Heterogeneous Hunter-Gatherer and Steppe-Related Ancestries in Late Neolithic and Bell Beaker Genomes from Present-Day France

Highlights

- Sequencing of 24 human genomes from France dated to ~3,400–1,600 cal. years BCE
- These genomes show heterogeneous hunter-gatherer and steppe-related ancestries
- Paris Basin Neolithic and Mesolithic groups admixed as recently as ~3,800 years BCE
- Admixture with steppe herders in southern France dated as early as ~2,650 years BCE

Authors

Andaine Seguin-Orlando,
Richard Donat, Clio Der Sarkissian, ..., Love Dalén, Jean Guilaine,
Ludovic Orlando

Correspondence

andaine.seguin@univ-tlse3.fr (A.S.-O.),
ludovic.orlando@univ-tlse3.fr (L.O.)

In Brief

The Late Neolithic to Bronze Age transition was marked by major genetic changes in Europeans but is poorly documented at the genomic level in France. With new extensive whole-genome time series for this period, Seguin-Orlando et al. extend the time frame during which steppe-related and hunter-gatherer ancestries entered and vanished in this region.
Heterogeneous Hunter-Gatherer and Steppe-Related Ancestries in Late Neolithic and Bell Beaker Genomes from Present-Day France

Andaine Seguin-Orlando,1,2,13,15,* Richard Donat,1,3 Clio Der Sarkissian,1 John Southon,4 Catherine Thèves,1 Claire Manen,1 Yaramila Tcherémissoff,5,6 Eric Crubézy,1 Beth Shapiro,7,8 Jean-François Deleuze,9 Love Dalén,10,11 Jean Guilaine,12 and Ludovic Orlando1,14,*

1Centre d’Anthropobiologie et de Génomique de Toulouse CAGT, CNRS UMR 5288, Université Toulouse III Paul Sabatier, Faculté de Médecine Purpan, Bâtiment A, 37 allées Jules Guesde, 31000 Toulouse, France
2Institute for Advanced Study in Toulouse IAST, Université Toulouse I Capitole, Esplanade de l’Université, 31080 Toulouse Cedex 06, France
3Institut National de Recherches Archéologiques Préventives INRAP, 561 Rue Etienne Lenoir, 30900 Nîmes, France
4Earth System Science Department, B321 Croul Hall, University of California, Irvine, Irvine, CA 92697-3100, USA
5Laboratoire Travaux et Recherches Archéologiques sur les Cultures, les Espaces et les Sociétés TRACES, CNRS UMR 5608, Université Toulouse II Jean Jaurès, Maison de la Recherche, 5 allées A. Machado, 31058 Toulouse Cedex 9, France
6Laboratoire Méditerranéen de Préhistoire Europe AFRIQUE LAMPEA, CNRS UMR 7269, Aix Marseille Université, 5 rue du Château de l’horloge, 13094 Aix-en-Provence, France
7Department of Ecology and Evolutionary Biology, University of California, Santa Cruz, 1156 High Street, Santa Cruz, CA 95064, USA
8Howard Hughes Medical Institute, University of California, Santa Cruz, 1156 High Street, Santa Cruz, CA 95064, USA
9Centre National de Recherche en Génomique Humaine CNRGH, Institut de Biologie François Jacob, Université Paris Saclay, CEA, 2 rue Gaston Crémieux CP 5721, 91057 Evry, France
10Centre for Palaeogenetics, Svante Arrhenius väg 20C, 10691 Stockholm, Sweden
11Department of Bioinformatics and Genetics, Swedish Museum of Natural History, Box 50007, 10405 Stockholm, Sweden
12Collège de France, 11, place Marcelin-Berthelot, 75005 Paris, France
13Twitter: @AndaineO
14Twitter: @LudovicLorlando
15Lead Contact
*Correspondence: andaine.seguin@univ-tlse3.fr (A.S.-O.), ludovic.orlando@univ-tlse3.fr (L.O.)

SUMMARY

The transition from the Late Neolithic to the Bronze Age has witnessed important population and societal changes in western Europe.1 These include massive genomic contributions of pastoralist herders originating from the Pontic-Caspian steppes2,3 into local populations, resulting from complex interactions between collapsing hunter-gatherers and expanding farmers of Anatolian ancestry.4–8 This transition is documented through extensive ancient genomic data from present-day Britain,9,10 Ireland,11,12 Iberia,13 Mediterranean islands,14,15 and Germany.8 It remains, however, largely overlooked in France, where most focus has been on the Middle Neolithic (n = 63),8,9,16 with the exception of one Late Neolithic genome sequenced at 0.05× coverage.16 This leaves the key transitional period covering ~3,400–2,700 cal. years (calibrated years) BCE genetically unsampled and thus the exact time frame of hunter-gatherer persistence and arrival of steppe migrations unknown. To remediate this, we sequenced 24 ancient human genomes from France spanning ~3,400–1,600 cal. years BCE. This reveals Late Neolithic populations that are genetically diverse and include individuals with dark skin, hair, and eyes. We detect heterogeneous hunter-gatherer ancestries within Late Neolithic communities, reaching up to ~63.3% in some individuals, and variable genetic contributions of steppe herders in Bell Beaker populations. We provide an estimate as late as ~3,800 years BCE for the admixture between Neolithic and Mesolithic populations and as early as ~2,650 years BCE for the arrival of steppe-related ancestry. The genomic heterogeneity characterized underlines the complex history of human interactions even at the local scale.

RESULTS AND DISCUSSION

Archaeological Sites and Radiocarbon Dates

A total of 30 ancient human petrosal bones, 5 post-cranial bones, and 18 teeth were prepared in ancient DNA facilities for shotgun sequencing on Illumina platforms (STAR Methods). Following shallow sequencing on the MiniSeq instrument, 26 (~49%) DNA extracts showing at least 10% of human DNA were selected for further sequencing on the NovaSeq and HiSeqX instruments (Data S1A). These correspond to remains excavated from...
collective burials associated with Late Neolithic, Bell Beaker, and Early Bronze Age archaeological contexts and located in the Paris Basin (Mont-Aimé) and southwestern France (Mas Rouge, Grotte du Rouquet, Grotte Basse de la Vigne Perdue, and Grotte des Tortues; Figures 1A–1C; STAR Methods). Direct radiocarbon dating indicated that the two Mont-Aimé hypogea (i.e., human-made subterranean burials) were active between ~3,400 and 2,900 cal. years (calibrated years) BCE (Figure 1D). All six individuals from Grotte du Rouquet were virtually contemporary to those from Mont-Aimé. Grotte des Tortues included one Late Neolithic individual dated to ~3,100–2,900 cal. years BCE (TORTD), and three individuals spanning the mid-3rd (TORTC and TORTE) and the first half of the 2nd (TORTF) millennium BCE. This indicates that the Grotte des Tortues cave was used opportunistically as a collective burial site over a time period encompassing the Late Neolithic to the Early Bronze Age, in line with previous archaeological evidence. The same was true for Grotte Basse de la Vigne Perdue, although only from the mid-3rd to the mid-2nd millennium BCE.

Genomic and Metagenomic Content

Metagenomic taxonomic profiles inferred from metaBIT and the MetaPhLAn2 database mainly contained contaminating bacterial species of unknown or environmental origin (Data S1B). Three out of 10 teeth from Mont-Aimé (2H10, 2H11, and 1H13), however, showed increasing fractions of human oral bacteria (4.8%–39.5%). These include the cariogenic Streptococcus mutans, as well as Treponema denticola, Porphyromonas endodontalis, and...
Campylobacter rectus that are associated with peri- and endodontal diseases\textsuperscript{21} but were also detected in healthy individuals today and in 120- to 5,700-year-old samples worldwide (Data S1D).\textsuperscript{22–34} The presence of S. mutans as early as the Neolithic adds to the evidence\textsuperscript{27,34,35} that this pathogen cannot provide a reliable marker for tooth decay following lifestyle changes in the Bronze Age and post-industrial era, despite earlier claims.\textsuperscript{36} The proximity of oral metagenomic profiles measured in 1H13 and modern individuals (Figure 2A) is in line with the reduced fraction of unclassified and environmental microbes in this sample but may also indicate that microscopic calculus remained undetected and was co-extracted with the tooth cementum.

Mitochondrial sequence alignments indicated minimal human DNA contamination in all individuals (Data S1E), except two from Mont-Aimé: 2H06, which is characterized with minimal sequence data (and excluded from downstream analyses), and 1H14, which shows 10\%–12\% contamination. X-to-autosomal sequence coverage revealed 13 males, for whom X chromosomal heterozygosity profiles confirmed minimal autosomal contamination (\% = 1.01\%; median = 0.12\%; Data S1F).

Figure 2. Microbial Profiling, DNA Methylation Analyses, and Phenotype Prediction

(A) Genus-level principal-coordinates analysis (PCoA) of bacterial profiles. Taxa supported by abundances strictly inferior to 1\% were disregarded.

(B) Correlation matrix of DNA methylation levels at gene bodies.

(C) Genotypes observed for the two Mont-Aimé samples sequenced to high coverage at 49 loci underlying important morphological, metabolic, behavioral, and medical phenotypes. The number of independent sequence counts is provided for each locus considered. Undetermined genotypes are indicated as “./.” See also Data S2 and S3.
Genetically identified as female, individual 1H14 could thus not be tested with this approach. Sequence data displayed post-mortem DNA degradation characteristic of DNA extracts partially treated with USER\textsuperscript{17} (Figure S1A), and genetic affinities were in line with those Mont-Aimé males showing minimal contamination. As mtDNA data do not necessarily reflect autosomal contamination rates,\textsuperscript{38} individual 1H14 was included in downstream analyses. Overall, we report a total of 24 ancient genomes at 0.20- to 23.88-fold average depth of coverage (median = 0.96-fold), including two at high depth (2H10 and 2H11, 13.91- and 23.88-fold, respectively). Supporting our data quality, error rates ranged between 0.057% and 0.083% substitutions per base (median = 0.069%), which is approximately half the average error rate estimated in ancient (high-coverage) genomes previously reported (Figures S1C–S1E).

Uniparental Markers and Kinship
No individuals from the same archaeological site carried identical mitochondrial haplotypes, indicating no maternal relatedness (Data S1E). In contrast, all males were assigned to the Y chromosome haplogroup I2a1, except one individual at Mont-Aimé belonging to haplogroup H2a1 and one ~4,400-year-old R1b1a1b1a1a2a1 individual from Grotte Basse de la Vigne Perdue (Data S1F). Close or incompletely covered but matching haplotypes were previously reported in contemporary Bell Beaker individuals (R1b1a1b for sample CBV95, La-Bouche-à-Vesie) and Bronze Age individuals from both northern (R1b1a1b1a1a2 for sample RX2, Rixheim-Zac du Petit-Prince; R1b1a1b1a1a2a5 for sample OBE3626-1, Obernai PAEI) and southern France (R1b1a1b1a1a for sample PIR3116B Rec de Ligno).\textsuperscript{16} This may reflect admixture from incoming steppe herders that, around that same time, have almost completely replaced other Y chromosome haplotypes in Iberian populations.\textsuperscript{13} Haplogroup I2a1 was previously found among various hunter-gatherer groups from Europe.\textsuperscript{4,6,12,13,38,40,41} This may indicate the persistence of western hunter-gatherer (WHG) ancestry in at least some Mont-Aimé individuals (2H11).

The apparently high frequency of I2a1 may reflect a general dominance of this haplogroup across Late Neolithic France. It may, however, also suggest communities organized patrilinearily, especially as individual sites showed no mitochondrial haplotypes shared between individuals. To explore this further, we assessed patterns of genetic relatedness at 1,240k autosomal SNP positions\textsuperscript{39} using pseudo-diploid calls in READ\textsuperscript{42} as well as genotype likelihood in lcMLkin\textsuperscript{43} (Data S1G and S1H). This indicated that five individuals analyzed at Mont-Aimé were genetically related. In hypogeum 2, a father and his son (2H10 and 2H17) were related to a lesser degree to a third male (2Hx05x196x1131). They shared similar Y chromosome haplotypes, confirming patrilinearity. However, we also identified a father-daughter relationship between two individuals anatomically determined as adults (2H11 and 1H06; Data S1A). That at least some women could remain in their native communities implies that no strict patrilocal rules were followed. Alternatively, this could also imply that at least a fraction of women were not involved in marital relationships or had their bodies repatriated to their father’s community after death. Extending the analyses to more individuals and integrating isotopic signatures informing on birth location will help assess the extent to which this also applied to other women in the community. Nonetheless, the presence of first-degree relationships between Mont-Aimé I and Mont-Aimé II supports that both hypogeae were used strictly contemporaneously and by the same group of people.

Inbreeding and Population Diversity
The fraction of the genome that is comprised within runs of homozygosity (ROHs) can indicate within-kin unions.\textsuperscript{12} It can also result in under-estimating genome-wide levels of heterozygosity, which inform on the population effective size.\textsuperscript{44} In order to both assess the presence of within-kin unions and estimate population effective sizes at Mont-Aimé, we applied ROHan\textsuperscript{45} to the two individuals sequenced to high depth (2H10 and 2H11). The ROH genome fraction was, however, limited (1.5%–2.0%; Data S2A) and on par with that observed among other Neolithic individuals, except at the megalithic site of Newgrange, where a brother-sister incestuous union was previously reported.\textsuperscript{12} This indicates limited, if any, within-kin unions at Mont-Aimé. Estimated heterozygosities outside ROHs were comparable to those found in other high-coverage Neolithic genomes, except Newgrange, but larger than those of west European Mesolithic hunter-gatherers (Loschbour and Smarem62; Data S2A). This is in line with Neolithic food production economies allowing population demographic expansion relative to hunting-gathering communities.\textsuperscript{45}

DNA Methylation and Phenotypic Inference
Post-mortem DNA decay, especially cytosome deamination, is known to have a differential impact on methylated and unmethylated CpGs.\textsuperscript{46} This provides an opportunity to map DNA methylation marks on ancient genomes, assuming USER treatment of DNA extracts\textsuperscript{47} and sufficient sequencing efforts.\textsuperscript{48} We leveraged the two high-coverage genomes characterized here and four others previously reported\textsuperscript{4,12} to identify genes that may have experienced important DNA methylation changes during the Mesolithic to Neolithic transition. Analyses were limited to teeth to avoid tissue-specific signatures. We found DNA methylation patterns within gene bodies generally more similar among pairs of Neolithic individuals than among Neolithic-Mesolithic pairs (Figure 2B). However, the Neolithic female (Stuttgart) was found closer to one Mesolithic male (Loschbour) prepared in the same laboratory than to the individual 2H10 prepared in our laboratory. This indicated technical batch effects impacting DNA methylation inference. To avoid these effects and sex-specific signatures, we further restricted the analyses to those male individuals characterized to highest coverage in different laboratories. We identified a total of 611 gene promoters in which DNA methylation shifted by at least 50% on average in both Neolithic males (Loschbour; Data S3A). Although not significantly enriched in particular functional categories, these included more instances undergoing hyper-methylation during the Neolithic than hypo-methylation (p = 1.6 × 10\textsuperscript{-15}). Examples include C1Qb, the first chain of the humoral immune complement response,\textsuperscript{49} and LCK, a key signaling molecule involved in T cell maturation.\textsuperscript{50} Further work is required to assess whether such signatures reflect regulatory changes in dental or blood tissues.

We next attempted to predict the age at death of the two individuals sequenced to high coverage following a previously
reported approach,\textsuperscript{51} leveraging DNA methylation levels inferred within 1 kb around 27,528 CpG positions forming the Illumina 27k methylation Bead Chip system. Although significantly correlated, the DNA methylation values returned by DamMet\textsuperscript{48} were consistently lower than those measured on a normalization gold standard panel used for epigenetic age inference (Data S3C). This precluded using the DNAmAge methylation clock predictor to precisely estimate age at death. The latter was instead estimated using DNA methylation levels at 11 clock-CpGs known to undergo DNA methylation changes in teeth with age.\textsuperscript{52} Despite cross-validation rates of 0.897–0.999 on modern DNA data, this methodology showed limited precision with ancient DNA data and provided confidence intervals spanning up to 46 years (Data S3C). Pending necessary methodological improvements, this approach suggested that 2H10 potentially died younger than 2H11 (29 years old versus 42 years old).

Although precise phenotypic inference was not possible using DNA methylation patterns, we used the genotypes measured for the two high-coverage individuals at 90 loci to infer morphological, pigmentation, behavioral, and medical phenotypes (Figure 2C; Data S2B). Both individuals were lactose intolerant and were not carriers of the increased risk allele for the celiac disease at rs653178, for increased addiction behavior at rs1800497 and rs2283265, or for elite endurance performance at rs4994. They likely had brown eyes and dark hair (prediction probabilities = 0.844–0.997) but did not carry alleles showing additive effects on hair thickness (rs4752566 and rs3827760). Individual 2H11 was most likely dark skinned (prediction probability = 0.960; Data S2B), although 2H10 did not have a (very) pale but intermediate to dark skin. Interestingly, the two individuals carried different haplotypes at the mono-amine oxidase A locus, which encodes for an enzyme involved in the oxidation of dopamine, norepinephrine, and serotonin neurotransmitters.\textsuperscript{53} These entail different combinations of alleles associated with higher (e.g., rs3027407 and rs909525) or lower (e.g., rs1137070, rs1465107, rs2072743, and rs6323) enzymatic production. This, and the existence of gene-environment interactions,\textsuperscript{54} make the exact phenotypic consequences difficult to predict. However, the genetic differences observed at this locus suggest different risks of developing aggressive and impulsive behavior in these individuals.\textsuperscript{55}

**Heterogeneous Mesolithic Ancestry among Late Neolithic Individuals at Mont-Aimé**

We next sought to identify genetic affinities between the 18 Late Neolithic individuals sequenced here and ~2,000 ancient individuals previously reported (STAR Methods). Projections of ancient individuals onto the principal components characterizing the genomic variation among 796 modern western Eurasians at 592,998 autosomal loci revealed that almost all Late Neolithic individuals from Esperstedt, Germany. This suggests multiple, sporadic contacts between Neolithic and Mesolithic groups during substantial time periods (at least 5 centuries) and up until ~3,800 B.C.E. Interestingly, the WHG ancestry estimated within the genomes of 1H06 and her father’s (2H11) indicate that her mother had an average 37.9% WHG ancestry, which is larger than the proportion estimated in other Mont-Aimé individuals presently sequenced (average = 21.9%; max = 29.9%). This supports late Neolithic communities, such as that of Mont-Aimé, as heterogeneous genetically, with WHG ancestors occupying various positions in their genealogies. Importantly, the most recent admixture date inferred here follows by an average of ~4,000 years the most recent admixture date previously estimated\textsuperscript{8} in western European scale (Figures 3F, S3A, and S3B). D-statistics\textsuperscript{57} of the form (Mbuti, WHG, X, 1H06 or 2H11) also confirmed an excess of WHG ancestry in 1H06 and 2H11, more pronounced in the latter, relative to all other “X” individuals sequenced in this study (Figure S4). D-statistics of the form (Mbuti, ANF, X, 1H06 or 2H11) revealed a reciprocal lack of ANF ancestry in these individuals.

Supervised ADMIXTURE\textsuperscript{58} further suggested 57.5% ± 3% and 65.7% ± 2.9% of WHG ancestry within 1H06 and 2H11, respectively (Figures 4B, S2B, and S2C). This contrasts with the 18.5% ± 1.6% to 28.8% ± 1.7% range estimated across the other Late Neolithic individuals reported here. Formal population modeling with qpADM,\textsuperscript{59} in which Neolithic individuals are composed of WHG and ANF ancestries, also supported an excess of the former for both 1H06 and 2H11 (50.6% ± 5.1% and 63.3% ± 4.1%, respectively; Figure 4A; Data S4A). This excess shows no equivalent in other Late Neolithic individuals (9.9%–29.9%) and is exceptional among Neolithic individuals from France, except the Early Neolithic PEN_B individual (51.6% ± 4.8%; Figure 4A). For individuals MAS15, ROUQW, TORTC, TORTD, and TORTE, simpler models consisting of ANF ancestry only were supported, confirming WHG ancestry at best limited. However, more complex modeling did not support the presence of GoyetQ2-like ancestry as a second, previously described genetic group of hunter-gatherers present in western Europe (Data S4A).\textsuperscript{5}

We next leveraged patterns of linkage disequilibrium (LD) decay in DATES\textsuperscript{50} to assess whether the significant excess of WHG ancestry within individuals 2H11 and 1H06 reflects recent or ancient and single or multiple admixture pulses. Modeling all Mont-Aimé individuals, except 2H11 and 1H06, as a combination of ANF and WHG sources, we inferred admixture dates ~39.5 ± 8.5 generations prior to their age distribution (~4,300 years B.C.E.; Figure 4E). Similar modeling indicated that the admixture occurred ~17.2 generations and ~6.2 generations later when considering individuals 1H06 and 2H11 (22.3 ± 4.6 and 33.3 ± 14.0 generations, respectively; i.e., ~4,100 and ~3,800 years B.C.E.). This suggests multiple, sporadic contacts between Neolithic and Mesolithic groups during substantial time periods (at least 5 centuries) and up until ~3,800 B.C.E. Interestingly, the WHG ancestry estimated within the genomes of 1H06 and her father’s (2H11) indicate that her mother had an average 37.9% WHG ancestry, which is larger than the proportion estimated in other Mont-Aimé individuals presently sequenced (average = 21.9%; max = 29.9%). This supports late Neolithic communities, such as that of Mont-Aimé, as heterogeneous genetically, with WHG ancestors occupying various positions in their genealogies. Importantly, the most recent admixture date inferred here follows by an average of ~4,000 years the most recent admixture date previously estimated\textsuperscript{8} in western European scale (Figures 3F, S3A, and S3B). D-statistics\textsuperscript{57} of the form (Mbuti, WHG, X, 1H06 or 2H11) also confirmed an excess of WHG ancestry in 1H06 and 2H11, more pronounced in the latter, relative to all other “X” individuals sequenced in this study (Figure S4). D-statistics of the form (Mbuti, ANF, X, 1H06 or 2H11) revealed a reciprocal lack of ANF ancestry in these individuals.
may indicate an acculturation process of hunter-gatherer groups following the arrival of farming in the region.

**Bell Beaker and Bronze Age Population Changes**

In addition to 18 Late Neolithic individuals, our dataset includes four Bell Beaker and two Early Bronze Age individuals from the Grotte des Tortues and Grotte Basse de la Vigne Perdue, both located in the La Clape massif, southern France (Figure 1C). Principal-component analysis (PCA) projections showed that three Bell Beaker individuals overlapped with other Late Neolithic individuals from France, except for the father-daughter pair (2H11 and 1H06; Figure 3A). One Bell Beaker individual (GBVPK) and...
the two Early Bronze Age individuals (GBVPO and TORTF), however, clustered with other previously reported Early to Middle Bronze Age individuals from France but were displaced along an axis showing closer affinity with Yamnaya steppe herders from Samara. D-statistics of the form (Mbuti, Yamnaya; X, GBVPK, GBVPO, or TORTF) also suggested a statistically significant excess of Yamnaya ancestry in these three individuals relative to some of the others from both sites (Figure S4).

Patterns of LD decay provided an estimate for the time when steppe-derived genetic ancestry entered southern France at around 9.2 ± 3.6 to 10.4 ± 3.5 generations prior to the age of the earliest individual showing Yamnaya ancestry (i.e., GBVPK; Figure 4E). This corresponds to ~2,650 years BCE, which is earlier than previous reports showing significant steppe-derived ancestry in Britain ~2,400 years BCE and in Iberia ~2,500–2,000 years BCE. Our estimate was, however, found
consistent, regardless of the Late Neolithic proxy considered in population modeling, including the Mont-Aimé individuals showing least WHG admixture, the individuals from Grotte du Rouquet, or those from the La Clape massif (Figure 4E). Our data also reveal the co-existence of individuals with no to significant Yamnaya-related ancestry within Grotte Basse de la Vigne Perdue. This and the absence of Yamnaya ancestry inferred in one contemporary Bell Beaker individual from the nearby site from Dolmen des Peiñères (individual PEI2) support a complex genetic composition of Bell Beaker groups in southern France involving non-ubiquitous admixture with individuals related to steppe herders. This is in line with previous reports in Central Europe but contrasts to Britain, where ~90% of the local gene pool was replaced by steppe-related ancestry by the second half of the 3rd millennium BCE.9

In this study, we recovered ancient genomes from 24 ancient human individuals in burial sites from present-day France to document genomic changes in Late Neolithic to Early Bronze Age populations. Bell Beaker groups from southwestern France included some individuals, but not all, showing steppe-derived genetic ancestry, with an earliest appearance of Yamnaya-related steppe ancestry around ~2,650 years BCE. Late Neolithic genomes from Mont-Aimé provide evidence of multiple, sporadic contacts between Neolithic and Mesolithic groups at a time when no Mesolithic cultural evidence are available. This may indicate that Mesolithic groups survived at least until the first quarter of the 4th millennium BCE in the region but followed an acculturation process.

STAR*METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.12.015.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES


# STAR METHODS

## KEY RESOURCES TABLE

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Critical Commercial Assays

- MiniElute PCR Purification kit (Qiagen) Cat# 28006
- TapeStation screenTape D1000 HS (Agilent) Cat# 5067-5584
- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) Cat# Q32854
- MiniSeq High Output Reagent Kit (150-cycles) (Illumina) Cat# FC-420-1002

Deposited Data

- Raw and analyzed data this study ENA: PRJEB41240

Software and Algorithms

- metaBIT 19 https://bitbucket.org/Glouvel/metabit/src/master/
- READ 42 https://bitbucket.org/tguenther/read/src/master/
- lCMLkin 43 https://github.com/COMBINE-lab/maximum-likelihood-relatedness-estimation
- ROHan 44 https://github.com/grenaud/rohan
- DamMet 48 https://github.com/KHanghoj/DamMet
- Admixtools 56 https://github.com/DRichLab/AdmixTools
- DATES 59 https://github.com/priyamoojani/DATES
- AdapterRemoval2 62 https://github.com/MikkelSchubert/adaptermoval
- Paleomix 63 https://github.com/MikkelSchubert/paleomix
- mapDamage2 64 https://ginothac.github.io/mapDamage
- ANGSd 65 https://github.com/ANGSD/angsd
- haplogrep 66 https://github.com/seppinho/haplogrep-cmd
- Schmutzi 67 https://github.com/grenaud/schmutzi
- Yleaf 68 https://github.com/genid/Yleaf
- HiRIsPlex-S 69 https://hirisplex.erasmusmc.nl/
- smartPCA 70 https://github.com/DRichLab/EIG/tree/master/POPGEN
- bamUIt 71 https://github.com/statgen/bamUIt
- Gene Organizer 72 http://geneorganizer.huji.ac.il/
- Panther 73 http://www.pantherdb.org/
- David 74 https://david.ncifcrf.gov/

RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources, material and reagents should be addressed and will be fulfilled by the lead contact, Andaine Seguin-Orlando (andaine.seguin@univ-tlse3.fr).

Materials Availability
Raw sequence data and alignments are available at the European Nucleotide Archive (ENA) under accession number ENA: PRJEB41240.
Data and Code Availability
The accession number for the genomic data reported in this paper is ENA: PRJEB41240. All other previously published genomic data used in this study is available at the sources referenced in the QUANTIFICATION AND STATISTICAL ANALYSIS section. The code used in this study is listed in the Key Resources Table.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Archaeological and anthropological information
Excavation inventory IDs are indicated in Data S1 for each sample analyzed in this study. The 27 remains that were not sequenced further but only screened for assessing the proportion of human DNA present in DNA extracts are not presented. They include three postcranial bones and eight teeth from the Mont-Aimé sites (endogenous content = 0.07%–8.27%), four petrosal bones from Grotte Basse de la Vigne Perdue (endogenous content = 0.01%–9.72%), three petrosal bones from Grotte des Tortues (endogenous content = 0.15%–6.52%), four petrosal bones from Grotte du Rouquet (endogenous content = 0.47%–5.24%) and five petrosal bones from Mas Rouge (endogenous content = 0.14%–3.26%).

Mont-Aimé I and II sites
The two Mont-Aimé hypogea are part of a wide complex of collective burials identified in the Marne region, France and including more than 160 artificial cavities, all organized in necropolises. They remain among the very few that were not emptied during early excavations by the end of the 19th century. Dug into Late Cretaceous chalk, the Mont-Aimé hypogea I and II are located only 30 m from one another, on the Southern side of the butte of Mont-Aimé. Both burials are oriented North-to-South. They open to the South, and show a very similar architecture consisting in an access corridor, an antechamber and a main funerary chamber of 16 to 17 m², split in two rooms by a lateral pillar. Within both burials, the skeletons were completely dislocated and dispersed.

The Mont-Aimé hypogoeum I (lat. 48.858874, long. 3.996466) was discovered and excavated in 1982 (Champagne-Ardenne Regional Archaeological Service) and contained the skeletal remains of at least 57 individuals (Minimum Number of Individuals MNI, 25 adults, 32 children and adolescents), as well as 272 artifacts consisting predominantly in shell ornaments, but also local flint arrowheads, bone and antlers artifacts and limestone beads.

The Mont-Aimé hypogoeum II (lat. 48.858472, long. 3.995887) was excavated in 1988 and 1989 and included the remains of at least 57 individuals (41 adults and 15 children and adolescents over 15 years old, 14 children). Many of the craniofacial blocks are missing and were most likely removed from the burial after decomposition, as both mandibles and atlas were well represented. To limit sampling bias between hypogoeum, we sampled teeth included in mandibles. In addition, two rib fragments 2HxC5x196 and 2HxC5x1131 were selected for DNA analysis as they showed cortical lesions compatible with tuberculosis infection. Grave goods were abundant (253 items) and included two axes in exogenous material, local flint arrowheads, bone and antlers artifacts, as well as shell ornaments.

Published radiocarbon dates suggested that the communities using both hypogoea as burials could be at least partially contemporaneous, and confirmed that the Mont-Aimé hypogoea are among the earliest collective burials in the region, marking the transition between Middle and Late Neolithic in the Paris Basin. Artifacts found in both hypogoea showed similar typology and technological characteristics, indicative of early and intermediate phases of the Late Neolithic period in the region. In particular, the ornaments presented similarities with those from the Horgen culture, observed in French Jura and in Switzerland, suggesting exchanges between the two localities.

Estimating an age-at-death on the basis of tooth wear in archaeological individuals is challenging due to the large variability in tooth wear, which increases with age and is influenced by sex. Difficulties also arise in absence of reference population (of known age and degree of wear), as is the case of European Neolithic populations. We therefore used a simple method, which is widely applied to populations from continental Europe and, and minimizes intra- and inter-observer variation. Teeth were classified according to five increasing tooth wear stages from 0 to 4. For infants and sub-adults, age estimation was performed according to teeth eruption. Determined dental ages are summarized in Data S1A.

Mas Rouge site
The Neolithic collective burial known as “Mas Rouge” (lat. 43.594016, long. 3.91929), located near Montpellier, Hérault, France, was excavated in 2013-2014 by Y. Tcherémissinnoff and colleagues. The excavation was part of a preventive archaeology program linked to railway and highway construction works and benefitted from extensive Geographic Information System documentation. The burial, on the edge of the contemporaneous La Cavalade village (Late Neolithic, Ferrières culture) is integrated into a fairly dense network of Neolithic occupations. The geographical context is that of a coastal plain in the proximity of a system of salt ponds. The burial consisted in a domestic cellar (6 m x 4 m x 1.7 m) whose elaborate internal architecture has been modified to host the remains of at least 189 individuals (91 adults and 98 immature individuals, of all age classes). The funerary architectural rebudding involved the implementation of complex infra- and superstructures (coated wooden floors, elevations in mud-bricks), which then formed a semi-substerranean burial, the emerged part of which constitutes a small, low-rise house with a light roof. The associated grave goods are not particularly prestigious, and mainly consist of necklace beads and everyday tools, as expected for the period. In addition to the radiocarbon date provided here, a total of 14 radiocarbon dates were published in the excavation report, indicating that Mas Rouge was used as a cellar during the 3,295-2,945 cal. years BCE interval and as a collective burial between 3,035 and 2,890 cal. years BCE. The sex and age-at-death anthropological determination, combined with the
elevated MNI, the narrow period of burial activity and the close proximity of the La Cavalaude habitat are compatible with the hypothesis of Mas Rouge being used as a community burial for the vast majority of the villagers over four to five generations.

**La Clape sites**
The limestone La Clape massif, located between Narbonne and the Mediterranean Sea (Aude, France), hosts more than a dozen of natural caves used as collective burials during the Late Neolithic and the Early Bronze Age, many of which were excavated during the first half of the 20th century by Théophile and Philippe Héléné. The remains are now part of the so-called ‘Héléné’ collection at the Archaeological Museum of Narbonne, France. Individuals from three of these caves (Grotte des Tortues, Grotte du Rouquet, and Grotte Basse de la Vigne Perdue) were analyzed in this study (Data S1F).

**Grotte des Tortues**
The Grotte des Tortues site (lat. 43.149777, long. 3.060539) is located near Les Monges vineyard, between Narbonne and Gruissan (Aude, France) and was excavated around 1920 by Théophile and Philippe Héléné. The 0.45 m by 0.75 m entrance of the cave was initially closed by a rock wall, blocking the access to a single 2.5 m wide chamber. Human skulls were arranged inside a recess and a short dead-end gallery, while dispersed bones were found in the main chamber, under large blocks. The total number of individuals was estimated to 22. The archaeological material is dated to the Late Neolithic to the Early Bronze Age period. Considering the radiocarbon dates obtained in this study, the individual TORTE (2,839-2,500 cal. years BCE) belongs to the classical phase of the Veraza culture (final Neolithic), predating the Bell Beaker phenomenon while the individual TORTC (2,578-2,472 cal. years BCE), also attributed to the classical phase of the Veraza culture, could correspond to the timing of the first intrusions of early Bell Beaker individuals (maritime/international style). The individual TORTF (2,008-1,825 cal. years BCE) corresponds to the post-Bell Beaker Early Bronze Age period. The individual TORTD was dated 3,261-2,918 cal. years BCE, which corresponds to a middle phase of the final Neolithic, as supported by the presence of limestone ‘à ailettes’ beads.

**Grotte Basse de la Vigne Perdue**
Also known as Grotte du Rouquet (lat. 43.149150, long. 3.060582), this site belongs to a series of natural caves in the Combe-Longue valley of the La Clape massif. Théophile and Philippe Héléné who excavated the site, also referred to this site as ‘Grotte de la Palette’ or ‘Grotte du Triton’ as they found among the grave goods a schist palette as well as a perforated triton’s shell. The limestone La Clape massif, located between Narbonne and the Mediterranean Sea (Aude, France), hosts more than a dozen of natural caves used as collective burials during the Late Neolithic and the Early Bronze Age, many of which were excavated during the first half of the 20th century by Théophile and Philippe Héléné. The remains are now part of the so-called ‘Héléné’ collection at the Archaeological Museum of Narbonne, France. Individuals from three of these caves (Grotte des Tortues, Grotte du Rouquet, and Grotte Basse de la Vigne Perdue) were analyzed in this study (Data S1F).

**Grotte du Rouquet**
Also known as “Grotte Vallée du Rouquet” (lat. 43.166302, long. 3.060582), this site belongs to a series of natural caves in the Combe-Longue valley of the La Clape massif. Théophile and Philippe Héléné who excavated the site, also referred to this site as ‘Grotte de la Palette’ or ‘Grotte du Triton’ as they found among the grave goods a schist palette as well as a perforated triton’s shell which could have been used as a horn. This cavity consisted of two galleries, one in the axis of the main entrance obstructed by rock blocks and the other, on the right hand-side, giving access to a lateral chamber whose entrance was closed by a dry-stone wall. This chamber hosted nine well-preserved heaps, each covered by a stone mound. Archaeological material is characteristic of the Late Neolithic and the Early-Middle Bronze Age. In particular hemispherical V-perforated buttons could be attributed to early Bell Beakers (Iberic influence). Two pottery fragments of the later Pyrenean style, as well as turtle-shaped V-perforated buttons were also present. Two double-ended copper awls of square cross-section and one barbed-and-tanged arrowhead could be attributed to any of these two early Bell Beaker phases.

**METHOD DETAILS**

**Radiocarbon dating**
AMS-radiocarbon dating was performed at the Keck Carbon Cycle Accelerator Mass Spectrometry Facility, Earth System Science Department, UC Irvine, California. Bone pieces between 0.5 and 2.15 g were sampled in the bone laboratory in the ancient DNA facilities of the CAGT laboratory and sent for subsequent isotopic measurements on ultrafiltered collagen. For the Mont-Aime remains, we sampled a fraction of the mandible bone holding the tooth analyzed for DNA to ensure that genomic and radiocarbon data correspond to the same individual. For individual 1H04, two aliquots were prepared and measured due to low collagen yields. Sample preparation backgrounds were estimated, and subtracted, based on measurements of 14C-free mammoth and whale bone. Calibration was carried out using OxCal online (OxCal 4.4) and the IntCal20 calibration curve. Values are provided in Data S1.
DNA extraction and sequencing

Drilling, DNA extraction and sequencing library building (pre-PCR amplification steps) were performed in the ancient DNA facilities of the CAGT laboratory (Toulouse, France). DNA was extracted from 80-430 mg of petrosal bone (samples from Mas Rouge, Grotte des Tortues, Grotte du Rouquet and Grotte Basse de la Vigne Perdue), rib bones (samples 2HxC5x196 and 2HxC5x1131 from Mont-Aimé hypogeeum II) or tooth cementum (all of the remaining Mont-Aimé samples). In short, after gentle surface abrasion using a drill, samples were pulverized using a manual mortar (for samples 2HxC5x196 and 2HxC5x1131 only) or a ball mill (Retsch MM 200). For teeth samples, the cementum was isolated using a procedure described in 95. DNA of the Mont-Aimé samples was extracted following the method described in 96, using 4 mL of lysis buffer (0.45 M EDTA, 0.25 mg/mL proteinase K and 0.5% N-lauryl Sarcosyl) for a pre-digestion step of 1 hour at 37°C, before fully digesting the pellet overnight at 42°C in 4 mL of fresh lysis buffer. For samples 2HxC5x196 and 2HxC5x1131, both pre-digestion and overnight digestion supernatants were further purified and built into sequencing libraries. For all the other samples, only the overnight digestion supernatants were further processed. Samples from Grotte des Tortues, Grotte du Rouquet and Grotte Basse de la Vigne Perdue were processed following a similar procedure, except that the lysis buffer volume was limited to 1 mL and that 250 μL of the supernatant obtained after overnight digestion was directly purified on a MinElute column (QIAGEN ©) and eluted in 23 μL of elution buffer (EB + 0.05% Tween 20). Of note, as the endogenous content of the first sequencing libraries built for samples TORTC and TORTD was relatively low (TORTC_AMIS_2_02252_U_i8_lr8 and TORTD_AMIS_2_02253_U_i10Ir10), a second bone powder aliquot was subjected to bleach pretreatment as described in 97 before proceeding with DNA extraction. After digestion in 1 mL lysis buffer as described above, DNA from the MAS15 sample was extracted using the combination of Buffer G and magnetic silica beads described in 98.

A 22.8 μL aliquot of each DNA extract was subjected to Uracil-Specific Excision Reagent treatment by incubation at 37°C for 3 hours with 7 μL of USER enzyme (NEB®), to limit the impact of nucleotide mis-incorporations in downstream analysis. Illumina sequencing libraries were built following a protocol first described in 37 and adapted in 99 introducing a unique index of seven nucleotides within adaptor P5 and within adaptor P7 before their ligation to the end-repaired sample’s DNA. Libraries were enriched and indexed by performing 11-12 PCR cycles in 25 μL reaction volumes using 1 unit of AccuPrime™ Pfx DNA polymerase, 4-6 μL of DNA library and with an overall concentration of 200 nM of each primer, including the InPE1.0 primer and one custom PCR primer. The latter includes a 6 nucleotides sequence tag index used for sequence demultiplexing. After purification using Agencourt Ampure XP beads (1.4:1 beads:DNA ratio) and elution in 20 μL EB + 0.05% Tween, library concentration and size were checked on a Tapestation 4200 instrument (Agilent Technologies) and on a QuBit HS dsDNA assay (Invitrogen). Up to 13 amplified libraries were obtained per sample.

Amplified libraries were pooled with other indexed libraries and sequenced on Illumina platforms using the Paired-End mode. Screening and library validation were carried out on a MiniSeq instrument (2x80 bp reads) at the CAGT laboratory. Libraries showing endogenous human DNA content higher than 10% were selected for deep-sequencing and sequenced on a HiSeqX (2x150 bp reads) at the CEA/CNGM (Evry, France) or on a NovaSeq S4 (2x150 bp reads) at SciLifeLab (Stockholm, Sweden). These represented 5/12 and 7/11 remains for Mont-Aimé hypogeea I and II respectively, 1/6 samples for Mas Rouge, 4/7 samples for Grotte des Tortues, 3/7 samples for Grotte Basse de la Vigne Perdue and 6/10 samples for Grotte du Rouquet.

Read processing and alignment

Illumina paired-end reads were demultiplexed on the basis of the seven-bp index sequences present at the starts of both read pairs 37, using a maximum edit distance of 1 mismatch for each index. Demultiplexed reads were then trimmed for adaptor sequences (–mm 5) and poor-quality ends (Phred quality scores ≤ 2), and collapsed into single reads using AdapterRemoval2 102. Collapsed, collapsed truncated and non-collapsed pairs were mapped against the human reference genome (GRCh37, hg19) using Bowtie2 (version 2.3.5.1, 105) and local sensitive mapping parameters, following the recommendations from 107. Aligned reads shorter than 25 bp and alignments showing mapping quality strictly inferior to 30 were disregarded. PCR duplicates were removed using MarkDuplicates from Picard Tools (version 2.18.0, 106). Local re-alignment around indels was carried out using GATK (version 3.8.1, 109). Read collapsing, trimming and mapping, as well as PCR duplicate removal and local realignment were carried out using the Paleomix automated computational pipeline (version 1.2.13. 65). Sequencing statistics, including numbers of sequencing reads, endogenous DNA content, clonality, and average sequence length and coverage are provided in Data S1A.

QUANTIFICATION AND STATISTICAL ANALYSIS

Post-mortem damage

The Paleomix pipeline automatically produced mapDamage2 profiles, which provide an assessment of post-mortem DNA damage through the analysis of nucleotide mis-incorporations and base composition within reads (and on flanking genomic regions for the latter). Nucleotide mis-incorporation profiles for each individual DNA library looked similar and as expected following partial USER enzymatic treatment. Illustrative examples showing a limited excess of C → T transitions at alignment ends, mirrored by an equivalent excess of G → A transitions at alignment ends, are provided in Figures S1A and S1B. The base composition within reads was uniform, while the genomic position preceding alignments was significantly enriched in C, due to the cleavage of ancient DNA templates at deaminated non-methylated Cytosines 46.
Error rates

Error rates were calculated using ANGSD (version 0.933–86–g3feddc4, with htslib: 1.1.0.2–106-g9c35744[65]) and the methodology fully described in previous work[104] using 3-way alignments including an outgroup genome (here, the ancestral human genome), a high-quality genome (here, the genome from a modern Sardinian individual, sequenced to 39.4-fold average depth-of-coverage by[105]; accession number SS6004474) and each ancient genome as the test genome. The relative difference between the branch length leading to the ancient genome and the high-quality modern genome provides a direct measurement of relative error rates in the ancient genome, both globally and for each individual substitution class (Figure S1C). The analysis was repeated on previously published genomes for comparison (Figures S1D and S1E). Overall, the global error rates of each individual genome characterized in this study ranged between 0.000566 and 0.000848 substitutions per base on average, which is lower than the error rates presented in a random selection of comparative studies[61,66,106–108].

Uniparental markers and Contamination estimates

Individual sex was inferred on the basis of X-to-autosomal sequence coverage, resulting in the identification of 12 women and 13 men (Data S1A). Mitochondrial haplotypes were called using haplogrep (version 2.2[66]), and minimal mapping and base quality thresholds of 30. Briefly, read alignments against the rCRS reference mitogenome (GenBank Accession Number NC_120920.1) were converted into a VCF file using bcftools (version 0.1.19–44428cd) mpileup, call (–ploidy 1) and filter modules, disregarding sites covered by less than 5 independent reads. The resulting VCF file was then directly processed through haplogrep (version 2.1.25), calculating the best 100 hits. Contamination rates based on mitochondrial data were estimated using Schmutz[67] and an identical base quality threshold. Contamination estimates were assessed to be within a 0%–2% confidence range for all individuals processed (Data S1E), except for 2H06 for whom extremely limited amounts of data were generated (mtDNA average coverage-of-depth = 1.25-fold) and 1H14 (confidence range 1%–12%). The former sample was disregarded in all other analyses. Sample 1H14 was, however, included and projected together with the majority of the other samples characterized at the Mont-Aimé site in PCA analyses. This sample also showed similar genetic profiles (e.g., f3- and D-statistics, ADMIXTURE, and PCA), supporting minimal nuclear contamination. Contamination rates were also estimated for males using heterozygosity measurements at polymorphic sites present on the X chromosome, following the methodology from[109] and implemented in ANGSD. Transition substitutions, and sites covered once or more than 200-times were disregarded. Contamination estimates were found to be extremely limited (median = 0.12%) and not greater than 1.01% (Data S1F). Y chromosome haplotypes were called using the Yleaf statistical package[68]. All the individuals analyzed belonged to haplogroup L2a1 (Data S1F), except individuals 2H07 and GBVPK who carried haplogroups H2a1a and R1b1a1b1a1a2a1, respectively.

Phenotype prediction

We assessed the genotypes of the two individuals sequenced to high depth-of-coverage, i.e., d2H11 and d2H10, d indicating that the high-coverage genome data (throughout the manuscript, pseudo-diploid genotypes called after random sampling one individual read per position are indicated using h as a prefix), for a total of 90 SNPs causative for or associated with a range of morphological, coloration, behavioral and health-related phenotypes (Figure 2C). Skin, eye and hair color was inferred using the 41 loci that are part of the HirisPlex-S, HirisPlex and IrisPlex sytems[39,69], available online at https://hirisplex.erasmusmc.nl/ (Data S2B). Individual SNP labels and their coordinates on the GRCh37 (hg19) reference genome are provided. Minimal mapping and base quality thresholds of 30 were required. Sites for which sequence coverage was above the 99.5% coverage quantile were disregarded to avoid any impact of mis-identified copy number variants. Bcftools mpileup, call and filter modules were assuming diploidy for autosomal SNPs and haploidy for those located on the X chromosome as both individuals were males.
Principal Component Analysis

Principal Component Analysis (PCA) was carried out using the Human Origin reference panel for 592,998 autosomal genotypes in 796 individuals to limit the possible impact of sequencing errors. Gated using f4-statistics. These statistics were calculated using qpDstat in Admixtools (version 5.0) in the form of (ANF, X; Y, Mbuti), showing greatest genetic affinities to those presented in this study are indicated with their original label and radiocarbon date (cal. years BCE).

Inbreeding and diversity estimates

Levels of inbreeding in the two individuals sequenced to high coverage were assessed using autosomal data in ROHan (−rohmu 2.0e-05). The program returned no evidence of inbreeding in any of the two individuals and returned largely overlapping diversity theta estimates for both individuals. Inbreeding and theta outside run-of-homozygosity were estimated using the same procedure on a comparative panel of five Neolithic and three Mesolithic high-coverage genomes previously reported (Stuttgart and Loschbour; NE139, Bar8 107; WC1 108; sf12 11, and; Jerpoint14 and Sramore62 12). The corresponding results are provided in Data S2A. The comparative panel was directly downloaded from public repositories as BAM files, except for individuals Bar8 and WC1 that were subject to the same read processing, mapping and filtering procedure as individuals d2H10 and d2H11. For samples Stuttgart and Loschbour, we noticed an excess of nucleotide mis-incorporations at the first and last sequence position of read alignment. These positions were trimmed using BAMtrim from the bamUtil repository (https://github.com/statgen/bamUtil) before running ROHan to limit the possible impact of sequencing errors.

f3-Outgroup and f4-statistics

f3-Outgroup statistics were carried out using q3Pop from Admixtools (version 5.0.56) to identify those ancient individuals previously characterized showing highest genetic affinities with each individual sequenced in this study, except 2H06 for whom insufficient data could be collected. Here, we used all 1,150,639 autosomal positions overlapping the 1240K panel and the comparative genotype panel provided on David Reich’s website (dataset v42.4.1240K_HumanOrigins, available at https://reichdata.hms.harvard.edu/pub/datasets/amh_repo/curated_releases/V42/V42.4/SHARE/public.dir/v42.4.1240K_HO.tar). We also included all those individuals recently reported in three recent studies5,12,16 and covering the Mesolithic, Neolithic and Bronze Age time periods. The individuals present in these publications were subjected to the same procedure as the individuals reported in this study, including read processing, mapping for those described by Brunel and colleagues16 and Cassidy and colleagues15, or starting from individual BAM files available from public repositories for those reported by Rivollat and colleagues8. The analysis was based on pseudo-diploid genotype calls for all the individuals presented in this study, except 2H06 for whom insufficient data could be collected. PCA was carried out using smartPCA from EIGENSOFT version 7.2.1, projecting ancient individuals onto the principal components (PCs) obtained from modern reference individuals (the lsgproject option was turned on). Projections on the first two PCs are provided in Figure 3A and projections PC2 and PC3 are provided in Figure S2A. In addition to pseudo-diploid genotype calls, the analysis was also carried out on truly diploid genotype calls for the two individuals sequenced to high coverage in order to confirm individual projections.
Admixture
Supervised ADMIXTURE (version 1.3.0\(^6\)) analyses were carried out to estimate different ancestry proportions in each individual sequenced in this study, except 2H06 for whom not enough data could be collected. Here, we followed a previously published procedure and used all autosomal positions overlapping those from the 1240K panel available for download from David Reich’s website (dataset v42.4.1240K, available at https://reichdata.hms.harvard.edu/pub/datasets/amh_repo/curated_releases/V42/V42.4/SHARE/public.dir/v42.4.1240K.tar). The same 36 ANF and 15 WHG individuals as described above were selected together with ten ancient Yamnaya_Samara individuals, following previous work\(^4,5\) (Yamnaya_Samara = I0370, I0441, I0444, I0359, I0357, I0429, I0438, I0443, I7489, I0231\(_{published}\)) that established that the genetic landscape of modern European individuals can be explained by various contributions of those three ancestries. Only sites that were covered in at least 50% of all individuals were considered. Termination criteria for convergence were based on a log-likelihood increase by a maximum of 0.0001 between iterations. Confidence intervals were estimated from 100 bootstrap pseudo-replicates. Analyses were repeated 10 times using 10 random seeds in order to assess cross-validate convergence. Individual profiles are provided in Figures S2B and S2C, averaging out the point estimates and confidence ranges obtained across the 10 analytical replicates. A ternary plot of the averaged point estimates is provided in Figure 4B.

D-statistics
Different combinations of D-statistics were calculated using qpDstat in Admixtools (version 5.0\(^5\)) in order to estimate whether pairs of ancient individuals were symmetrically related to ANF, WHG and/or Yamnaya_Samara individuals previously reported (and defined as indicated in the previous section). Calculations were carried out on the 1240K dataset and using four Mbuti individuals as the outgroup (S_Mbuti-3.DG, B_Mbuti-4.DG, S_Mbuti-2.DG, S_Mbuti-1.DG). The topologies investigated were in the form of (Outgroup, ANF or WHG or Yamnaya_Samara; Ancient_Individual1, Ancient_Individual2), respectively. Only those combinations represented by a minimal number of 10,000 covered sites were considered. The returned values of the different D-statistics calculations are provided in Figure S4 for each individual reported in this study, except 2H06 for whom not enough data could be collected. Negative values indicate closer genetic affinities between H3 (i.e., WHG, ANG and Yamnaya_Samara) and H2, while positive values indicate closer genetic proximity to H1.

Population modeling
Affinities between various population groups were assessed using the 1240K dataset and qpAdm from Admixtools (version 5.0\(^5\)). Analyses were aimed at assessing the respective proportions of ANF and WHG ancestries in the individual genomes presented in this study as well as in the genome-scale data previously reported for other contemporary ancient individuals from France\(^3,8,16\). This represented a total of 121 ancient individuals from France (I1381, I1382, I1388, I1390, I1391\_published, I1392, I2575\_published, I3874, I3875, I4030, I4034, I4035, I4038, d2H10, d2H11, h1H04, h1H06, h1H07, h1H13, h1H14, h2H07, h2H10, h2H11, h2H17, h2HxC5x196x1131, hBERG032-2, hBERG157-2, hBERG157-7, hBIS130, hBIS385, hBLP10, hBUCH2, hCBV95, hCRE20D, hEUG311, hEs97-1, hFLR001, hFLR002, hFLR003, hFLR004, hFLR006, hFLR007, hFLR010, hFLR013, hFLR014, hGBVPR, hGBVPL, hGBVPO, hGRG003, hGRG008, hGRG015, hGRG016, hGRG019, hGRG022, hGRG023, hGRG025, hGRG027, hGRG028, hGRG032, hGRG035, hGRG043, hGRG047, hGRG049, hGRG050, hGRG052, hGRG056, hLBR001, hLBR002, hLBR003, hLBR004, hLBR005, hMAS15, hMDV248, hMor6, hOBE3626-1, hOBE3722, hOBE3061, hOBN002, hOBN004, hOBN005, hOBN006, hOBN007, hOBN008, hOBN010, hOBN011, hPE10, hPE12, hPEN001, hPEN003, hPERS037AB, hPERS116B, hPHER001, hPHER006, hPSS4170, hPSS4693, hQUIN234, hQUIN58, hRX15, hRX2, hRX4, hRS0102, hRS0245, hRS057, hRS058, hROUQCC, hROUQEE, hROUQFF, hROUQHH, hROUQOV, hROUQWQ, hSch72-15, hSchw432, hTORTC, hTORTD, hTORTF, hWET370). In all models tested, we followed Haak and colleagues\(^7\), Olalde and colleagues\(^9\) and Brunel and colleagues\(^16\) and used Mbuti (n = 4; S_Mbuti-3.DG, B_Mbuti-4.DG, S_Mbuti-2.DG, S_Mbuti-1.DG\(^15\)), Papuans (n = 15; S_Papuan-1.DG, S_Papuan-5.DG, S_Papuan-13.DG, S_Papuan-10.DG, B_Papuan-15.DG, S_Papuan-12.DG, S_Papuan-2.DG, S_Papuan-9.DG, S_Papuan-7.DG, S_Papuan-8.DG, S_Papuan-3.DG, S_Papuan-14.DG, S_Papuan-4.DG, S_Papuan-6.DG, S_Papuan-11.DG), Onge (n = 6; mondAL_ONG-1.SG, mondAL_ONG-12.SG, mondAL_ONG-14.SG, mondAL_ONG-4.SG, mondAL_ONG-8.SG, mondAL_ONG-9.SG\(^15\)), Han (n = 106; S_Han-1 to S_Han-3, NA18525.SG, NA18526.SG, NA18528.SG, NA18530.SG-NA18539.SG, NA18541.SG-NA18550.SG, NA18552.SG, NA18553.SG, NA18555.SG, NA18557.SG-NA18567.SG, NA18570.SG-NA18574.SG, NA18577.SG, NA18579.SG, NA18582.SG, NA18591.SG-NA18593.SG, NA18595.SG-NA18597.SG, NA18599.SG, NA18602.SG, NA18603.SG, NA18605.SG, NA18606.SG, NA18608.SG-NA18648.SG, NA18740.SG, NA18747.SG-NA18749.SG, NA18757.SG), Kartiana (n = 4; B_Kartiana-3.DG, S_Kartiana-1.DG, S_Kartiana-2.DG, B16.SG\(^12,14,15\)) modern individuals as outgroup (‘right’) populations together with Mota (n = 1; moto.SG\(^15\)), Ust-Ishim (n = 1; Ust_Ishim\_published.DG\(^15\)), Malta Ma-1 (n = 1; MA1.SG\(^18\)), Koros (n = 1; 11507\(^7,109\)), Vestonice (n = 1; Vestonice16), Natufian (n = 1, I1072\(^1\)) and Goyet-116 (n = 1; GoyetQ116-1\_published\(^1\)). This selection was limited to those individuals showing data sequence for a minimum of 700,000 SNPs. The same coverage filter was applied to the list of ANF, WHG and Yamnaya_Samara individuals described above, leaving a final number of 18, 5 and 10 individuals, respectively.

In a first model, each ancient individual from France was grouped together with WHG and ANF as ‘left’ populations. This provided WHG and ANF ancestry estimates in each ancient individual considered and assessed the validity of the model relative to models in which only one of the two possible ancestries modeled was considered (Data S4A).

A second model was aimed at assessing the possibly different contributions of WHG by adding the Goyet-Q2 individual\(^20\) to the list of ‘left’ populations (Data S4A), as previous work identified the presence of a cline of ancestry related to this individual in some
hunter-gatherer populations from Western Europe. This provided the opportunity to assess whether an additional and Goyet-Q2-related ancestry was necessary to explain the genetic profiles of the different ancient individuals from France analyzed at the genetic level. This analysis was complemented with a third model, which corresponded to the second model, except that the individual Goyet-Q2 was now considered as an additional outgroup (i.e., among the so-called ‘right’ populations; Data S4A). Since this modeling largely supported the conclusions obtained the previous two models, this allowed us to confirm the absence of Goyet-Q2 ancestry in the individuals analyzed.

Finally, a fourth model was assessed in order to estimate the proportion of Yamnaya_Samara ancestry in each ancient individual considered. This was done using a list of ‘left’ populations including each ancient individual from France, as well as Yamnaya_Samara individuals, WHG and ANF (Data S4B).

Admixture dating
The different analyses presented supported the presence of an excess of WHG ancestry in two individuals at Mont-Aimé (2H10 and 1H06) and an excess of Yamnaya_Samara ancestry in one individual at Grotte Basse de la Vigne Perdue (GBVPK). We used LD-decay profiles and DATES (93, https://github.com/priamoorjani/DATES) to estimate the timing of underlying admixture events. For timing the dates of admixture between WHG and ANF ancestors in the genealogy of 2H11and 1H06 individuals, DATES was run on the 1240K dataset, restricting the analyses to those two individuals and the 36 ANF and 15 WHG individuals defined in the f3Outgroup, f4-statistics and ADMIXTURE sections. Specifically, each of these individuals was modeled as a combination of the two ANF and WHG possible sources. The same analyses were repeated in a third run where all the other Mont-Aimé individuals for which at least 10,000 SNPs were covered and no particular excess of WHG ancestry was identified (n = 8, h1H04, h1H07, h1H13, h1H14, h2H07, h2H10, h2H17, h2HxC5x196x1131). Similarly, the timing of admixture with Yamnaya-related individuals in the genealogy of the GBVPK individual was assessed modeling this individual as a combination of two possible sources: on the one hand, a Late Neolithic population from France, and on the other hand, the ten Yamnaya_Samara individuals defined above. In these analyses, three proxies were used as Late Neolithic groups from France: (1) the individuals from Grotte du Rouquet, (2) the Late Neolithic individuals from the La Clape massif sequenced in this study, and including Grotte du Rouquet as well as MAS15 and TORTD, and; (3) those individuals from Mont-Aimé showing no particular excess of WHG ancestry, as described above. Weighted LD-decay profiles are provided in Figure 4E, together with average and standard deviation estimates for the respective times of admixture inferred.

Microbial profiling
Microbial taxonomic profiles of each individual DNA library were determined using the metaBIT automated computational package19, and restricting analyses to the fraction of collapsed reads (i.e., collapsed truncated and uncollapsed pairs were disregarded), and ignoring virus and eukaryotes. This package leverages the MetaPhLn2 diversity database that comprises a total of 1,036,027 biomarkers including 896,781 markers for 12,926 bacterial taxa. By-library profiles were merged per individual, and bacterial taxa supported by abundances lower than 1% were disregarded. Principal Coordinate Analyses were carried out based on Bray-Curtis distances to compare these taxonomic profiles to those obtained using a similar methodology: 15 profiles from soil samples31 and 689 profiles published by the Human Microbiome Project Consortium32 for the mouth (n = 382), skin (n = 26), nose (n = 87) and vagina (n = 56) environments.

DNA methylation
The characterization of two individual genomes at high-coverage allowed us to assess DNA methylation patterns in Late Neolithic individuals from France. DNA methylation was inferred using DamMet48 within genomic windows of 1,000, 1,500 and 2,000 bases centered around 27,578 genomic positions corresponding to the Illumina 27k methylation Bead Chip system. The age-at-death of the two ancient individuals was then estimated using these profiles and leveraging the DNA Methylation Age Calculator statistical model available at http://dnamage.genetics.ucla.edu/submit, which integrates age-dependent DNA methylation profiles at 353 sites to return age estimates. This analysis, and all following analyses, were repeated for an extended comparative panel including two Neolithic and two Mesolithic individual genomes also sequenced to high-coverage-of-depth (Stuttgart and Loschbour), as well as Jerpoint14 and Sramore62; note that sequence data generated in the absence of USER treatment were disregarded. These analyses were restricted to those individuals characterized from teeth in order to avoid possible DNA methylation differences across tissues. Despite returning point estimates for the age-at-death, calculations also indicated poor correlation with a Gold standard panel of DNA methylation values used internal for data normalization (Data S3B). They were, thus, considered only indicative.

Independent estimates for the age-at-death of this panel of individuals were obtained considering the methylation data reported by Bekaeft and colleagues53 for 11 individual loci (ASPA: 17:3379066-3380067; PDE4C: 19:18343388-18344389, 19:1834400-18344401, 19:18343414-18344415; EDARADD:1:236557181-236558182; ELOVL2: 6:11044376-11045375, 6:11044372-11045373). Eight additional loci analyzed in the original publication were disregarded either as they could not be positioned on the GRCh37 reference unambiguously or as their DNA methylation profiles were not associated with age. Following the original publication, we developed multiple regression models between the age of 207 modern individuals and either linear or quadratic values of their DNA methylation profiles at the 11 loci. Cross-validation using the R crossval library indicated excellent prediction performance (0.9378 and 0.9366 for the linear and quadratic multiple regression models, respectively). We applied similar models to the panel of ancient individuals, considering sites
for which the DNA methylation value estimate returned by DamMet showed a confidence range where at least one boundary was included within the 0-1 frequency range, so as to include only those sites where sufficient data may be collected. The resulting individual age-at-death estimates are provided in Data S1A and S3C.

Finally, we estimated DNA methylation profiles within promoter regions and gene bodies. Promoters were defined as the one kilobase upstream of the Transcription Start Site (TSS) while gene bodies were restricted to the two kilobases downstream. TSS and gene models were downloaded from Ensembl (GRCh37). Only the longest transcript was considered at a given locus. A total of 10,659 gene bodies provided DNA methylation values with confidence ranges where at least one boundary was included within the 0-1 interval for all six individuals analyzed (i.e., d2H10, d2H11, Stuttgart, Loschbour, Jerpoint14 and Sramore62). Pairwise correlations varied between 0.40 and 0.62, but were lower for those two individuals sequenced to lower coverage (Sramore62, 13.68X and d2H10, 13.90X respectively; Figure 2B). This indicated that inferred DNA methylation values may be of lower accuracy in those two individuals, due to limited sequence data. Additionally, the Stuttgart and Loschbour individuals, which represent a Neolithic woman and a Mesolithic man genome-sequenced in the same laboratory4, showed correlation values of 0.55 which is superior to those observed in Neolithic pairs from the same site (d2H10 and d2H11: 0.53) or from different sites (d2H10 and Stuttgart: 0.52). This indicated possible technical batch effect impacting on DNA methylation inference. To limit such technical batch artifacts, we restricted the following analyses to only three individual genomes generated in different laboratories, and including one Mesolithic individual. These include only males, and display the maximal pairwise correlation values observed within gene bodies (0.62, Figure 2B). Although the exact sources of those technical batch artifacts are unknown, we noticed that the procedures underlying data production encompass a range of experimental conditions, both at the levels of DNA extraction, USER treatment, library construction, sequencing and sequence post-processing. For example, reduced efficacy of USER treatment at terminal ends or in silico trimming of read termini is expected to affect those sequence positions that are the most subject to post-mortem Cytosine deamination123. This, in turn, is expected to directly impact DNA methylation measurements48. For example, fully efficient USER treatment leaves CpG → TpG substitutions only at CpG sites that were methylated, while treatments of lower efficacy leave variable proportions of CpG → TpG substitutions at CpG sites that were not methylated. The latter procedures will, thus, limit the specificity and sensitivity of any statistical method aimed at DNA methylation inference based on CpG → TpG counts. In silico trimming of read ends is expected to reduce sensitivity only, as it removes the total number of occurrences of CpG → TpG substitutions. Further work is required to quantify the exact importance of those possible sources of bias in the inference of DNA methylation patterns in ancient individuals.

DNA methylation profiles for a total of 6,355 promoters showing credible confidence range (see above) were compared between the two pairs of Mesolithic and Neolithic individuals (i.e., Loschbour versus d2H11 on the one hand, and Loschbour versus Jerpoint14 on the other hand). Those promoters showing (1) average DNA methylation shifts of at least 50% across pairs, (2) average DNA methylation shifts at least twice as large as the standard deviation across pairs, and (3) that showed consistent directions were short-listed as a candidate list of promoters that experience significant DNA methylation changes during the Mesolithic to Neolithic transition. Functional enrichment in Gene Organizer (71, http://geneorganizer.huji.ac.il/), Panther (70, http://www.pantherdb.org/) and David 6.8 (72, https://david.ncifcrf.gov/), however, indicated that no particular functional category was significantly over-represented in the 611 promoter shortlisted relative to the full background of 6,355 promoters (Data S3A). We noted that the list of candidates included significantly more gene promoters that were hyper-methylated in Neolithic individuals, than in Mesolithic individuals (404 versus 207, chi-square test \( p\)-values = 1.59.10^{-15}).

**ADDITIONAL RESOURCES**

Our study has not generated or contributed to a new website/forum and it is not part of a clinical trial.