefore, the cavity was
69 reoccupied sporadically during the Fontbousse period at the end of the 3rd millennium BCE
70 and the final stage of the Bronze Age (Final Bronze Age II; ~1,200-900 BCE) (12). The

71 succession of these main Neolithic horizons raises the questions of continuity versus
72 discontinuity of communities using the cavity and the evolution of funerary rites at the end of
73 the Neolithic in eastern Languedoc.

74 At the genomic scale, extensive documentation on the end of the Neolithic period is still lacking
75 in France. Recent genome-wide studies revealed 39 individuals for the whole French territory,
76 spanning ~3,600 to 2,000 BCE (13–17). Despite the heterogeneity of cultural contexts and the
77 variability of population dynamics documented during this time transect in the modern-day
78 French territory, no local or microregional focus has been conducted thus far.

79 Our multidisciplinary study was articulated around several lines of research. First, we aimed to
80 document the biological identity of the deceased and to discuss the entwinement of the
81 genomic results and archaeoanthropological observations. Second, we questioned the
82 potential of combining aDNA studies and radiocarbon dating to decipher the dynamics of
83 sepulchral cavity use, notably by addressing the question of continuity vs. discontinuity
84 regarding deceased deposits. In a third line of research, we also addressed the genomic
85 results obtained at the population level by examining how the individuals represented at the
86 Aven de la Boucle fit into the regional and pan-European picture.

87

88 **Results**

89 ***Overview of the Aven de la Boucle dataset***

90 In this study, we present genome-wide data for 37 individuals from the Aven de la Boucle,
91 including ten previously sequenced individuals from this cavity (18). Among the 37 individuals
92 analyzed, 27 are directly dated, we report 17 new radiocarbon dates, complementing ten dates
93 previously reported (18, Figure 1B, Table 3). The dataset is represented by 34 Neolithic
94 individuals (3,800-2,900 BCE) and three Bronze Age individuals (Table 1, 2 & 3).

95 For palaeogenomic analyses, we sampled 41 left petrous bones to retrieve bone powder from
96 which DNA was extracted. Libraries were then constructed, and postmortem deamination
97 patterns consistent with aDNA origin were examined. The first screening via shallow shotgun
98 sequencing allowed us to select libraries from 39 individuals who passed quality filtering (Table
99 2, mapped reads and more than 0.1% of endogenous DNA). Ten libraries with low proportions
100 of endogenous DNA content (0.3-13.5%) were enriched for 1.2 million SNPs using targeted in-
101 solution capture (“1,240k” SNP capture, (19)), while 29 libraries with satisfactory proportions
102 of endogenous DNA content (10.3-71.3%) were selected for whole-genome shotgun
103 sequencing. After excluding one individual (BOU26) with a coverage of 0.04X, we obtained
104 genome-wide data for nine samples with a mean coverage between 0.08X and 2.7X as well
105 as 29 partial genomes with a coverage between 0.05X and 0.68X (Table 1 & 2). We estimated

106 potential contamination of the nuclear genome by examining the heterozygosity of polymorphic
107 sites on the X chromosome in males. Assuming a contamination threshold of ~5%, we
108 excluded one individual (BOU43) from downstream analyses (Table 5,(20)).

109 ***Genetic sex determination***

110 We confidently identified eight female individuals (XX) and 28 male individuals (XY) (Table 4,
111 SI, Figure C10). For two additional individuals, genetic sex determination provided ambiguous
112 results (BOU29, Y reads ratio 0.0287-0.0299, most likely female; BOU21 Y reads ratio 0.0564-
113 0.0585, most likely male). Additionally, individual BOU12 displayed a Y/autosome ratio of 0.84,
114 while the X/autosome ratio was within the expected range for a male individual, and X-
115 contamination was low. Therefore, it is likely that this individual carried an XYY karyotype
116 (Table 4, SI, Figure C10). This type of chromosomal anomaly is known as 47,XYY syndrome
117 (21). With a prevalence of one in every 1000 births, it is a relatively rare type of trisomy that
118 can affect male individuals without phenotypic implications. It can also manifest as symptoms
119 such as tall stature, learning disability and infertility.

120 ***Uniparental markers***

121 Uniparentally inherited markers were identified using mitochondrial and Y-chromosome data
122 (Table 6, 7, & 8, SI).

123 The Aven de la Boucle individuals carry mitochondrial subhaplogroups H (N=13), K (N=8), U
124 (N=9), T (N=4), X2b (N=2) and V (N=1), which were previously reported among Neolithic
125 groups from Western Europe and represent maternal ancestors of both Anatolian farmers and
126 Western European Hunter-Gatherers (Table 6, 7, & 8). Indeed, six individuals carried
127 haplogroups derived from U5b, and three individuals carried haplogroups U2e1c1, U4 and U8,
128 which are likely attributable to the Mesolithic ancestry of Western Europe (22–24).

129 Neolithic male individuals were found to carry Y chromosome haplogroups derived from I2a1
130 (N=4), H2a1 (N=2), G2 (N=19) and R1b1b (N=1). Except R1b1b, such haplogroups are
131 relatively common in Western European Neolithic communities from both continental and
132 Mediterranean expansion routes, i.e., France (15,16,18,25,26) Switzerland (17), Germany
133 (26–29) and the Iberian Peninsula (13,14,28,30–33).

134 Although rare in Neolithic communities from western Europe, the haplogroup R1b1b carried
135 by BOU38 (3,626–3,369 BCE) has been previously identified in Western Mediterranean Early
136 Neolithic communities from Els Trocs, Cueva de Chaves and Grotta Continenza (19,32,34).

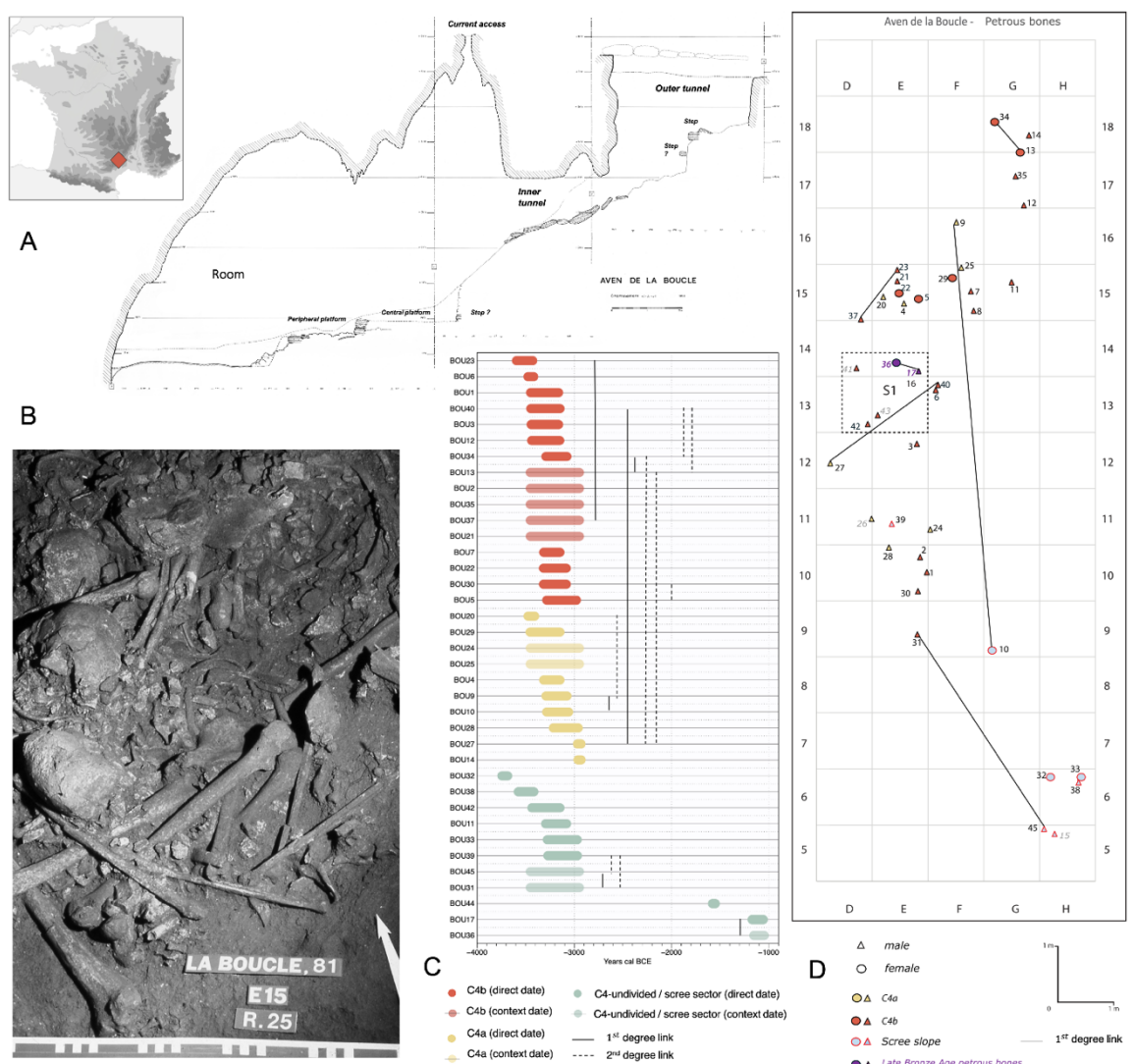
137 The high frequency of the G2a lineage (73%) is similar to what has been previously described
138 for the contemporary cave of Les Treilles (90% of the G2a lineage; Lacan et al., 2011; Aveyron,
139 France). This may reflect a general predominance of this haplogroup in southwestern France
140 at the end of the Neolithic (31,35). BOU44 and BOU17, dated to the second half of the 2nd

141 millennium BCE, both carry haplogroup R1b1a1b, consistent with previous records regarding
 142 the genetic impact of Pontic steppe herders (27).

143 **Radiocarbon dating**

144 Regarding radiocarbon dating, 25 individuals ranged between ~3,800 and 2,900 BCE and
 145 confirmed that the collective burial was mainly active during the second half of the 4th and
 146 beginning of the 3rd millennium BCE, which broadly corresponds to the bounds of the Ferrières
 147 culture in the region (36). Additionally, five undated individuals can be attributed to this
 148 chronological range because they are involved in first- or second-degree biological kinship with
 149 directly dated individuals (Figure 1C).

150 In the scree slope sector and within *Sondage 1* (“S1”, Figure 1D), remains can be attributed to
 151 sporadic use of the cavity during the Bronze Age. Thus, individual BOU44 ranged from ~1,600
 152 to 1,500 BCE, corresponding to the Middle Bronze Age, whereas individual BOU17 was
 153 directly dated to the Late Bronze Age, between ~1,200 and ~1,000 BCE (Table 8, Figure 1C).



155 *Figure 1: Geographical and chronological description of the Aven de la Boucle. A) Location of*
156 *the site in modern-day France and longitudinal section plan of the cavity. B) Skulls and long*
157 *bones alignments gathered in bundles near the west wall of the cavity, photo: H. Duday, 1981.*
158 *C) Chronological distribution of samples displaying directly dated individuals and individuals*
159 *dated through contextual date. Biological relations between individuals are indicated by the full*
160 *lines (1st degree) and the dashed lines (2nd degree), colors indicate stratigraphic attributions*
161 *(Table 1, 3 & 8). D) Plan and location of sampled petrous bones, samples without genomic*
162 *results are marked in grey, grids indicate the denomination of excavated sectors within the*
163 *cavity, colors indicate stratigraphic attributions, first-degree relations are signaled by full lines.*
164

165 ***Integration of the Aven de la Boucle group into macro-regional variability***

166 To explore genetic variation among our individuals, we constructed a Principal Component
167 Analysis based on a set of modern-day Eurasians, on which we projected the Aven de la
168 Boucle samples along with previously published ancient individuals (Figure 2). The Neolithic
169 group of the Aven de la Boucle falls within the general variability of Western European Neolithic
170 individuals and, more specifically, with previously reported individuals from France and the
171 Iberian Peninsula dated from the 5th to the late 3rd millennium. Nevertheless, we detected two
172 outliers, BOU20 and BOU29, which displayed a very distinct shift upwards in PC2 variation
173 from the main cluster. These individuals cluster with previously reported Bell Beaker individuals
174 from Central Europe and the Iberian Peninsula as well as Early to Middle Bronze Age samples
175 from France, despite being contemporaneous with the rest of the Neolithic group from this
176 sepulchral cavity (13,15,16,37).

177 The results of qpWave analysis supported the outlier status of Neolithic individuals BOU20 and
178 BOU29 as well as individual BOU6, who carry additional WHG ancestry, as already described
179 (Table 9) (18). The remaining Neolithic individuals formed a clade with a common genetic
180 background (Figure 2B, Table 9).

181 At the regional scale, qpWave results highlight the genetic clustering of the Aven de la Boucle
182 Neolithic group with Southern France individuals dating from the late 4th to the mid-3rd
183 millennium and lacking steppe ancestry (Table 9, Figure C20). Conversely, outlier individuals
184 BOU20 and BOU29, as well as the three Bronze Age individuals, cluster with Bell Beaker (La
185 Fare, Grotte des Tortues, Grotte Basse de la Vigne Perdue; (13,16)) and Bronze Age
186 individuals from southern France (Villard, Le Pirou, St Eugène, Quinquiris; (15,37)). This
187 clustering was also supported by the pairwise outgroup-f3 test of the f3 form (Mbuti; Individual
188 1; Individual 2) (Table 10, Figure C16).

189 Mirroring these findings, outgroup f3 statistics of the form f3 (Mbuti; *Test*, BOU), using various
190 West European Neolithic groups between ~5,600 and 2,500 BCE as *Test*, permitted us to
191 explore the geographical affinities of the Aven de la Boucle group and outlier individuals with

192 contemporaneous Western European Neolithic individuals (Table 11, Figure C19). The results
193 indicate that the Aven de la Boucle Neolithic group has local variability, as it shows the highest
194 genetic affinities with neighboring groups originating from collective burials at Les Peirières
195 (2,900-2,650 BCE;(15)) and Collet Redon (3,600-3,400 BCE;(13)). The Neolithic outlier
196 individuals BOU20 and BOU29 display lower f_3 values and therefore lower genetic affinities
197 with local groups. Individual BOU20 shares more affinities with contemporaneous individuals
198 from Murcia, Spain (2,900-2,600 BCE), and BOU29 shares the highest genetic affinities with
199 the late Neolithic individual TGM008 from Tangermünde, Germany (13,26).

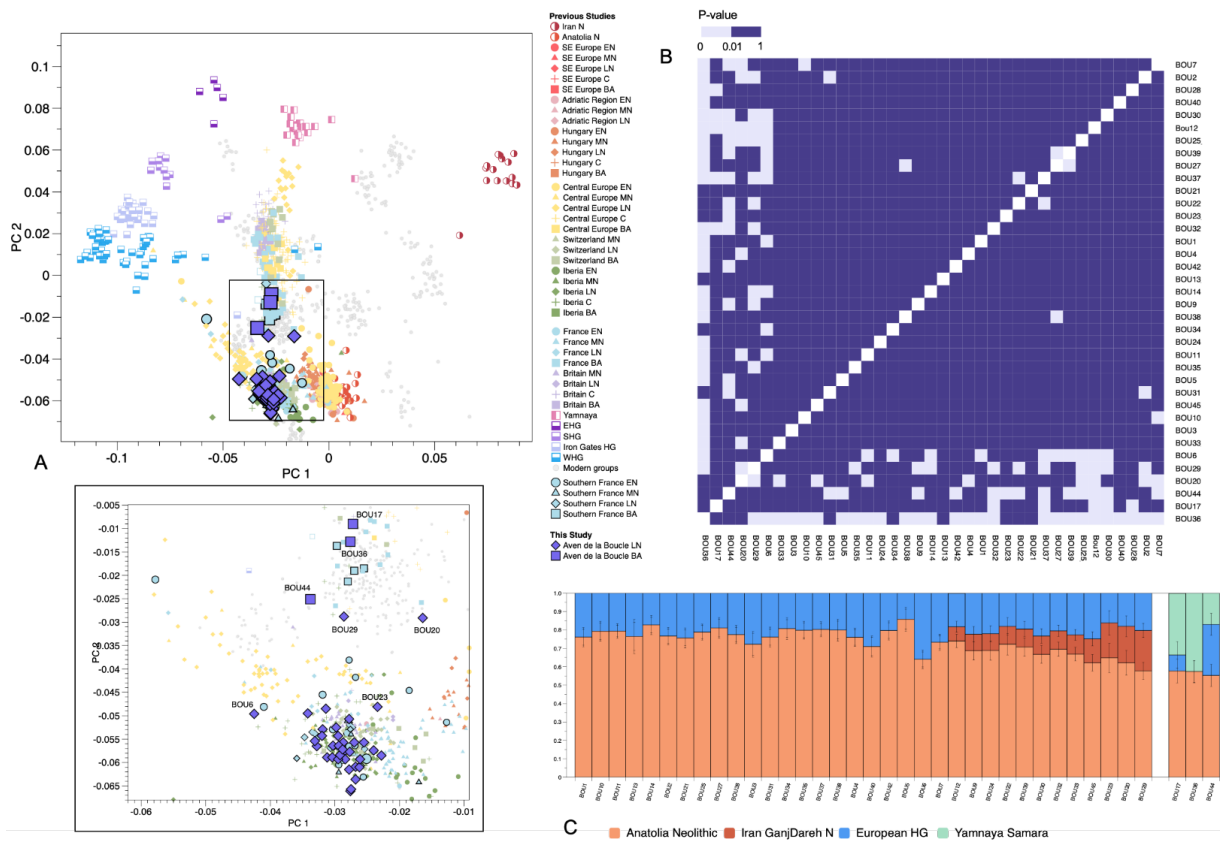
200 We used qpAdm to explore sources of genetic ancestries in the Aven de la Boucle community.
201 First, we modelled the Neolithic individuals as a two-way mixture of Anatolia farmers and
202 European HG ancestries (Table 12). As previously reported, the Neolithic individuals from the
203 Aven de la Boucle carry various amounts of the European HG genetic component (14.7-36.2%;
204 Table 12), consistent with heterogeneous HG legacy and heterogeneous admixture processes
205 between human groups throughout the Neolithic (16,18,26).

206 Drawing from the observations reported in previous studies on the differential distribution of
207 various types of HG and early farmer ancestries among Western Europe Neolithic groups, we
208 explored alternative models. We aimed to trace the presence of so-called Magdalenian-related
209 ancestry, as previous studies highlighted the persistence of this type of genetic component
210 among South-Western Europe Neolithic groups. Notably, we sought to test whether an
211 additional GoyetQ2 component could be a candidate for explaining the outlier status of BOU29
212 and BOU20 (15,18,31,32). We found no evidence of additional Goyet-Q2-like ancestry, as all
213 Neolithic individuals can be modelled with Oberkassel ancestry only (formal Villabruna
214 ancestry (38); Table 14, Model 3). In another test, we modelled the Aven de la Boucle Neolithic
215 group by adding “Iran_Ganj_Dareh_Neolithic” as a third source of the ancestral component to
216 the first model (Table 15, Model 4). This additional genetic component was previously
217 highlighted in several recent studies, appearing sporadically alongside the main Neolithic
218 ancestry component in the Anatolian and Levantine groups from the 6th millennium BCE as
219 well as within European Neolithic communities mainly stemming from the Western
220 Mediterranean (31,35,39). This additional source seems to slightly improve the fit of the model,
221 notably for BOU29 ($p = 0,984886$). We note that several individuals can be modelled as a
222 three-way mixture of European HG, Anatolia and Iran Neolithic ancestries ($N=12$, 7.7-19.9%
223 Iran_Ganj_Dareh_N ancestry). This could therefore indicate that an additional ancestral
224 component maximized in Iranian Neolithic individuals from Ganj Dareh could have contributed
225 to the genetic ancestry of the Aven de la Boucle individuals to various degrees (Table 15,
226 Figure 2C). Individuals BOU20 and BOU29 carry the highest levels of this type of genetic

227 ancestry, as they can be modelled with 19.9 and 21.9% of Iran_Ganj_Dareh ancestry,
 228 respectively. This point is also reflected in the results of the outgroup-f3 statistics of the f3
 229 (Mbuti; Ind, Iran_Ganj_Dareh) form, with BOU20 and BOU29 displaying the highest f3 values
 230 among the groups (SI, Figure C18).

231 It has been hypothesized that farmers spreading towards Europe along the Mediterranean
 232 were initially characterized by subtle variation in Near-Eastern ancestry with different
 233 proportions of Levantine/Western Caucasus components as opposed to Western Anatolian
 234 ancestry. However, we cannot exclude the possibility that this genetic component was
 235 implemented in the Western Mediterranean gene pool through exchanges with later groups
 236 originating from the Near East (31).

237



238 *Figure 2: Population genetics analyses. A) PCA presenting the genetic variability of previously*
 239 *published ancient individuals from Eurasia and 37 individuals from the Aven de la Boucle*
 240 *collective burial (newly reported and published) projected onto modern populations from*
 241 *western Eurasia. B) Heatmap of pairwise qpWave clustering of the Aven de la Boucle*
 242 *individuals. Pairs represented by light purple squares correspond to p-values below 0.01 and*
 243 *were rejected based on Fernandes et al., 2020. Matrix details are provided in Table 9. C)*
 244 *qpAdm analysis of BOU individuals using Anatolian Neolithic, European_HG, Iranian Neolithic*
 245 *or Yamnaya Samara groups as ancestral sources (Table 12, 13 & 15).*

246

247 **Bronze Age Individuals**

248 The three Bronze Age (BA) samples originating from the Aven de la Boucle correspond to one
249 female (BOU36) and two male individuals (BOU17 and BOU44) belonging to the Y-
250 chromosome haplogroup R1b1a1 (Tables 4 & 7), which has been linked to the dispersion of
251 Pontic steppe herders who moved westwards at the end of the Neolithic Age/beginning of the
252 Bronze Age (13,15,16,27). The projection of the three new BA samples from the Aven de la
253 Boucle collective burials on the PCA overlapped with previously published early to late Bronze
254 Age samples from France and southwestern Europe and clustered more specifically with early
255 to late Bronze Age individuals from southern France (Quinquiris, Le Pirou, Grotte des Tortues,
256 Saint Eugène) (Figure 2A).

257 Using qpAdm, we were able to model these individuals as a three-way mixture of Anatolia
258 Farmers (Anatolia_N), European HG and steppe-related (Russia_Yamnaya_Samara) ancestry
259 (Table 13, qpAdm Model 2, Figure 2C). The three individuals exhibited medium to low amounts
260 of steppe-related ancestral components, with estimates ranging between 17 and 43% of
261 Yamnaya_Samara ancestry. These observations fit previous records of the local impact of
262 steppe migrations, as lower ancestry proportions were reported for South-Western Europe late
263 Neolithic and Bronze Age communities (13,40).

264

265 ***Biological relatedness, funerary and social functioning of the Neolithic community***

266 The genetic sex analysis highlighted a strong underrepresentation of female individuals, as
267 only eight were identified as opposed to 26 male individuals, providing a sex-ratio significantly
268 imbalanced in favor of male individuals representing 76% of the Neolithic group ($P=0.0432$;
269 Table 1 & 4, SI, Figure C10). The morphoscopic examinations of coxal bones also suspected
270 a preponderance of male subjects, although proper quantification could not be performed.

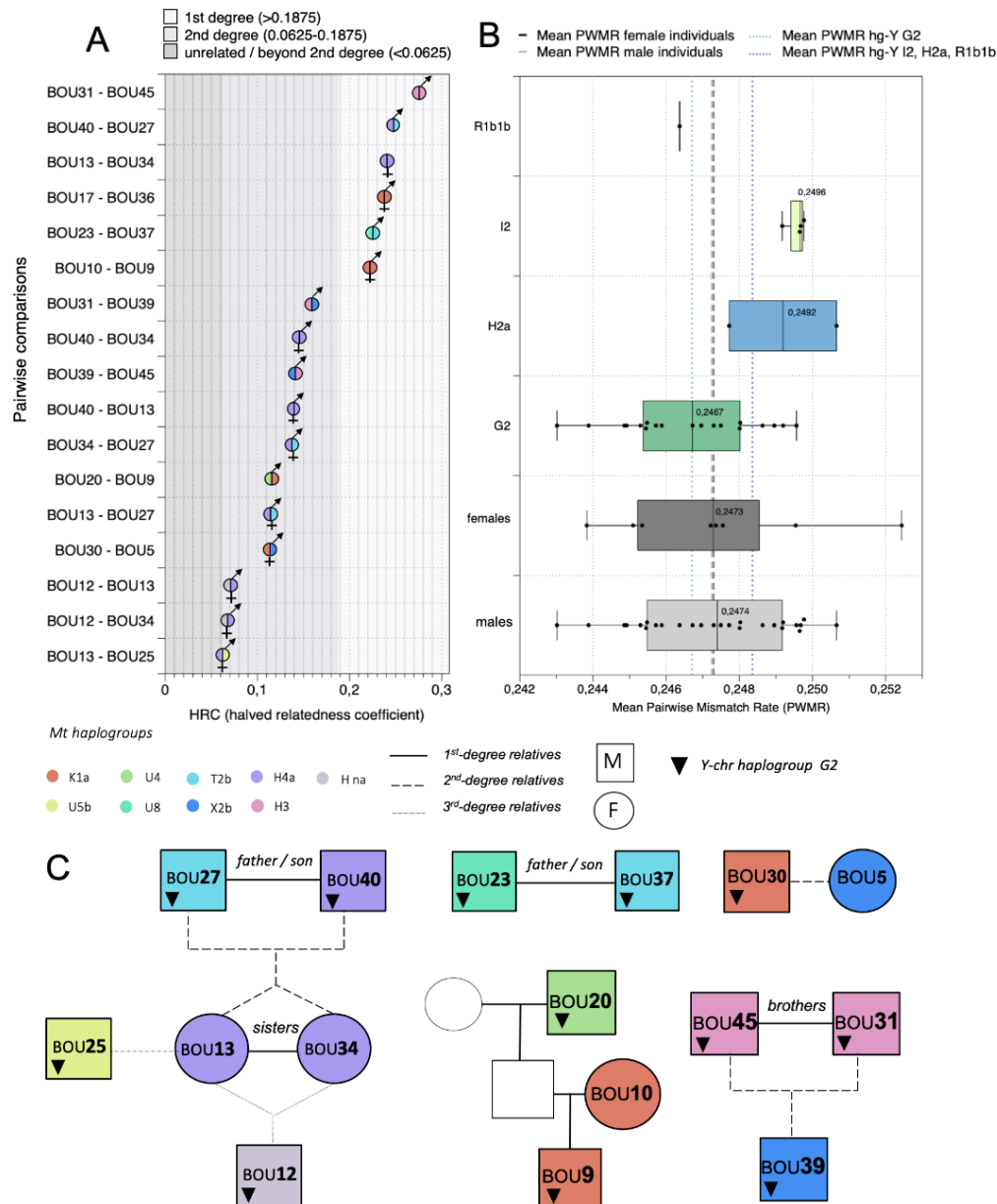
271 Among male individuals, the analysis of uniparentally inherited markers underlines the
272 overrepresentation of the Y-chromosome haplogroup G2a2 ($N= 19/26$ males; 73%).
273 Conversely, mitochondrial haplogroups exhibit a more diverse distribution, as frequently
274 observed among Neolithic groups, which is often interpreted as evidence of female mobility
275 and the practice of female exogamy in a patrilocal system (Table 6 & 7;(41)).

276 We analysed the genetic relatedness between all Neolithic individuals and identified 16 pairs
277 of biologically related individuals to the first, second and third-degree relationship (Table 8,
278 Figure 3, SI, Figure C11-C15). Interestingly, biological relatedness involves both male and
279 female individuals despite the marked male-biased selection identified. The only two related
280 female individuals, BOU13 and BOU34, were found in close proximity in the northern part of
281 the cavity. These individuals carry haplogroup H4a and share second-degree relations with
282 several individuals. Combining these observations with sex and uniparental marker

283 attributions, different pedigree scenarios were explored and are described in the SI (Figure 3,
284 Figure C11-C15). Although we cannot infer precise pedigrees with a satisfactory resolution,
285 the results of biological relatedness and pairwise outgroup-f3 analysis are consistent with a
286 close-knit family group using the cavity as a collective burial between ~3,600 and ~2,900 BCE
287 (Table 8 & 10, SI, Figure C15). Moreover, the anthropological study of the Aven de la Boucle
288 collective burial identified a high frequency of an anatomical variation called *patella*
289 *emarginata*. Indeed, 75% of the patellae display pronounced emargination of the upper lateral
290 edge, equal to or exceeding a length of 10 mm (SI, Figure C5). Interestingly, out of 11
291 individuals exhibiting an emargination exceeding 16 mm in length, eight individuals were
292 associated with the C4a unit (SI, Figure C5). Given the unusually high frequency of this
293 anatomical variation, the hypothesis of a high level of biological proximity between the
294 deceased was originally proposed (7,8,10).

295 To assess the parental relatedness of the deceased individuals deposited in the Aven de la
296 Boucle, we analysed runs of homozygosity through hapROH (Table 16, Figure C21,(42)).
297 Three individuals displayed long ROH (BOU31, BOU28, BOU35), whose length and
298 distribution could suggest that their parents were related as second cousins. These
299 observations aside, the overall limitation of long ROH could indicate that this funerary group
300 represents a population in which close kin unions were limited. The pattern of ROH distribution
301 observed at the Aven de la Boucle echoes those of contemporaneous Neolithic groups and
302 individuals reported in Western Europe (Table 16, Figure C21).

303 To confirm female exogamy practices within this Neolithic community, we tested whether the
304 few female individuals buried at the Aven de la Boucle had fewer close relatives at the site
305 than male individuals (Figure 3B, Table 8, SI, Figure C15, approach described in (31)). The
306 observed mean pairwise mismatch rates between male and female individuals are highly
307 similar; therefore, biological relatedness between Neolithic individuals is unlikely to be sex
308 biased (Figure 3B). We tested for a significant difference in genetic proximity between male
309 and female individuals with f3 statistics for the f3 (Mbuti; female, female), f3 (Mbuti; female,
310 male), and f3 (Mbuti; male, male) forms (SI, Figure C17). We found no significant difference in
311 f3 values between the female-female and male-male groups (p value=0.13) or between the
312 female-female and female-male groups (p value=0.146). This does not support a significant
313 difference in genetic proximity between females with other female individuals and males with
314 either male or female individuals (SI, Figure C17). These observations contrast with the female
315 exogamy signal expressed by uniparental markers and could be linked to the biased selection
316 of individuals who were granted access to the collective burial.



317

318 *Figure 3: Biological relatedness analysis. A) First, second/third-degree relations using*
 319 *TKGWV2 results. For each pair, mt haplogroups are indicated by the color. B) Mean pairwise*
 320 *mismatch rate extracted from READ for the 34 Neolithic individuals, colors indicate Y*
 321 *chromosome haplogroups for male individuals C) Pedigrees reconstructed for 16 individuals*
 322 *involved in biological relatedness, first-degree relations are represented by full lines, second-*
 323 *degree by dashed lines (Table 8, SI, Section V).*
 324

325 **Chronological modelling**

326 We conducted Bayesian modelling of the radiocarbon dates obtained for 26 Neolithic
 327 individuals analysed on the genomic scale (Figure 4, Table 17 & 18, SI, Section III). We
 328 examined the chronological distribution of different clusters of individuals defined according to
 329 stratigraphic attributions. The *a posteriori* modes of phases beginning and ending (43) were
 330 retained here as relevant to describe the phases of site utilization (Table 18;(44)).

331 Both models constructed place the C4b unit ("Recent Neolithic"/Pre-Ferrières/Final Chasséen
332 Culture) between 3,600 and 3,185 BCE, with a marked density of activity at approximately
333 3,350 BCE, and the C4a unit (Ferrières) between 3,140 and 2,880 BCE, with a plateau of
334 activity between ~3,100 and 2,910 BCE (Figure 4).

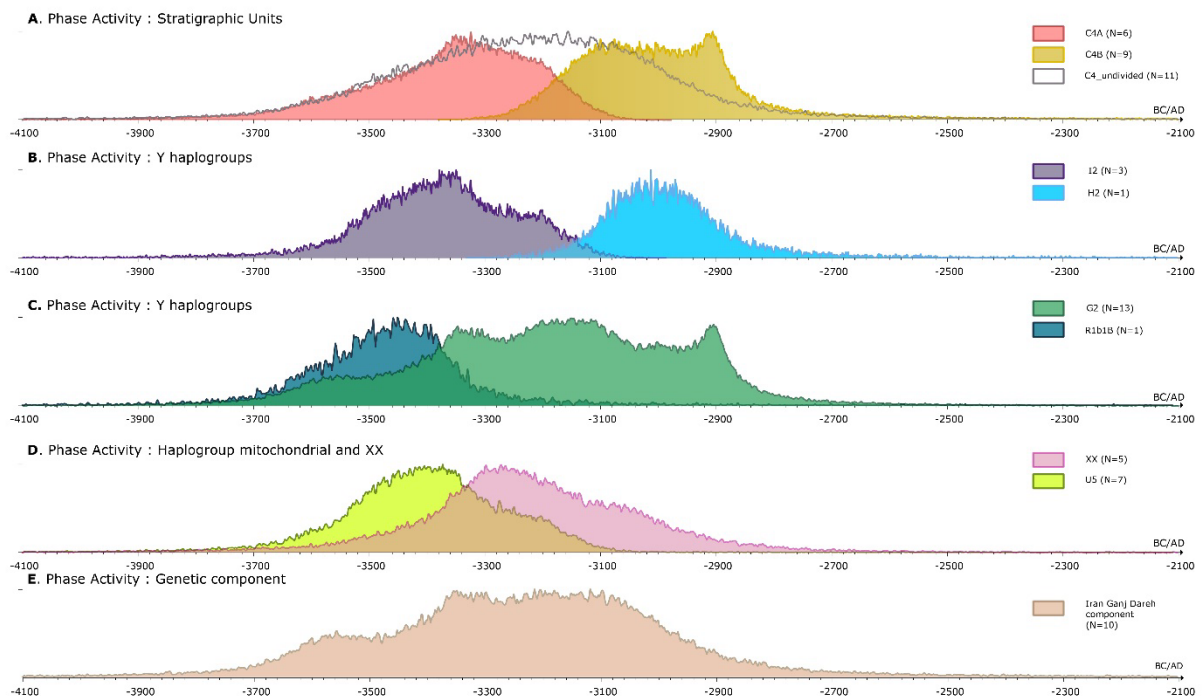
335 The combined observations of stratigraphic attribution and radiocarbon dating of cranial
336 remains therefore reveal no clear break between the first ("Recent Neolithic"/Final Chasséen
337 Culture, C4b unit) and second phases of occupation (Ferrières culture, C4a unit). To an extent,
338 these observations could argue in favour of the continuous use of collective burial throughout
339 the cultural transition reflected in the archaeological deposits rather than a hiatus between
340 distinct phases.

341 We also examined the traces of activity *a posteriori* of different clusters of individuals defined
342 according to genetic criteria, i.e., males vs. females, males carrying different Y chromosome
343 haplogroups, mitochondrial haplogroup U5 or "Iran Ganj Dareh" ancestry (Table 1, Table 6,
344 Table 7, Table 15; SI, Figure C8 & C9, Table C1). From these models, several observations
345 and hypotheses can be discussed. Traces of Y chromosome haplogroup I2 and mt haplogroup
346 U5 are strongly correlated with each other (results of pairs overlap correlation - hereafter
347 res.OCPairs -: 0.91) and with the activity of the C4b unit (res.OCPairs: 0.86 and 0.82,
348 respectively) but not with C4a (res.OCPairs: 0.14 and 0.13, respectively, Figure 4, SI, Figure
349 C9, Table C1). Moreover, several individuals belonging to the Y chromosome haplogroup G2
350 cluster contribute to the beginning of the C4b phase (res.OCPairs: 0.59, SI, Figure C9, Table
351 C1), but while the activity traces of haplogroup I2 disappear after 3,100 BCE, the chronological
352 distribution of individuals carrying G2 is wider and vastly extends to the C4a unit (res.OCPairs:
353 0.57, SI, Figure C9, Table C1). Indeed, if we consider the distribution of the cluster formed by
354 individuals carrying the G2 paternal lineage, as well as the female individual cluster (XX), they
355 both significantly contribute to the C4a unit (and secondarily individuals carrying Y-H2 at the
356 end of the phase).

357 Additionally, individuals who can be modelled with "Iran Ganj Dareh" ancestry (GD, Table 15)
358 are correlated with the distribution of the G2 cluster (BOU12, 23 and 30) and the whole female
359 cluster (res.OCPairs: 0.91 and 0.84, respectively; SI, Figure C9, Table C1). These individuals
360 appear to contribute equally to the C4b and C4a units (res.OCPairs: 0.67 and 0.49,
361 respectively). They appear at the beginning of C4b, and even as early as the late Chasséen,
362 during the first half of the 4th millennium, if we consider the genetic profile and date of BOU32
363 (Table 3, Table 17, Table 18).

364 It is perhaps necessary to consider the division of C4b into two stages, the earliest more
365 strongly anchored in the late Chasséen and the next marked by the contribution of individuals

366 with uniparental markers related to Mesolithic ancestry (Y chromosome haplogroup I2 and mt
367 haplogroup U5), whose visibility fades afterwards (Figure 4, SI, Figure C8 & C9, Table C1).



368
369 *Figure 4: Bayesian modelling of the chronological phases of the Aven de la Boucle collective*
370 *burial. The diagram illustrates the a posteriori chronology described by activity curves*
371 *(probability densities of events within the different phases and clusters; Table 17 & 18; SI,*
372 *Section III).*

373 374 **Discussion and perspectives**

375 A major challenge in the study of collective burials and commingled assemblages is to decipher
376 the preestablished rules of selection and identify the biological and/or social principles
377 according to which an individual is entitled to access the grave. The Aven de la Boucle
378 sepulchral cavity is one of the most thoroughly documented among the 4th millennial collective
379 burials in the Languedoc region. The assemblage that characterizes this site, in which two
380 funerary sequences are discriminated by material culture, is also a unique opportunity to
381 address the notions of cultural versus biological continuity or discontinuity during the late
382 Neolithic period.

383 The genomic results highlighted a marked sex-biased selection, manifested by the
384 overrepresentation of male individuals deposited inside the cavity. Within collective burials,
385 morphological sex is frequently inaccessible or unreliable due to commingling and
386 fragmentation of pelvic bones. Therefore, ancient DNA is an important contribution in this
387 regard as reflected in this study.

388

389 This marked imbalance in sexual representation argues in favor of the presence of a male
390 biased selection that could have been added to the selection of adults previously identified
391 (9,10). A differential preservation of less robust craniofacial blocks with a female deficit
392 (through fragmentation or the action of water) appears highly unlikely as the Neolithic levels of
393 the cavity have yielded a high number of much more fragile human bone pieces, such as hyoid
394 bones and ossified thyroid cartilage (9).

395 In light of the secondary gestures, the cranial remains represent a sub-sample of the dead
396 population (56%, SI). Therefore, several questions can be raised regarding the
397 underrepresentation of female individuals. First, this biased representation could illustrate that
398 the primary deposition inside the cavity was preferentially granted to male individuals, whereas
399 only a limited number of female individuals were allowed or chose to be included in the
400 collective burial. A second hypothesis to explain the overrepresentation of males could be that
401 cranial and coxal remains belonging to female individuals were preferentially removed from
402 the cavity after decomposition occurred and moved elsewhere. Within the ethnographic record,
403 the practice of temporary access to a collective funerary structure is, for instance, attested in
404 West Sumba, where individuals awaiting their own funerary monument will temporarily be
405 granted access to a collective burial without properly fitting the selective patterns (45).
406 Occurrences of temporary burials are also found among the Merina of the Antananarivo region
407 in Madagascar, where remains can be temporarily placed within a collective tomb before the
408 traditional exhumation of the bones is performed during the *famadihana* ceremonies (46–48).
409 At the Aven de la Boucle, the hypothesis of temporary female burials would presuppose that
410 those bones remained identifiable, even after skeletonization. Considering these observations,
411 the living would have had to recover both craniofacial blocks and coxal bones from a large
412 number of women. Biased selection in favour of male subjects could therefore be more likely
413 than selective recovery of many female remains after decomposition.

414 Among the Neolithic community, the selection of the deceased could therefore include age at
415 death and biological sex criteria, but not in a totally fixed and strict manner insofar as a minority
416 of female and immature individuals still accessed to the cavity.

417 Kinship ties, both biologically and socially defined, are often a strong candidate to explain the
418 successive gathering of the deceased in collective burials. At the Aven de la Boucle, the
419 biological proximity of the deceased suspected on the basis of nonmetric traits echoes the
420 patterns of biological relatedness highlighted by genomic results. The overrepresentation of
421 male individuals, as well as the predominance of the G2a2 paternal lineage, which is also the

422 only lineage represented among closely related individuals, could indicate that selection
423 criteria regarding access to the cavity were also anchored in a patrilineal descent (49).

424 These observations are somewhat reminiscent of the selection pattern described in the group
425 buried inside the Treilles I cave, dated to 3,000 BCE and located ~70 km away from the Aven
426 de la Boucle. This sepulchral cave also contains the remains of predominantly male individuals,
427 most of which were found to carry the Y-chromosome lineage G2a (25). Similar patterns have
428 been reported recently in more geographically distant megalithic collective contexts, notably in
429 the British Isles, and have been interpreted as relating to the transgenerational appropriation
430 of burial grounds by patrilineal structured communities (50).

431 Furthermore, Bayesian modelling of the chronological distribution of the cranial remains
432 indicate that the use of this collective burial started in a “Recent Neolithic” (pre-Ferrières) phase
433 corresponding to the C4b unit and included a group dominated by male individuals
434 characterized by higher paternal lineage diversity. Without a clear hiatus separating phases,
435 the results indicate that the use of the cavity as a collective burial continued during a period
436 associated with the Ferrières and corresponding to the C4a unit. However, during this phase,
437 a modification of the funerary functioning, or at least of the group using the cavity, is perceived
438 as the G2 paternal lineage becoming largely dominant and almost exclusive. This finding
439 appears to be correlated with the inclusion of several female subjects and the importance of
440 close biological relatedness. Overall, these observations could illustrate a phase during which
441 the male-mediated transmission of social status is more exclusive than during the previous
442 phase. The affiliation with a specific male lineage appears to become a preponderant selection
443 factor that would not be incompatible with the inclusion of certain women tied in this filiation
444 system. These observations might also indicate that women could choose their burial location
445 (52).

446 However, both available materials and funerary gestures are important biases to consider, as
447 they undoubtedly impede the ability to make a clear representation of the full living community
448 or communities involved in the utilization of this sepulchral cavity. This representation bias has
449 to consider both the retrieval of bone pieces and funerary selection employed by the living.

450 In southern France, genomic studies of late Neolithic megalithic tombs are scarce and have
451 been restricted to a small number of individuals per site, with a focus on population genetics
452 and migration dynamics. The present study represents the first extensive archaeogenomic
453 documentation of a collective burial in France and a mean to explore the funerary selection
454 processes from a different perspective. Additional data retrieved from different contexts,
455 notably dolmens, would therefore be instrumental for properly comparing groups displaying
456 different expressions of collective burial within a restricted region.

457

458 **Materials and Methods**

459 ***Archaeological context and anthropological observations***

460 The Aven de la Boucle is a natural cavity located near Corconne in the Gard region in southern
461 France. The excavations were conducted under the supervision of H. Duda and S. Cours
462 between 1974 and 2002.

463 The oldest archaeological deposits, attributed to a transitional stage postdating the late
464 Chasséen stage (also known as the “Recent Neolithic” or Pre-Ferrières), correspond to
465 stratigraphic unit C4b. A second group of deposits, corresponding to the C4a unit, represents
466 a later stage of this late Neolithic horizon and can be attributed to the Ferrières culture.

467 Archaeo-anthropological analysis revealed that approximately 75 individuals remained, with a
468 massive underrepresentation of immature individuals; quantitative osteology revealed a
469 notable deficit of the largest bones (craniomaxillofacial blocks, long bones of the limbs) (SI,
470 Figure C2).

471

472 ***Radiocarbon dating and chronological modelling***

473 Four initial radiometric measurements obtained on charcoal attributed the funerary occupation
474 of the Aven de la Boucle between the 37th and 25th centuries BCE.

475 During this study, 28 dates were obtained by AMS on human bone collagen. Among them, 26
476 refer to Neolithic collective burials and two to punctual introductions of deceased in the cave
477 during the Middle and Final Bronze Age.

478 We conducted Bayesian modelling of ¹⁴C dates retrieved on petrous bones ((53) SI, Section
479 III). To do so, we first constructed different clusters of individuals defined according to
480 stratigraphic attributions

481 We then constructed different clusters of dated individuals defined according to genetic criteria:

482 - males vs females

483 - male individuals carrying different Y-chromosome haplogroups (Y-G2, Y-H2, Y-R1b1b, Y-I2;
484 Table 6, Table 18)

485 - individuals carrying mitochondrial haplogroup U5 (Table 7, Table 18)

486 - individuals that we were able to model with “Iran Ganj Dareh” ancestry (see Table 15, qpadm
487 model 4).

488 The models are built only from the 26 individuals dated to the Neolithic; indeed, those from the
489 Bronze Age are too distant in time to constrain the earlier dates.

490 Two Bayesian models were constructed, taking (BCL_M1) or not taking into account (BCL_M2)
491 an individual (BOU32) whose 14C date and stratigraphic position suggest that the cave was
492 used for funerary purposes during the Late Chasséen period.

493 Table 18 presents a general overview of the clusters built for Bayesian modelling. Additional
494 information is available in the SI, Section III.

495

496 **Palaeogenomic analyses**

497 ***Sample preparation and DNA extraction***

498 All experiments were conducted in dedicated clean rooms of the ancient DNA facilities at the
499 Laboratory of *Anonymous*. We targeted a large number of petrous bones available (N=42) to
500 maximize the achievement of usable genomic results and to extensively document the
501 group(s) represented in this sepulchral cavity.

502 We sampled 42 human petrous bones to retrieve between 100 and 200 mg of bone powder
503 per sample. Steps of decontamination were conducted before sampling. Petrous bones were
504 irradiated with ultraviolet light (UV) for 30 minutes on each side, bone surface was scraped on
505 the area targeted for sampling and tools were cleaned before drilling into the cochlea to retrieve
506 bone powder (54).

507 All 42 bone powder samples originating from the Aven de la Boucle were processed according
508 to a two-step extraction procedure and purification followed a silica-based method using a
509 MinElute column (QIAGEN) (15).

510

511 ***Library preparation and sequencing***

512 For each DNA extract, double stranded libraries were produced according to a protocol
513 adapted from (15,55), using a partial UDG treatment (UDG half) to remove deaminations while
514 preserving ancient DNA damage patterns (56). Libraries were pooled and sequenced on an
515 Illumina NextSeq 500 at *Anonymous* using a NextSeq 500/550 High Output Kit v2.5 (150
516 Cycles).

517

518 ***Read processing, alignment and post-mortem damage***

519 Raw sequenced data were processed after demultiplexing through EAGER (v 1.92.37) using
520 the following steps (57). Adaptors sequences were trimmed and processed into single reads
521 with Clip&Merge, trimmed sequences were then mapped to Human Reference Genome
522 hs37d5 using BWA v.0.7.12 (58,59). Duplicate reads were removed with DeDup and to observe
523 characteristic aDNA damage patterns we used mapDamage v.2.06, excluding reads with a
524 mapping quality <30 (60). Sequencing results are presented in Table 2.

525

526 **Genotyping**

527 All our bam files were trimmed for two bases on each side using trimBam function from BamUtil
528 package and were genotyped by using PileupCaller. We considered the human genome as
529 pseudo-haploid, randomly calling one allele for each position according to the Human Origin
530 (~600,000 SNPs) and 1240k panels (28,61–63). Individuals having at least 19,000 SNPs on
531 the 1240k panel were considered for further analysis and coverage information for the 37
532 individuals retained are given in Table 1.

533

534 **Genetic sex determination**

535 We determined genetic sex using the method described in (64), based on the estimations of
536 reads ratios mapping to X and Y chromosomes compared to reads mapping to the autosomes.
537 We determined a threshold of Y ratio based on the method published by (64). We used an
538 upper threshold of 0.016 of the ratio of sequence mapping to the Y chromosome for females
539 and a lower bound of 0.077 for males (Table 4). For the ten capture samples, we also calculated
540 the coverage for the X and Y chromosomes and compared to the relative coverage observed
541 for the autosomes (65)(Table 4, SI, Figure C10).

542

543 **Contamination estimations**

544 We used ANGSD (Analysis of Next Generation Sequencing Data) package to test the degree
545 of heterozygosity of polymorphic sites on the X chromosome and estimate contamination
546 levels in all male individuals (20). Considering a contamination threshold of ~5%, we excluded
547 one individual (BOU43 – 13.7%) of downstream analyses (Table 5).

548

549 **Uniparental markers**

550 Reads were mapped to the revised Cambridge Reference Sequence (rCRS) and VCF files
551 were constructed using bcftools before being processed through HaploGrep 2 and Phy-Mer to
552 determine mitochondrial haplotypes (66,67). Mitochondrial sequences were also visually
553 inspected through Geneious to reinforce mitochondrial lineages attributions, presented in Table
554 7 and Table 1.

555 For each male individual, Y chromosome haplotypes were inferred using both Yleaf software
556 package (v.2.1,(68)) and manual assignment through an in-house script. Results for the 28
557 male individuals are provided in Table 6.

558

559 **Biological relatedness**

560 Degrees of genetic relatedness between all individuals included in this study were estimated
561 by applying a combination of four methods, TKGWV2, READ (Relationship Estimation from
562 Ancient DNA), IcMLkin and KIN to infer pairwise relationships up to the second and third
563 degree (SI, Section V; (69–72).

564

565 ***Statistical exploration of biological relatedness patterns***

566 We tested the overall mean relatedness for each Neolithic individual by extracting the pairwise
567 mismatch rate (PWMR) from READ for each pair of individuals. We then calculated the mean
568 PWMR per individual, considering each of the 33 pairs of individuals possible. We also
569 confronted the mean PWMR obtained for all female individuals to the one calculated for all
570 male individuals (Table 8, Figure C15).

571

572 ***Population genetic analyses***

573 For population genetic analyses, we co-analysed our data with published ancient and modern
574 individuals from Eurasia. To qualitatively explore the genetic variability of our dataset, we
575 conducted a PCA using the HO dataset and the program smartpca (Eigensoft) (73). PCA was
576 constructed based on the genetic variation of 796 modern individuals from Eurasia on which
577 ancient individuals (>10,000 SNPs) were then projected (lsqproject: YES, shrinkmode: YES,
578 Figure 2).

579

580 ***Inbreeding and population size***

581 We assessed runs of homozygosity and levels of inbreeding in our samples using the 1000
582 Genome as a reference panel, calling SNPs for each individual chromosome by chromosome,
583 through hapROH (42). We were able to retrieve data for all our samples (Table 16, SI, Figure
584 C21).

585

586 ***Acknowledgement***

587 *We thank Olivier Gorgé and the Molecular Biology team of the Département des Plateformes*
588 *et Recherche Technologique at Institut de Recherche Biomédicale des Armées for processing*
589 *samples for Next-generation sequencing. We are grateful to the genotoul bioinformatics*
590 *platform Occitanie (Bioinfo Genotoul, <https://doi.org/10.15454/1.5572369328961167E12>) for*
591 *providing help, computing, and storage resources. We thank Wolfgang Haak for discussions*
592 *and for providing funding for radiocarbon dating. We are grateful to Frédéric Santos at PACEA*
593 *for discussions and insights into computational analysis.*

594 *We thank Muriel Gandelin and Vincent Ard for discussions regarding archaeological data and*
595 *cultural background. We thank Etienne Patin at the Institut Pasteur for discussions and insights*
596 *into computational analysis. The authors are grateful to Dominique Castex, Fanny Mendisco,*
597 *Juliette Henrion, Eliza Orellana-González as well as other members from PACEA lab for*
598 *discussions. The authors thank the Service Régional de l'Archéologie Occitanie as well as the*
599 *Institut National de Recherches Archéologiques Préventives through the PAS ANCESTRA*
600 *(Coord. M. Pruvost and F. Maziere) for granting access to archaeological material.*
601 *This project is a part of a PhD Research project carried out at the University of Bordeaux,*
602 *supervised by Marie-France Deguilloux and Mélanie Pruvost and funded by the Ministère de*
603 *l'Enseignement Supérieur et de la Recherche. Research was conducted in the PACEA lab*
604 *(UMR 5199 CNRS). For radiocarbon dating, this study received funding from the PACEA lab*
605 *following the ANCOR actions (Actions Nouvelles et Collectives de Recherche).*
606 *This project has received funding from the French National Research Agency (ANR) and*
607 *Deutsche Forschungsgemeinschaft (DFG) under both the ANR project ANCESTRA, Grant*
608 *ANR15-CE27-0001 coordinated by Mélanie Pruvost and the Franco-German Call in*
609 *Humanities and Social Sciences project ANR-DFG INTERACT, grant ANR-17-FRAL-0010,*
610 *DFG-HA-5407/4-1, 2018-2023, coordinated by Marie-France Deguilloux and Wolfgang Haak.*
611 *This research benefited from the scientific framework of the University of Bordeaux's IdEx*
612 *"Investments for the Future" program / GPR "Human Past".*

613

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