

Published in final edited form as:

*Nat Genet.* 2013 April ; 45(4): 450–455e1. doi:10.1038/ng.2536.

## Sequencing ancient calcified dental plaque shows changes in oral microbiota with dietary shifts of the Neolithic and Industrial revolutions

Christina J Adler<sup>1,2</sup>, Keith Dobney<sup>3</sup>, Laura S Weyrich<sup>1</sup>, John Kaidonis<sup>4</sup>, Alan W Walker<sup>5</sup>, Wolfgang Haak<sup>1</sup>, Corey JA Bradshaw<sup>6,7</sup>, Grant Townsend<sup>4</sup>, Arkadiusz Softysiak<sup>8</sup>, Kurt W Alt<sup>9</sup>, Julian Parkhill<sup>5</sup>, and Alan Cooper<sup>1</sup>

<sup>1</sup>Australian Centre for Ancient DNA, School of Earth and Environmental Sciences and Environment Institute, The University of Adelaide, South Australia 5005, Australia <sup>2</sup>Institute of Dental Research, Faculty of Dentistry, University of Sydney, Sydney, New South Wales, Australia <sup>3</sup>Department of Archaeology, University of Aberdeen, Aberdeen AB24 3UF, Scotland, United Kingdom <sup>4</sup>School of Dentistry, The University of Adelaide, South Australia 5005, Australia <sup>5</sup>The Wellcome Trust Sanger Institute, Hinxton, Cambridgeshire, CB10 1SA, United Kingdom <sup>6</sup>The Environment Institute and School of Earth and Environmental Sciences, The University of Adelaide, South Australia 5005, Australia <sup>7</sup>South Australian Research and Development Institute, PO Box 120, Henley Beach, SA 5022, Australia <sup>8</sup>Department of Bioarchaeology, Institute of Archaeology, University of Warsaw, Poland <sup>9</sup>Institute for Anthropology, Johannes Gutenberg University of Mainz, Mainz, Germany

### Abstract

The importance of commensal microbes for human health is increasingly recognized<sup>1-5</sup>, yet the impacts of evolutionary changes in human diet and culture on commensal microbiota remain almost unknown. Two of the greatest dietary shifts in human evolution involved the adoption of carbohydrate-rich Neolithic (farming) diets<sup>6,7</sup> (beginning ~10,000 years BP<sup>6,8</sup>), and the more recent advent of industrially processed flour and sugar (~1850)<sup>9</sup>. Here, we show that calcified dental plaque (dental calculus) on ancient teeth preserves a detailed genetic record throughout this period. Data from 34 early European skeletons indicate that the transition from hunter-gatherer to farming shifted the oral microbial community to a disease-associated configuration. The composition of oral microbiota remained surprisingly constant between Neolithic and Medieval times, after which (the now ubiquitous) cariogenic bacteria became dominant, apparently during the Industrial Revolution. Modern oral microbiota are markedly less diverse than historic

---

**Corresponding Author:** Alan Cooper; Australian Centre for Ancient DNA, University of Adelaide, North Terrace Campus, South Australia 5005, Australia; Phone 61-8-8303-5950/3952; Fax 61-8-8303 4364 alan.cooper@adelaide.edu.au.

**Author Contributions** C.J.A., A.C., K.D., A.W.W., J.P., K.W.A., G.T., J.K., and W.H. designed the study, C.J.A., K.D., K.W.A., A.S., W.H. A.C. and J.K. collected samples, C.J.A. and L.S.W. extracted and amplified DNA from dental calculus, C.J.A. and L.S.W. analysed sequence data, A.W.W. performed 454 sequencing, C.J.A.B. performed alpha diversity bootstrapping analyses, and C.J.A., A.C. and K.D. wrote the paper. All authors discussed the results and contributed to writing the manuscript.

Sequence data have been deposited in Genbank under accession ERP002107.

The authors declare no competing financial interests.

populations, which might be contributing to chronic oral (and other) disease in post-industrial lifestyles.

---

Commensal microbiota comprise the majority of cells in the body and play a key role in human health<sup>1-5,10</sup>. However, their evolution remains poorly understood, and detailed genetic records from commensal bacteria have yet to be recovered from the archaeological record. Dental calculus is ubiquitous in both present-day and ancient human populations<sup>11</sup>, and microscopic analysis has shown that it accurately preserves bacterial morphology over millennia<sup>12-14</sup>. Dental calculus develops when dental plaque, an extremely dense bacterial biofilm<sup>15</sup>, becomes mineralised with calcium phosphate<sup>16</sup>. Bacteria in calculus become locked in a crystalline matrix similar to bone<sup>16</sup> (Supplementary Figure 1), with deposits occurring both above and below the gum or gingiva (supra- and subgingivally)<sup>17</sup>. Calculus represents one of the few sources of preserved human and hominid microbiota, and genetic analysis has the potential to create a powerful new record of past dietary impacts, health changes, and oral pathogen genomic evolution deep into the past. In addition, oral bacteria are transferred vertically from the primary caregiver(s) in early childhood<sup>18</sup> and horizontally between family members later in life<sup>18,19</sup>, making archaeological dental calculus a potentially unique means of tracing population structure, movement and admixture between ancient cultures, as well as the spread of diseases.

The increased consumption of domesticated cereals (wheat and barley in the Near East) beginning with the Neolithic was associated with a marked increase in prevalence of dental calculus and oral pathology<sup>20</sup>. These oral diseases include dental caries (tooth decay)<sup>1</sup> and periodontal disease (an infection causing damage to the supporting connective tissues of the tooth and resorption of bone)<sup>21</sup>, both of which were rare in pre-Neolithic hunter-gatherer societies<sup>20</sup> and early hominins<sup>22</sup>. Caries and periodontal disease are both polymicrobial, plaque-mediated infections, thought to result from perturbation of a healthy, ecologically balanced oral biofilm<sup>23,24</sup> that can occur from dietary changes, such as the increased consumption of fermentable carbohydrates<sup>25,26</sup>. Caries has become a major endemic disease, affecting 60-90% of school-aged children in industrialised countries, whilst periodontal disease occurs in 5-20% of the adult population worldwide<sup>27</sup>. Notably, oral bacteria are also associated with many systemic diseases including arthritis<sup>28</sup>, cardiovascular disease<sup>3</sup> and diabetes<sup>4</sup>, in addition to diseases of the oral cavity<sup>1,2</sup>.

We collected a mixture of supra- and subgingival calculus samples (determined morphologically, Supplementary Note) from the teeth of 34 prehistoric European human skeletons (11 males, 11 females and 12 of unknown sex, ranging in age from < 20 to > 60 years at death, Supplementary Table 1), dating from before farming (Mesolithic) to Medieval periods. The samples included individuals from the last hunter-gatherers in Poland, the earliest farming culture in Central Europe (the Linear Pottery Culture – LBK), as well as late Neolithic (Bell Beaker Culture), early and later Bronze Age and medieval rural and urban populations (Table 1, see Supplementary Note for description). All ancient DNA work was conducted in a physically isolated, specialist laboratory dedicated to ancient environmental and bacterial DNA research at the Australian Centre for Ancient DNA, using strict decontamination and authentication protocols (Supplementary Note). We extracted

bacterial DNA from sterilized ancient calculus samples ( $n = 34$ ) and generated PCR amplicon libraries of the 16S rRNA gene, targeting three hypervariable regions (V1, V3 and V6) with barcoded primers (Supplementary Tables 2 and 3). In addition, primers specific to *Streptococcus mutans* and *Porphyromonas gingivalis* were used to detect oral pathogens in ancient dental calculus (Supplementary Table 2). We compared the ancient samples to modern calculus ( $n = 6$ ) and plaque ( $n = 13$ ) samples that were extracted and sequenced in an analogous manner (Methods, Supplementary Tables 3 and 4). We also extracted and sequenced bacterial DNA from within the teeth that provided the calculus samples, to determine the background bacterial contribution of the post-mortem depositional environment ( $n = 6$ , Supplementary Note). Amplicons generated from extracted samples and multiple extraction blanks were sequenced using both conventional and pyrosequencing technology. Of the 998,575 sequences generated, we discarded around ~ 50% following quality filtering and de-noising, to remove sequences containing PCR and sequencing errors<sup>29</sup>, leaving 451,241 sequences (Supplementary Table 4).

At the phylum level, the bacterial composition of ancient calculus was similar to modern oral samples and sequences from the Human Oral Microbiome Database (HOMD)<sup>30</sup>, but markedly distinct from laboratory reagents (extraction blanks), environmental samples (soils, sediments and water), and within the ancient teeth themselves (Figure 1, Supplementary Fig. 2 and Supplementary Note). The archaeological calculus was dominated by Firmicutes (33% for the V3 region, which was the most phylogenetically informative fragment, see Supplementary Note), which is comparable to percentages found in both the HOMD (37%), and modern oral samples (average 50%)<sup>1,2,21,31</sup>. Again, this was clearly distinguishable from the bacterial sequences obtained from extraction blanks (6% Firmicutes,  $p = 0.003$ ), environmental samples (1.6% Firmicutes,  $p < 0.001$ ) and within the ancient teeth (8% Firmicutes,  $p < 0.001$ ), which were all dominated by Proteobacteria (73%  $p = 0.2$ , 56%  $p < 0.001$  and 31%  $p = 0.005$ , respectively). The sequences from the extraction blanks were typical of bacterial communities found in clean-room environments<sup>32</sup> and non-template controls<sup>33</sup>. In addition to Firmicutes, the ancient dental calculus samples contained all 15 phyla commonly found in the modern human oral cavity<sup>30</sup>, with high percentages of Actinobacteria (19%) as is observed in modern calculus deposits (7%). We have shown that dental calculus from samples that are thousands of years old preserve representative and informative microbial signatures of past human-associated microbiota.

Phylogenetic analyses of the  $\beta$ -diversity, which measures the number of operational taxonomic units (OTUs) that are unique between the groups (Supplementary Note), confirmed the V3 sequences from ancient calculus were clearly more similar to those from modern dental calculus, plaque, and saliva samples<sup>1,2,21,31</sup> than environmental samples<sup>34-40</sup> (Figure 2a and b, Supplementary Fig. 4 and Supplementary Table 5). Similar patterns were observed for sequences from V1 and V6 (Supplementary Fig. 5, Supplementary Note). Furthermore, the bacterial composition of the ancient calculus samples clustered separately from the sequences present within the ancient teeth (Supplementary Fig. 6,  $p = 0.002$ ). Overall, these results strongly suggest that the DNA sequences from the ancient calculus samples are not derived from contamination in the post-mortem environment.

The temporal transect of ancient dental calculus samples provides the first views about the timing and nature of changes in human oral bacterial composition and diversity over the last 7,500 years. The composition of oral microbiota underwent a distinct shift with the introduction of farming in the early Neolithic (Figures 2c and d), with the earlier hunter-gatherer groups displaying fewer caries- and periodontal disease-associated taxa (Figure 3). This is consistent with skeletal evidence showing marked increases in periodontal disease<sup>41</sup> following the transition to an agricultural diet, suggesting a major impact on the human oral ecosystem around this time. This is thought to be caused by increased amounts of soft carbohydrate foods compared with hunter-gatherer diets<sup>26</sup>. After the transition to agriculture in the early Neolithic there was a notable consistency in the composition of bacteria through the Medieval period (~ 400 years BP) (Figure 3), in parallel with the broad similarity of food-processing technologies during these times<sup>9</sup>. In contrast, today's oral environment is much less biodiverse and dominated by potentially cariogenic bacteria (Figure 3a and b, e.g., *S. mutans*, Supplementary Figs. 7-9 and Supplementary Table 7).

Random forest (RF) analysis was used to identify the taxa which discriminate the different time periods (Supplementary Note). RF revealed that Clostridia taxa, such as *Clostridiales* (importance score =  $0.014 \pm 0.002$ ) and non-pathogenic oral microbial family *Ruminococcaceae* (importance score =  $0.0035 \pm 0.0009$ ), were predictive of hunter-gatherer microbial communities compared to early agriculturists (ratio baseline error to observed error = 3.5, Supplementary Table 8). Farming groups from the Neolithic and Medieval periods were discriminated by both non-pathogenic taxa such as *Clostridiales incertae sedis* (importance score =  $0.014 \pm 0.003$ ) and decay-associated *Veillonellaceae* (importance score =  $0.012 \pm 0.0038$ ). Farming populations also displayed more periodontal disease-associated taxa, including *P. gingivalis*, *Tannerella* and *Treponema*, than hunter-gatherers. While there is also a strong association between periodontal disease and individual age at death<sup>42</sup>, we found periodontal disease-associated taxa across a range of ages, including the youngest individual (3-4 years old, ID 8247).

RF analysis revealed that only a limited number of taxa distinguished modern oral environments from farming groups in the Medieval and Neolithic periods (ratio baseline error to observed error = 4.0, Supplementary Table 9). These taxa include decay-associated *Veillonellaceae* (importance score =  $0.021 \pm 0.004$ ), in addition to *Lachnospiraceae* (importance score =  $0.019 \pm 0.007$ ) and *Actinomycetales* (importance score =  $0.0013 \pm 0.0005$ ). Notably, the frequency of *S. mutans* is significantly higher in modern samples than pre-industrial, agricultural samples (Figure 3b, Supplementary Note), indicating that caries-associated bacteria have only become dominant after Medieval times. This change is most likely associated with the onset of the Industrial Revolution which began some 200 years ago and represents the largest change in food production and processing technology since the shift to farming<sup>9</sup>. The Industrial Revolution saw the production of refined grain and concentrated sugar from processed sugar beet and cane<sup>9</sup>, generating mono- and disaccharides, which are the main substrates for microbial fermentation that lowers plaque pH and cause enamel demineralization<sup>26</sup>.

Overall, it is clear that modern Europeans have much lower oral microbial diversity than either Mesolithic or pre-Industrial Neolithic groups ( $p < 0.001$ , Supplementary Table 7),

including fewer bacteria associated with good health (*Ruminococcaceae*), periodontal disease-associated taxa (e.g., *Porphyromonas gingivalis*, *Tannerella* and *Treponema*) similar to early agriculturists, and a markedly higher abundance of (now ubiquitous) pathogens such as *Streptococcus mutans* (Figure 2, Supplementary Note). Perhaps more importantly, the decline in overall oral microbial diversity indicates that over the past few hundred years, the human mouth has become a substantially less-biodiverse ecosystem. In both a human-associated microbiota<sup>43-45</sup> and macroecological<sup>46,47</sup> context, higher phylogenetic diversity is associated with greater ecosystem resilience and productivity. Therefore, the modern oral environment is likely to be less resilient to perturbations<sup>48</sup> in the form of dietary imbalances or invasion<sup>49</sup> by pathogenic bacterial species.

Major changes in carbohydrate intake in human history appear to have impacted the ecosystem of the mouth, opening up pathological niches for periodontal disease in the early Neolithic and caries in the recent past. These data are potentially important for assessing current associations with systemic diseases; for example, it has been proposed that periodontal disease might contribute to the development of diabetes and heart disease<sup>25</sup> through the production of a prolonged inflammatory state<sup>3</sup>. However, whilst the frequency of these systemic diseases has risen over the last few decades<sup>25</sup>, our data show that the abundance of periodontal disease-associated bacteria has been relatively stable since the introduction of farming (e.g., Figure 3, *P. gingivalis*). This indicates that although periodontal disease might contribute to pathogenesis, it is probably not a factor in the rising incidence of these systemic diseases.

Our research has identified a powerful new avenue for bio-anthropological research, which promises to provide the first detailed genetic records of the evolution of human microbiota. This provides the potential to directly examine the effects of nutritional and cultural transitions (see Figure 4, Supplementary Figs. 10 and 11 and Supplementary Note) on human health through time and to record the genomic evolution of human commensals and pathogens.

## Methods

The ancient dental calculus sample details, including archaeological information, preparation methodology and authentication criteria, are described in Supplementary Note.

### DNA extraction, PCR, cloning and sequencing

We extracted DNA from 0.05–0.2 g of sterilized and powdered ancient dental calculus, and included a non-template control every three extractions. The ancient dental calculus deposits, modern calculus and plaque samples, and non-template controls were lysed in 1 ml of lysis buffer, containing 0.5 M EDTA (pH 8), SDS (10%) and Proteinase K (20 mg/ml). The samples and lysis buffer were rotated for 24 hours at 55 °C. Following sample lysis, DNA was isolated using the QIAamp DNA Investigator Kit (Qiagen). DNA was eluted to a final volume of 100 µl, and extracts were stored at 4 °C. The tooth samples were extracted using protocols described previously<sup>51</sup>. Independent extractions were not possible due to the small size of samples; commonly, only one calculus deposit per individual was available for DNA analysis.

PCR was used to amplify microbial DNA in the ancient dental calculus samples, modern oral samples, tooth samples and extraction controls using both universal 16S rRNA gene microbial primers and specific primers for the oral pathogens, *Streptococcus mutans* (GtfB gene) and *Porphyromonas gingivalis* (16S rRNA gene) (Supplementary Table 2). We also attempted (unsuccessfully) to amplify human mtDNA from the ancient dental calculus samples. For all primer sets, the PCR conditions were as follows; Amplitaq Gold (Applied Biosystems) at 2 U in 25  $\mu$ l volumes using 1 $\times$  Buffer Gold, 2.5 mM MgCl<sub>2</sub>, 0.25 mM of each dNTP (Fermentas), 400  $\mu$ M of each primer, 1 mg/ml RSA (Sigma-Aldrich), ShrimpDNase (Affymetrix) at 0.002 U/ $\mu$ l and 2  $\mu$ l of DNA extract. ShrimpDNase was used to remove microbial contamination from PCR reagents prior to the amplification reaction, and was added to the PCR mixture (minus the extract), incubated at 37 °C for 15 minutes, and then inactivated by heating the mixture to 65 °C for 15 minutes. For the specific primers, the thermocycling conditions consisted of an initial enzyme activation at 95 °C for 6 minutes, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and elongation at 72 °C for 30 s and a single final extension step at 60 °C for 10 minutes. Only 40 cycles were used to amplify 16S rRNA universal sequence, and the annealing temperature was 50 °C. Each set of PCRs included multiple extraction and PCR blanks. All PCRs were visually examined by electrophoresis on 3.5% agarose TBE gels. Specific PCR products were purified using 5  $\mu$ l of amplified product, exonuclease I (0.8 U/ $\mu$ l) and shrimp alkaline phosphatase (1 U/ $\mu$ l). The mixture was heated to 37 °C for 40 minutes and then heat inactivated at 80 °C for 10 minutes. The purified amplicons were sequenced bidirectionally using PCR primers and the BigDye Terminator 3.1 Kit (Applied Biosystems), according to the manufacturer's instructions. Sequencing products were purified using a Multiscreen<sub>HTS</sub> Vacuum Manifold (Millipore), according to the manufacturer's protocol. Sequencing products were separated on the 3130 $\times$ 1 Genetic Analyzer (Applied Biosystems), and the resulting sequences were edited using Sequencher (version 4.7).

We cloned the 16S rRNA gene universal amplicons to monitor contamination within the ancient samples and non-template controls and to assess the suitability of calculus samples for 454 sequencing. The PCR products were purified using Agencourt Ampure (Beckman Coulter) according to the manufacturer's instructions, and cloned using a StrataClone PCR cloning Kit (Stratagene). The clones were added directly to the colony PCR mix, which contained 10 $\times$  Hotmaster Buffer (Eppendorf), Hotmaster Taq 0.5 U/ $\mu$ l (5Prime), forward and reverse M13 primers (10  $\mu$ M) (Supplementary Table 2) in a 25  $\mu$ l reaction. The thermocycling conditions consisted of 94 °C for 10 minutes and 35 cycles of 94 °C for 20 s, 55 °C for 10 s and 65 °C for 45 s, with a single extension of 65 °C for 10 minutes. Colony PCRs were visually inspected on 2% agarose TBE gels. The colony PCR products were purified and sequenced using the same protocols as described for the products of the specific primers.

#### 454 GS FLX Titanium Sequencing

Pyrosequencing (GS-FLX Titanium) was used to examine 34 ancient dental calculus samples, 19 modern oral samples (six calculus and 13 plaque), six tooth samples and two extraction blanks. For the ancient calculus, modern oral samples and non-template controls, three hypervariable regions of the 16S rRNA gene (V1, V3 and V6) were amplified, using



the conditions described above. For the tooth samples, only the V3 region was amplified, again using the same conditions. The forward and reverse primers contained 454 Lib-L kit A- and B-adaptors, respectively, at the 5' end. The forward primer also contained sample-specific barcodes (Supplementary Table 3) that were developed by the Human Microbiome Project ([www.hmpdacc.org/tools\\_protocols/tools\\_protocols.php](http://www.hmpdacc.org/tools_protocols/tools_protocols.php)). The barcode sequences had not previously been used in either the Australian Centre for Ancient DNA or the Wellcome Trust Sanger Institute, where the 454 sequencing was performed. Hence, all sequences retrieved that did not contain a barcode were assumed to be contaminants and discarded. Each region of the 16S rRNA gene was amplified twice (on different days), and the duplicates were pooled for 454 sequencing to minimize the potential impact of preferential sequence amplification.

### Filtering, OTU picking, alignment and taxonomic assignment of 454 sequences

The sequences from the GS FLX Titanium platform were processed using the QIIME (version 1.5.0) software package<sup>52</sup>. Quality filtering was performed to remove sequences which were either under 60 bp (potential primer dimers), contained ambiguous bases, had primer or barcode mismatches, contained homopolymers which exceeded six bases or had an average quality score below 25. The remaining sequences ranged between 60 and 210 bp in length. The quality-filtered sequences were denoised<sup>53</sup> to remove sequences containing errors produced during the PCR (single base errors) and pyrosequencing, which resulted in the removal of ~ 50% of the sequences that were identified as having ambiguous flow data (Supplementary Table 4). However, we found that sequence classifications and beta diversity analyses were comparable between the dataset on which only quality-filtering had been performed and the de-noised dataset, as has previously been shown<sup>53</sup>. Similar sequences were binned into OTUs using optimal UCLUST<sup>54</sup> at a 95% likeness. Clustering is more commonly performed at 97%; however, a 95% cut-off has been found to classify OTUs more accurately for closely related, short sequences<sup>55</sup>. Representative sequences from each OTU were aligned using PyNAST<sup>52</sup> against the GreenGenes core set, with a minimum length of 60 bp and identity of 75%. PyNAST aligns the short GS FLX generated sequences (60-210 bp) against the full 16S rRNA gene. Columns which solely contained gaps were removed from the alignment prior to building phylogenetic trees. To overcome the difficulty in aligning highly variable 16S rRNA gene sequences, it is common to hide or lane-mask regions where at least 50% of the base composition is not conserved<sup>56</sup>. We did not hide variable regions because lane-masked alignments can 'mute' the phylogenetic diversity observed<sup>55</sup>. The gap-filtered sequences were taxonomically assigned using the RDP classifier and nomenclature<sup>57</sup>.

Detailed descriptions of the analyses performed on the ancient dental calculus, modern oral and extraction blank sequences are described in the Supplementary Note:  $\beta$ -diversity, alpha-diversity, Random Forest and discriminant analyses.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank N. Gully and S. Bent for critical discussions, J. Soubrier for bioinformatics assistance, the Australian Research Council, Wellcome Trust (WT092799MA and WT076964) and the Sir Mark Mitchell Foundation for funding support. We thank Harald Meller from the State Heritage museum of Saxony-Anhalt, Germany for prehistoric samples and members of the Australian Centre for Ancient DNA for practical help, and providing samples of plaque and calculus. We thank several anonymous reviewers whose comments have considerably improved the manuscript.

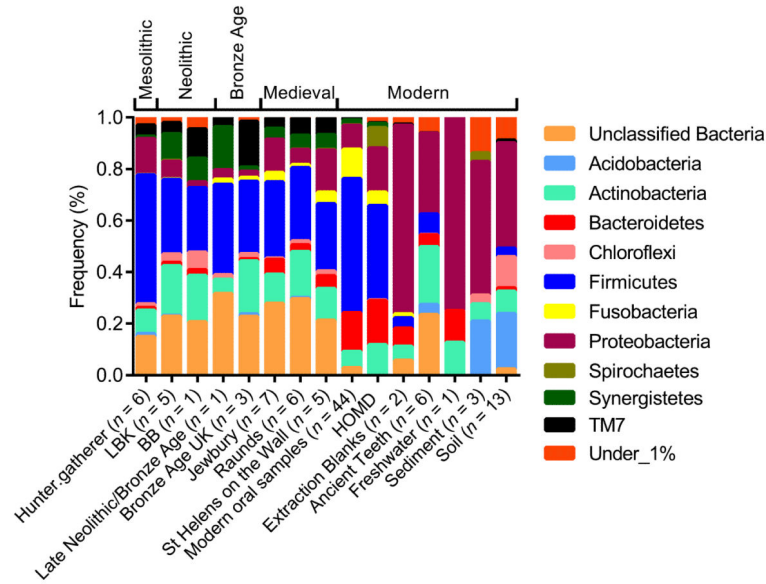
## References

1. Aas JA, et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. *J Clin Microbiol.* 2008; 46:1407–17. [PubMed: 18216213]
2. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol.* 2005; 43:5721–32. [PubMed: 16272510]
3. Dave S, Van Dyke T. The link between periodontal disease and cardiovascular disease is probably inflammation. *Oral Dis.* 2008; 14:95–101. [PubMed: 18302669]
4. Grossi SG, Genco RJ. Periodontal disease and diabetes mellitus: a two-way relationship. *Ann Periodontol.* 1998; 3:51–61. [PubMed: 9722690]
5. Structure, function and diversity of the healthy human microbiome. *Nature.* 2012; 486:207–14. [PubMed: 22699609]
6. Braidwood RJ, Howe B, Reed CA. The Iranian Prehistoric Project: New problems arise as more is learned of the first attempts at food production and settled village life. *Science.* 1961; 133:2008–10. [PubMed: 17754651]
7. Oelzea VM, et al. Early Neolithic diet and animal husbandry: stable isotope evidence from three Linearbandkeramik (LBK) sites in Central Germany. *Journal of Archaeological Science.* 2011; 38:270–279.
8. Childe, VG. *The dawn of European civilisation.* Kegan Paul; London: 1925.
9. Cordain L, et al. Origins and evolution of the Western diet: health implications for the 21st century. *Am J Clin Nutr.* 2005; 81:341–54. [PubMed: 15699220]
10. Savage DC. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol.* 1977; 31:107–33. [PubMed: 334036]
11. Scott GR, Poulson SR. Stable carbon and nitrogen isotopes of human dental calculus: a potentially new non-destructive proxy for paleodietary analysis. *Journal of Archaeological Science.* 2012; 39:1388–1393.
12. Lilley, J.; Stroud, G.; Brothwell, D. The Jewish burial ground at Jewbury. In: Addyman, PV.; Kinsler, VA., editors. *The Archaeology of York.* Vol. Vol. 12. Council for British Archaeology; York: 1994.
13. Preus HR, Marvik OJ, Selvig KA, Bennike P. Ancient bacterial DNA (aDNA) in dental calculus from archaeological human remains. *Journal of Archaeological Science.* 2011
14. Vandermeersch B, et al. Middle Palaeolithic dental Bacteria from Kebara, Israel. *C.R. Acad Sci. Paris.* 1994; 319:727–731.
15. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. *Periodontol 2000.* 2002; 28:12–55. [PubMed: 12013340]
16. Jin Y, Yip HK. Supragingival calculus: formation and control. *Crit Rev Oral Biol Med.* 2002; 13:426–41. [PubMed: 12393761]
17. Lieverse AR. Diet and the aetiology of dental calculus. *International Journal of Osteoarchaeology.* 1999; 9:219–232.
18. Asikainen S, Chen C, Slots J. Likelihood of transmitting *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in families with periodontitis. *Oral Microbiol Immunol.* 1996; 11:387–94. [PubMed: 9467371]
19. Van Steenberghe TJ, Menard C, Tjihof CJ, Mouton C, De Graaff J. Comparison of three molecular typing methods in studies of transmission of *Porphyromonas gingivalis*. *J Med Microbiol.* 1993; 39:416–21. [PubMed: 8246259]



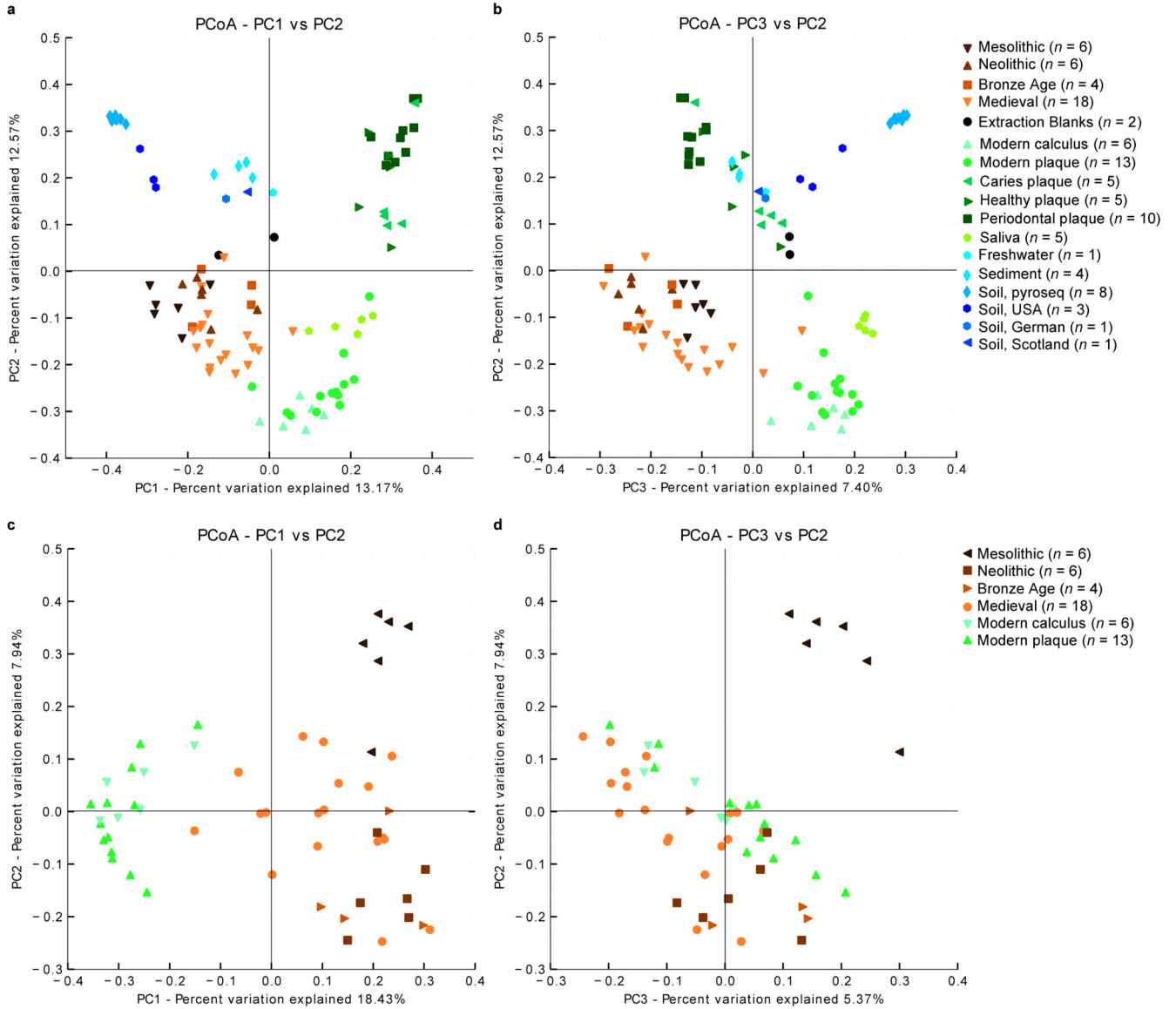
20. Aufderheide, AC.; Rodriguez-Martin, C.; Langsjoen, O. The Cambridge encyclopedia of human paleopathology. Cambridge University Press; Cambridge: 1998.
21. Faveri M, et al. Microbiological diversity of generalized aggressive periodontitis by 16S rRNA clonal analysis. *Oral Microbiol Immunol.* 2008; 23:112–8. [PubMed: 18279178]
22. Grine FE, Gwinnett AJ, Oaks JH. Early hominid dental pathology: interproximal caries in 1.5 million-year-old *Paranthropus robustus* from Swartkrans. *Arch Oral Biol.* 1990; 35:381–6. [PubMed: 2196866]
23. Marsh PD. Sugar, fluoride, pH and microbial homeostasis in dental plaque. *Proc Finn Dent Soc.* 1991; 87:515–25. [PubMed: 1775479]
24. Marsh PD. Are dental diseases examples of ecological catastrophes? *Microbiology.* 2003; 149:279–94. [PubMed: 12624191]
25. Hujoel P. Dietary carbohydrates and dental-systemic diseases. *J Dent Res.* 2009; 88:490–502. [PubMed: 19587153]
26. Marsh PD. Microbiology of dental plaque biofilms and their role in oral health and caries. *Dent Clin North Am.* 2010; 54:441–54. [PubMed: 20630188]
27. Petersen PE, Bourgeois D, Ogawa H, Estupinan-Day S, Ndiaye C. The global burden of oral diseases and risks to oral health. *Bull World Health Organ.* 2005; 83:661–9. [PubMed: 16211157]
28. Mercado FB, Marshall RI, Klestov AC, Bartold PM. Relationship between rheumatoid arthritis and periodontitis. *J Periodontol.* 2001; 72:779–87. [PubMed: 11453241]
29. Quince C, et al. Accurate determination of microbial diversity from 454 pyrosequencing data. *Nat Methods.* 2009; 6:639–41. [PubMed: 19668203]
30. Dewhirst FE, et al. The human oral microbiome. *J Bacteriol.* 2010; 192:5002–17. [PubMed: 20656903]
31. Lazarevic V, Whiteson K, Hernandez D, Francois P, Schrenzel J. Study of inter- and intra-individual variations in the salivary microbiota. *BMC Genomics.* 2010; 11:523. [PubMed: 20920195]
32. La Duc MT, Kern R, Venkateswaran K. Microbial monitoring of spacecraft and associated environments. *Microb Ecol.* 2004; 47:150–8. [PubMed: 14749906]
33. D'Costa VM, et al. Antibiotic resistance is ancient. *Nature.* 2011; 477:457–61. [PubMed: 21881561]
34. Beier S, Witzel KP, Marxsen J. Bacterial community composition in Central European running waters examined by temperature gradient gel electrophoresis and sequence analysis of 16S rRNA genes. *Appl Environ Microbiol.* 2008; 74:188–99. [PubMed: 18024682]
35. Schloss PD, Handelsman J. Toward a census of bacteria in soil. *PLoS Comput Biol.* 2006; 2:e92. [PubMed: 16848637]
36. Ellis RJ, Morgan P, Weightman AJ, Fry JC. Cultivation-dependent and - independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. *Appl Environ Microbiol.* 2003; 69:3223–30. [PubMed: 12788719]
37. Nogales B, et al. Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. *Appl Environ Microbiol.* 2001; 67:1874–84. [PubMed: 11282645]
38. Elshahed MS, et al. Novelty and uniqueness patterns of rare members of the soil biosphere. *Appl Environ Microbiol.* 2008; 74:5422–8. [PubMed: 18606799]
39. Tringe SG, et al. Comparative metagenomics of microbial communities. *Science.* 2005; 308:554–7. [PubMed: 15845853]
40. Will C, et al. Horizon-Specific Bacterial Community Composition of German Grassland Soils, as Revealed by Pyrosequencing-Based Analysis of 16S rRNA Genes. *Applied and Environmental Microbiology.* 2010; 76:6751–6759. [PubMed: 20729324]
41. Kerr NW. Prevalence and natural history of periodontal disease in prehistoric Scots (pre-900 AD). *J Periodontol Res.* 1998; 33:131–7. [PubMed: 9651874]
42. Albandar JM, Brunelle JA, Kingman A. Destructive periodontal disease in adults 30 years of age and older in the United States, 1988-1994. *J Periodontol.* 1999; 70:13–29. [PubMed: 10052767]

43. Bailey MT, et al. Stressor exposure disrupts commensal microbial populations in the intestines and leads to increased colonization by *Citrobacter rodentium*. *Infect Immun*. 2010; 78:1509–19. [PubMed: 20145094]
44. Lawley TD, et al. Antibiotic treatment of *clostridium difficile* carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. *Infect Immun*. 2009; 77:3661–9. [PubMed: 19564382]
45. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. *Nature*. 2012; 489:220–30. [PubMed: 22972295]
46. Cadotte M, Dinnage R, Tilman GD. Phylogenetic diversity promotes ecosystem stability. *Ecology*. 2012
47. Zhang Y, Chen HYH, Reich PB. Forest productivity increases with evenness, species richness and trait variation: a global meta-analysis. *Journal of Ecology*. 2012; 100:742–749.
48. Petchey O, Gaston K. Effects on ecosystem resilience of biodiversity, extinctions, and the structure of regional species pools. *Theoretical Ecology*. 2009; 2:177–187.
49. Loreau, M., et al. A new look at the relationship between diversity and stability. In: Loreau, M.; Naeem, S.; Inchausti, P., editors. *Biodiversity and Ecosystem Functioning. Synthesis and Perspectives*. Oxford University Press; Oxford: 2002. p. 79-91.
50. Faith DP. Conservation evaluation and phylogenetic diversity. *Biological Conservation*. 1992; 61:1–10.
51. Haak W, et al. Ancient DNA from European early neolithic farmers reveals their near eastern affinities. *PLoS Biol*. 2010; 8:e1000536. [PubMed: 21085689]
52. Caporaso JG, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*. 2010; 7:335–6. [PubMed: 20383131]
53. Reeder J, Knight R. Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. *Nat Methods*. 2010; 7:668–9. [PubMed: 20805793]
54. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. 2010; 26:2460–1. [PubMed: 20709691]
55. Schloss PD. The effects of alignment quality, distance calculation method, sequence filtering, and region on the analysis of 16S rRNA gene-based studies. *PLoS Comput Biol*. 2010; 6:e1000844. [PubMed: 20628621]
56. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol*. 1991; 173:697–703. [PubMed: 1987160]
57. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol*. 2007; 73:5261–7. [PubMed: 17586664]



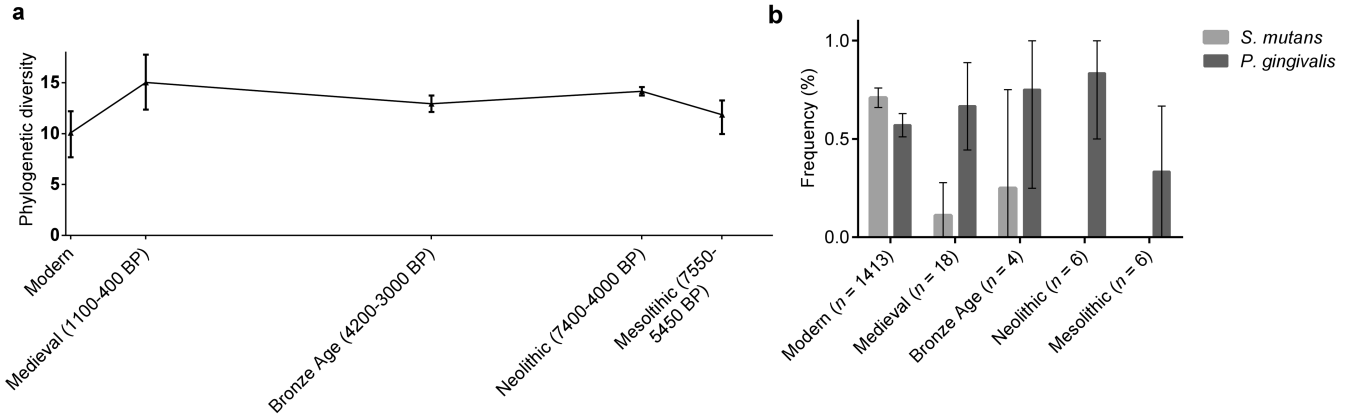
**Figure 1. Phylum level microbial composition of the ancient dental calculus deposits**

This is similar to modern oral samples, and distinct from non-template controls (EBC), ancient human teeth and environmental samples. The phylum frequencies for the V3 region are presented for the ancient calculus samples (LBK; Linear Pottery Culture, BB; Bell Beaker), modern oral samples, which included pyrosequenced (calculus, plaque and saliva<sup>31</sup>) and cloned (plaque<sup>1,2,21</sup>) data, non-template controls (or extraction blanks), ancient human teeth and environmental samples (freshwater, sediments and soils<sup>34-40</sup>) (Supplementary Table 1). Phylum frequencies from the HOMD were generated from partial and full-length sequences of the 16S rRNA gene. The phyla with a frequency < 1% include: ABY1\_OD1, AD3, Armatimonadetes, BRC1, CCM11b, Chlamydiae, Chlorobi, Cyanobacteria, Elusimicrobia, Euryarchaeota, Fibrobacteres, GAL15, Gemmatimonadetes, GN02, GN04, GOUTA4, KSB1, Lentisphaerae, NC10, Nitrospirae, NKB19, OP11, OP3, OP9, PAUC34f, Planctomycetes, SBR1093, SC3, SC4, SM2F11, SPAM, Spirochaetes, SR1, Tenericutes, Thermi, TM6, Verrucomicrobia, WPS-2, WS3 and ZB2.



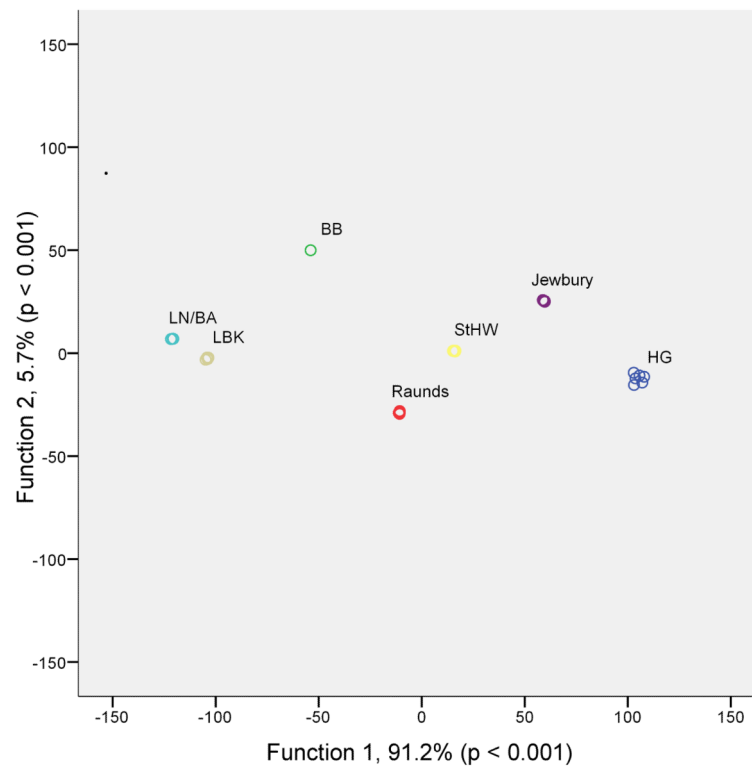
**Figure 2. Principal components plot of  $\beta$ -diversity**

PCoA reveals a close phylogenetic relationship between ancient dental calculus and modern oral samples, both of which are distinct from the non-template controls and environmental samples.  $\beta$ -diversity was calculated for all samples (Supplementary Note) using the UniFrac metric for the V3 region, and PCoA was applied to the unweighted, UniFrac distances. The plot of the first and second components (PC1 and PC2) (a) and the first and third components (PC1 and PC3) (b) of the PCoA clustered the ancient dental calculus samples with the modern oral pyrosequenced data (calculus, plaque and saliva), which were separated from the environmental samples and extraction blanks. A restricted PCoA plot of PC1 and PC2 (c) and PC1 and PC3 (d) that only includes ancient and modern oral pyrosequencing samples separated the hunter-gatherer (Mesolithic) individuals from modern, Medieval, and Neolithic samples.



**Figure 3. Changes in the diversity and composition of oral microbiota**

(a) For the V3 region sequences, we estimated the phylogenetic diversity<sup>50</sup> (Supplementary Note) of the archaeological dental calculus samples ( $n = 34$ ) and compared them to modern calculus ( $n = 6$ ) and plaque ( $n = 13$ ). We estimated phylogenetic diversity from only classified, Gram-positive bacterial sequences to minimize the influence of taphonomic bias (see Supplementary Note). Diversity was calculated at a depth of 34 sequences and bootstrapped to assess the robustness of the pattern. Error bars represent bootstrapped frequencies generated by sampling 255 replicates without replacement. (b) Specific primers were used to amplify sequences unique to the oral pathogens *S. mutans* (light) and *P. gingivalis* (dark).



**Figure 4. Discriminant analysis of  $\beta$ -diversity**

Discriminant analysis was applied to the principal coordinates generated from the unweighted UniFrac distances calculated from the V3 region sequences. Each individual is represented by a circle and coloured according to archaeological grouping (HG; Hunter-Gatherer, LBK; Linear Pottery Culture, BB; Bell Beaker, LN/BA; Late Neolithic/Bronze Age, StHW; St. Helen-on-the-Walls). The majority of phylogenetic variation (91.2%) was described by the first discriminant function, showing that individuals from the same archaeological groups cluster according to microbial composition



**Table 1**  
**Archaeological and anthropological samples used in the study.**

Sample ID	Museum#	Group/Culture	Period (years BP)	Location
12011	VI-1 B	Hunter-Gatherer	Mesolithic/para Neolithic (7550 - 5450)	Dudka, Poland
12012	VI-7 A	Hunter-Gatherer	Mesolithic/para Neolithic (7550 - 5450)	Dudka, Poland
12013	VI-14 A	Hunter-Gatherer	Mesolithic/para Neolithic (7550 - 5450)	Dudka, Poland
12015	VI-2 D	Hunter-Gatherer	Mesolithic/para Neolithic (7550 - 5450)	Dudka, Poland
12016	VI-1 A	Hunter-Gatherer	Mesolithic/para Neolithic (7550 - 5450)	Dudka, Poland
12017	VI-7 A	Hunter-Gatherer	Mesolithic/para Neolithic (7550 - 5450)	Dudka, Poland
8215	HK2000:4083a, 613.1	LBK	Neolithic (7400 – 6725)	Halberstadt-Sonntagsfeld, Germany
8240	HK2000:4228a, 861	LBK	Neolithic (7400 – 6725)	Halberstadt-Sonntagsfeld, Germany
8247	HK2000:4233a, 870	LBK	Neolithic (7400 – 6725)	Halberstadt-Sonntagsfeld, Germany
8275	HK2000:7374a, 1324	LBK	Neolithic (7400 – 6725)	Halberstadt-Sonntagsfeld, Germany
8277	HK2000:4014b, 413.1	LBK	Neolithic (7400 – 6725)	Halberstadt-Sonntagsfeld, Germany
4331	HK2004:9463a, 6255.1	Bell Beaker	Neolithic (4450 - 4000)	Quedlinburg XII, Germany
9436	HK, 43	LN/BA	LN/BA (4150 – 3600)	Benzingerode-Heimburg, Germany
8890	T82GF	Bronze Age	Bronze Age	Yorkshire, England
8891	14Barrow 163	Bronze Age	Bronze Age	Yorkshire, England
8894	T98	Bronze Age	Bronze Age	Yorkshire, England
8326	2095	Jewbury	Late Medieval (750 - 650)	York, England
8330	2106	Jewbury	Late Medieval (750 - 650)	York, England
8332	4440	Jewbury	Late Medieval (750 - 650)	York, England
8477	2357	Jewbury	Late Medieval (750 - 650)	York, England
8482	2654	Jewbury	Late Medieval (750 - 650)	York, England
8814	4161	Jewbury	Late Medieval (750 - 650)	York, England
8863	4485	Jewbury	Late Medieval (750 - 650)	York, England
8333	R5287	Raunds Furnells	Early Medieval (1100 – 850)	Northamptonshire, England
8335	R5252	Raunds Furnells	Early Medieval (1100 – 850)	Northamptonshire, England
8337	R5136	Raunds Furnells	Early Medieval (1100 – 850)	Northamptonshire, England
8341	R5206	Raunds Furnells	Early Medieval (1100 – 850)	Northamptonshire, England
8868	R5157	Raunds Furnells	Early Medieval (1100 – 850)	Northamptonshire, England
8869	R5229	Raunds Furnells	Early Medieval (1100 – 850)	Northamptonshire, England
8873	5228	StHW	Late Medieval (1000 - 400)	York, England
8874	5241	StHW	Late Medieval (1000 - 400)	York, England
8877	5113	StHW	Late Medieval (1000 - 400)	York, England
8878	5203	StHW	Late Medieval (1000 - 400)	York, England
8883	5244	StHW	Late Medieval (1000 - 400)	York, England
1	NA	European Descent	0	Adelaide, Australia
2	NA	European Descent	0	Adelaide, Australia
3	NA	European Descent	0	Adelaide, Australia
4	NA	European Descent	0	Adelaide, Australia
5	NA	European Descent	0	Adelaide, Australia

Sample ID	Museum#	Group/Culture	Period (years BP)	Location
6	NA	European Descent	0	Adelaide, Australia
7	NA	European Descent	0	Adelaide, Australia
8	NA	European Descent	0	Adelaide, Australia
9	NA	European Descent	0	Adelaide, Australia
10	NA	European Descent	0	Adelaide, Australia

Abbreviations: LBK - Linear Pottery Culture; LN/BA - Late Neolithic/early Bronze Age; StHW - St. Helen-on-the-Walls. Mesolithic/para-Neolithic is the terminology used for the transitional cultures of the forest zone of Eastern Europe. See Supplementary Table 1 for further information about the ancient calculus, and modern plaque and calculus samples.