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Title:
Dental calculus and the evolution of the human oral microbiome

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Abstract
Characterizing the evolution of the oral microbiome is a challenging, but increasingly feasible, task. Recently, dental calculus has been shown to preserve ancient biomolecules from the oral microbiota, host tissues, and diet for tens of thousands of years. As such, it provides a unique window into the ancestral oral microbiome. This article reviews recent advancements in ancient dental calculus research and emerging insights into the evolution and ecology of the human oral microbiome.

Introduction
The human oral cavity is home to a diverse ecology of microorganisms, collectively known as the oral microbiome. Composed of more than six hundred taxa[1], the oral microbiota plays a central role in dental health, and increasingly it has been shown to influence extra-oral organs and tissues, as well as general health[2]. Investigating the evolution of this rich microbial ecology is challenging, but it is critical for understanding the origins and prevalence of oral and systemic disorders as diverse as dental caries, periodontal disease, rheumatoid arthritis, cardiovascular disease, respiratory illness, and a range of infectious diseases.

Research on what constitutes a healthy or normal oral microbiome has expanded dramatically over the past decade[3], in large part due to the advent of high-throughput DNA sequencing[4, 5] and major public funding initiatives, such as the National Institutes of Health's Human Microbiome Project (HMP)[6]. However, the fact that the oral microbiome will cause dental disease in a majority of individuals during their lifetimes has been argued to suggest that even the "healthy" or "normal" oral microbiome today is already in an altered dysbiotic state[7-9], and likely has been for some time[10-12].

To better understand the oral microbiome and its associations to both dental disease and other so-called "diseases of civilization," it is useful to investigate how our vulnerability to disease is related to human evolutionary history. Evolutionary medicine, a field that integrates both

medicine and evolutionary biology, provides a framework for rethinking conventional models of oral health and disease[13], and biological anthropology, a field that encompasses primatology, paleoanthropology, bioarchaeology, and human biology, provides a context in which to characterize the ancestral state of the human microbiome[14]. Together, these fields provide pathways to better understanding the oral microbiome, and ultimately to restoring and better maintaining oral health in the future.

One of the most exciting recent discoveries in biological anthropology has come from an unlikely quarter—dental calculus (Figure 1). Since at least the 1980s, it has been known that archaeological dental calculus contains preserved cellular structures of oral bacteria[15-17], but it was only recently discovered that it is also a robust and long-term reservoir of well-preserved DNA and proteins[17-20]. Advances in ancient DNA and paleoprotein technologies now allow detailed characterization of these ancient biomolecules, enabling direct comparisons between ancient and modern oral microbial communities[18, 20]. Host proteins preserved within ancient dental calculus additionally provide insights into past microbial virulence and host immune response[20], and food particles entrapped within dental calculus offer unprecedented insights into the diets of past populations[20-23].

This article provides an overview of recent ancient dental calculus research and discusses four areas in which the investigation of ancient oral microbiota is poised to make significant contributions to an evolutionary perspective on human health and disease: 1) dental caries; 2) periodontal disease, rheumatoid arthritis, and cardiovascular disease; 3) respiratory infections and meningitis; and 4) major infectious diseases.

Dental calculus and ancient biomolecules

Dental calculus (tartar) is a complex, mineralized bacterial biofilm that forms from dental plaque on the surfaces of teeth[24, 25]. It is found in all known past and present human populations, and it is nearly ubiquitous among adults without active dental hygiene[17]. The amount of dental calculus buildup on the dentition varies widely among populations[26, 27] and may result from a complex combination of factors related to subsistence, dietary abrasiveness, dental hygiene practices, and genetic predisposition. However, prior to modern dentistry it is not uncommon to observe heavy calculus deposits in excess of 100 mg, especially from post-Neolithic periods (Figure 1). Importantly for archaeology, dental calculus preserves over time at least as well as bone and dentine, and it has even been found on the teeth of Neanderthals[28] and australopithocenes[29], in addition to humans. Among primates, the oldest known calculus to date was reported on the dentition of a Miocene orangutan ancestor and dates to roughly twelve million years ago[30] (Figure 2). Dental calculus is thus noteworthy for its availability in the fossil record throughout the entirety of human evolution.

Dental calculus formation occurs when dental plaque undergoes periodic mineralization events, although the timing and triggers for this process are not well understood[17]. During mineralization, calcium phosphate ions from saliva and gingival crevicular fluid precipitate within the dental plaque matrix, at once killing the microbiota and calcifying the microbial cells and other debris *in situ* (Figure 3a). Organic structures preserve well in this environment (Figure 3b), and bacterial

cell walls with functional cell surface protein epitopes, as well as delicate dietary microfossils such as starch granules, have been found to remain intact for more than 10,000 years[28, 31]. Genetic material also survives within dental calculus (Figure 3c), in part because the hydroxyapatite mineral within dental calculus strongly binds DNA[17]. Genetic analyses of archaeological dental calculus have found that it typically contains 10-1,000-fold more DNA than bone or dentine from the same individual, making it the richest source of ancient DNA yet identified in the archaeological record[17].

A number of recent studies have explored the potential of dental calculus to reconstruct aspects of ancient health and disease (Figure 2) and have shown it to be an area of great potential. Made possible by dramatic advances in ancient DNA[32] and paleoprotein[33] technologies over the past decade (Figure 4), these studies have uncovered specific aspects of individuals' health states[18-20], diets[20, 23, 28], ancestry[34], and even craft activities[35] using a combination of genetic, proteomic, and microscopic analyses of dental calculus. This type of specificity and the ability to address issues that typically leave no lasting mark in the macro-archeological record provide opportunities to address questions that were previously thought to be unanswerable. By using these new and emerging techniques, dental calculus can be used to address fundamental questions about the evolution of human oral health.

Oral microbiota and disease—targets for discovery

Dental caries

Dental diseases, such as caries and periodontal disease, are among the most prevalent diseases affecting industrialized societies[7]. In the 1960s, the average number of decayed, missing, and filled teeth (DMFT index) among 12-year-old children in Western Europe was greater than 5, and by age 15 the average DMFT exceeded 10[36]. Thus, a European child reaching adulthood in the mid-20th century could expect one third of their dentition to be compromised by dental decay. From the 19th century to the mid-20th century, dental decay was perceived as so inevitable that complete dental extraction and replacement with dentures became a popular gift for young women and brides in Western Europe and parts of North America[37-39]. Extensive dental public health interventions and the introduction of fluoridated dentrifices over the past 50 years have significantly reduced caries rates[36], but even today dental caries affect more than 40% of children and 90% of adults in the United States[40].

Dental caries are easily observed in the archaeological record, and they have been systematically studied for more than a century[41]. Extensive data have now been collected on paleoanthropological and archaeological populations around the world, spanning time periods from the Pleistocene[42] to the 19th century[43]. It is clear from these studies that diet is the major driver of caries frequency[12]. Although caries are observed during all time periods, and are in fact also present in non-human primates[44, 45], caries frequencies vary remarkably through time and space. The most salient patterns relate to the transition between foraging (hunting and gathering) and agriculture[46], and later to the widespread availability of refined flour and sugar, especially sucrose, during the 18th century[41].

Numerous studies have documented increases in caries frequency with the onset of agriculture[12, 41]. The Eastern Woodlands of North America is perhaps the most intensively studied region, and analysis of caries frequency at 180 sites reveals that during the 2,000 years preceding the introduction of maize agriculture the percentage of teeth affected by dental caries was approximately 2-5%. After the introduction of maize ca. 500 CE, caries frequencies increased steadily from 14% during the Late Woodland period to 18% during the late Prehistoric period to 22% during the early Contact period[12] (Figure 5). Although not all regions saw such sharp increases, a general trend toward increased caries frequency in agricultural populations is observed globally[12, 41], with relatively few exceptions[47-50].

The origin of today's extreme levels of dental decay, however, lies not with agriculture but with the introduction of refined flours and sugars during the Early Modern period (ca. 1450-1800 CE; Figure 2). In Western societies, tooth decay was so severe during this time that smiling is all but absent from European and American portraiture of the period[51]. Since then, consumption of refined carbohydrates has been met with increasingly aggressive oral hygiene regiments and prophylactic dental care as necessary measures to prevent premature tooth loss[24]. The need to continuously engage in such behavior has been argued to be a sign that even the "healthy" oral microbiome today is in an altered state of dysbiosis[7, 11].

One question that emerges with these observations is to what degree the oral microbiota of these populations changed in step with subsistence practices. Recent research on the gut microbiome has identified major changes in microbial ecology associated with foraging, agricultural, and industrial lifestyles[52], but less is known about the oral microbiome of traditional societies[53, 54]. A recent study of ancient oral microbial communities spanning the past 8,000 years reported evidence for slight, phylum-level microbial shifts correlated with the onset of agriculture and industrialization[18]; deeper sequencing and a larger sample size in future studies is anticipated to clarify these associations and provide greater taxonomic resolution during these transitions.

Direct investigation of ancient carious lesions using ancient DNA techniques has identified the presence of the cariopathogen *Streptococcus mutans*[55], but reconstructing the full polymicrobial community contributing to such lesions[56] is challenging because dentine is highly susceptible to postmortem alteration by environmental microbes[20]. By contrast, dental calculus is much better preserved[20]. Genetic sequences from *S. mutans* have been detected in the dental calculus of diverse populations[18-20], but interestingly it has not been detected in samples prior to the Bronze Age (ca. 2200-1000 BCE)[18] (Figure 2). Recent phylogenetic analysis of modern *S. mutans* strains has estimated that *S. mutans* underwent an exponential expansion ca. 10,000 years ago (95% confidence interval: 3,268–14,344 years ago), suggesting an association with the onset of agriculture in the Near East[57]. Future ancient DNA investigations of dental calculus using whole-genome capture enrichment technologies[32] may be able to reveal the natural history of *S. mutans* and determine the major drivers of its evolution and functional role within the oral cavity of humans.

Periodontal disease, rheumatoid arthritis, and cardiovascular disease

Periodontal diseases (gingivitis and periodontitis) affect up to 90% of the worldwide population[9], and moderate to severe chronic periodontitis is estimated to affect 13% of US adults over age 30[58], although new diagnostic criteria suggest that this may be a gross underestimate[59]. In addition to dental morbidities, periodontitis is also associated with increased risk of a wide range of so-called "diseases of civilization," including type II diabetes, obesity, rheumatoid arthritis, cardiovascular disease, stroke, and pulmonary disease[9, 60-65]. This association may reflect a partially causal relationship. For example, *Porphyromonas gingivalis*, an oral bacterium strongly associated with periodontitis[66], has recently been implicated in the development and progression of rheumatoid arthritis[67, 68], and treatment of periodontitis has been found to alleviate rheumatoid arthritis symptoms[69, 70]. Periodontitis is also associated with a 19% increase in the risk of cardiovascular disease[71], and a recent study of cardiovascular specimens found that >80% of diseased heart valves and >90% of aortic aneurisms have been infected with cariogenic or pathogenic periodontal bacteria[62].

Although there is ample evidence for periodontitis in the archaeological record, periodontal diseases are very poorly studied. The most widely used laboratory guide for osteological analysis, *Standards for Data Collection from Human Skeletal Remains*[72], contains recording metrics for age estimation from dental development and wear, enamel hypoplasia measurement, caries scoring, and dental calculus quantification, but no information on how to record or measure periodontal disease in archaeological dentitions. As a result, it is rarely noted in archaeological reports. When periodontal disease is mentioned, the descriptions are usually qualitative and limited to small case reports[12]. At present there have been no large-scale, systematic studies of periodontal disease prevalence in the past, leaving many gaps in our understanding of the antiquity, prevalence, and significance of periodontal disease in human evolutionary history.

Although clinical metrics[73] are difficult to apply to archaeological specimens, molecular characterization of periodontal disease is increasingly feasible. Recent conceptual changes in periodontology over the past fifty years now point to disrupted microbial communities and host-mediated inflammatory tissue destruction as the proximate causes of periodontal disease[74, 75], and new molecular approaches, such as metagenomics and metaproteomics, allow culture-free investigation and comparison of oral microbiota and host immune response from both clinical and archaeological dental samples (Figure 2). For example, a recent study of medieval dental calculus found that periopathogens common today were also associated with suspected periodontal disease cases nearly a thousand years ago[20]. Frequencies of the periodontal pathogens *P. gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Filifactor alocis* were found to be highly elevated in dental calculus collected from archaeological dentitions with generalized moderate or severe attachment loss. Additionally, virulence factors expressed by these taxa and host innate immune proteins were found at high abundance among the proteins recovered from these samples, strongly suggesting periodontitis-associated inflammation. In dental calculus from one individual, genetic sequences for *T. forsythia* were so abundant that a near complete ancient genome for this pathogen could be reconstructed. The ancient strain was found to harbor the same fourteen virulence proteins found in modern *T. forsythia*, but it lacked several mobile elements, including a putative antibiotic resistance gene, tetQ, found today in the *T. forsythia* reference strain.

Two additional studies have begun to look at temporal changes in periodontal pathogens. In a study of ancient Chilean and Argentinian dental calculus, *P. gingivalis* was detected in most time periods from 2500 BCE to the present[19], and in a study of ancient Europeans, *P. gingivalis* was detected in all major periods dating back to the Mesolithic, ca. 5550 BCE–3450 BCE[18] (Figure 2). Interestingly, the latter study found that *P. gingivalis* frequencies were lower in Mesolithic hunter-gatherers than in post-Neolithic farmers, a finding consistent with observations that hunter-gatherer societies in the recent past and today appear to have lower rates of periodontal disease than agricultural and industrialized societies[11, 76]. However, further research is necessary to ensure that this finding is not simply an artifact of more advanced DNA decay in older samples[77].

The investigation of *P. gingivalis* in archaeological dental calculus may also have implications for the study of rheumatoid arthritis. In 1990, rheumatologist Bruce Rothschild proposed a radical hypothesis—that rheumatoid arthritis is a vector-transmitted infectious disease that originated in central North America and spread to the Old World after European contact in the region ca. 1750[78, 79]. Among the evidence he assembled, he noted that the first documented case of rheumatoid arthritis in Europe dates to 1800, while cases in North America are known from archaeological remains up to 6,500 years old, and that Native Americans today have unusually high rates of the autoimmune disease (more than 5-fold higher than the U.S. general population[80]). Although aspects of this hypothesis are now quite dated[81], the recent link made between rheumatoid arthritis and *P. gingivalis*[67, 68] has rekindled interest in a possible microbial origin for this disease. By using ancient DNA from dental calculus along with osteological data, it may be possible to unravel the historic biogeography of virulent *P. gingivalis* strains and their relationship to autoimmunity.

Finally, the recent link made between cardiovascular disease and periodontal and cariogenic taxa[82] raises questions as to the antiquity of oral involvement in atherosclerotic plaques. Cardiovascular disease is conventionally viewed as a disease of modernity, but archaeological evidence now confirms its presence in diverse ancient cultures, from the elites of ancient empires to prehistoric hunter-gatherers. Calcific atherosclerosis has been identified, first by autopsy and later by x-ray and computed tomography (CT), in the coronary and peripheral arteries of mummies originating from ancient cultures in Egypt, the Alps, Peru, the American Southwest, the Aleutian Islands, and Korea[83, 84]. Moreover, radiological scans suggest that the prevalence of atherosclerosis was relatively high in prehistory, and, in the case of Egypt, no significant difference was found in the incidence or prevalence of atherosclerotic plaques between today and 3,500 years ago[85].

A collaboration of cardiologists, radiologists, molecular biologists, and archaeologists known as the Horus Team has been at the forefront of ancient atherosclerosis research since 2008[83]. Because many of the risk factors associated with cardiovascular disease today do not apply to ancient populations, they have proposed that the high rates of atherosclerosis in the past may have resulted from chronic systemic inflammation caused by long-term infection by gastrointestinal parasites or repeated exposure to microbial or viral pathogens[83, 86]; however, in the absence of direct evidence for such infections, they note that other yet-to-be discovered risk factors may also play a role. Poor oral health may be just such a risk factor. Untreated caries, heavy calculus

deposits, and alveolar recession suggest both dysbiotic oral microbial communities and sustained inflammation were prevalent in many ancient populations. A promising future direction in this research would be to genetically test ancient calcific atherosclerotic plaques directly for the presence of oral taxa. Systematic CT scanning has identified atherosclerotic plaques in more than fifty ancient mummies from around the world, and biopsies of these plaques could be analyzed and compared to microbial profiles generated from dental calculus collected from the same individuals. Taxonomic matches would be strong evidence for a long-term role of oral involvement in the initiation of cardiovascular disease.

Respiratory infections and meningitis

The oral microbiome is the natural reservoir for a large number of pathobionts (endogenous potential pathogens), including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pyogenes*, *Corynebacterium diphtheriae, Bordetella pertussis*, and *Neisseria meningitidis*[1], that cause a wide range of acute and potentially life-threatening respiratory and meningeal infections, especially in children. Importantly, asymptomatic carriage of these microbes is relatively prevalent, although it varies widely among populations. For example, reported carriage rates of *S. pneumonia* range from 1% in parts of Scandinavia to more than 80% in parts of France and Gambia, while *H. influenzae* carriage rates range from 3% in Sweden to 88% in Costa Rica, and carriage rates for *M. catarrhalis*, a common cause of middle ear infections, range from 2% in parts of Sweden to 82% in parts of the Netherlands[87]. In Europe, *N. meningitidis* is typically carried by an average of 24% of teenagers and 8-13% of adults[88], and nearly one fifth of American schoolchildren carry *S. pyogenes* at any given time[89]. For pathogens with widespread vaccine programs, such as *Corynebacterium diphtheriae* and *Bordetella pertussis*, carriage rates are generally low[90-94], but were presumably once much higher. In addition to these pathobionts, even commensal oral taxa pose serious health risks among the elderly and immunocompromised. Aspiration of common oropharyngeal taxa can result in aspiration pneumonia[95, 96], the leading cause of death and the second most common cause of hospitalization among nursing home patients[96]. Additionally, it is common for the elderly to acquire extra-oral pathobionts, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, in their dental plaque, which also contribute to aspiration pneumonia.[2]

To date, multiple pathobionts have been identified in archaeological dental calculus, including *S. pneumoniae*, *H. influenzae*, *S. pyogenes*, *C. diphtheriae*, and *N. meningitidis*[20]. Additionally, genetic sequences consistent with *Bordetella parapertussis*, an organism responsible for persistent cough in children that is similar to pertussis[97], and several species of *Moraxella* were also identified. These pathobionts were detected in the dental calculus of two individuals excavated from the medieval St. Petri cemetery in Dalheim, Germany (ca. 1100 CE). Although there is no evidence that these organisms were causing disease in these individuals, they nevertheless provide the first direct evidence of respiratory pathobiont carriage in the oral cavity before the 20th century[20].

Only a century ago, respiratory infections were the leading causes of death in the U.S.[98], and studies of periosteal rib lesions in skeletal collections[99] and soft tissue changes in South American mummies[100-102] suggest that pneumonia and/or other respiratory infections were a major cause of

human mortality in prehistory[99]. The discovery that dental calculus is a reliable source of genetic material from common respiratory pathobionts opens up the possibility of conducting epidemiological studies of past carriage rates, and also presents the opportunity to investigate the evolution of these taxa through time.

Dental calculus may also help to date the origin of infectious diseases that have recently emerged from the oral cavity, such as bacterial meningitis and gonorrhea. Phylogenetic studies of *Neisseria*, a genus of bacteria that colonize mucosal surfaces in animals, indicate that *N. meningitidis* and *N. gonorrhoeae* belong to a recently diverged pathogenic clade in humans that is most closely related to the common nasopharynx commensal *Neisseria lactamica*[103-105]; however, the timing and context of this divergence are not well understood. Both *N. meningitidis* and *N. gonorrhoaeae* are obligate human taxa, indicating that this divergence must have occurred since the chimp-human split approximately 6 million years ago, and whole-genome comparisons with other *Neisseria* species suggest that *N. meningitidis* may have undergone a population bottleneck and acquired its virulence genes for polysaccharide capsule synthesis very recently, perhaps only a few centuries ago[105]. Non-specific endocranial meningeal reactions are often found in skeletons[106], and with the recent identification of putative *N. meningitidis* genetic sequences in archaeological dental calculus, this hypothesis has become testable. Future ancient DNA investigations using whole-genome capture enrichment technologies[32] hold great promise for resolving the origins and evolution of *N. meningitidis*, a pathobiont whose mortality rate from bacterial meningitis and septicemia continues to exceed 10% even in developed nations[104].

Major infectious diseases

Finally, although not true members of the oral microbiome, several opportunistic and obligate pathogens can be found transiently within dental plaque, buccal mucosa, and saliva[1]. These include the causative agents of tuberculosis (*Mycobacterium tuberculosis*), leprosy (*Mycobacterium leprae*), plague (Yersinia pestis), syphilis (*Treponema pallidum*), gastritis (*Helicobacter pylori*), and smallpox (Variola virus), among others. *M. tuberculosis,* for example, is present in sputum and regularly comes into contact with the oral cavity throughout the entire course of the disease[107]. In addition to sputum, *M. tuberculosis*, has also been detected in 92% of dental plaque samples from infected patients using PCR-based techniques[108]. Leprosy involves the oral cavity in up to 60% of cases, and multiple oral structures may develop lesions and ulcers, including the hard and soft palate, the gingiva, tongue, lips, and buccal mucosa[109, 110]. During outbreaks of bubonic plague, *Y. pestis* that escapes the lymphatic system and infects the lungs causes pneumonic plague, a highly infectious form of the disease that results in lethal fulminant pneumonia[111]. *T. pallidum* is known to cause oral lesions during the secondary phase of syphilis infection, which may last for many years[112], and this provides ample opportunity for passive or active incorporation and preservation in calcifying dental plaque biofilms. Finally, *H. pylori* is readily found in the saliva and dental plaque of infected individuals[113], and smallpox causes oropharyngeal lesions[114].

Infectious diseases have played a major role in shaping human history, and many pathogens continue to present serious challenges to public health. Little is known, however, about the origins or evolutionary history of most human infectious agents. Ancient DNA research has

contributed greatly to what is known about the origins of a handful of pathogens, including *M. tuberculosis*[115, 116], *Yersinia pestis*[117-119], and *H. pylori*[120], and it has been used to confirm the presence of several additional pathogens in ancient infections, including *M. leprosy*[121], *T. pallidum*[122], and variola virus[123].

However, genetic detection rates for most ancient pathogens are low, even in remains with overt and relatively diagnostic paleopathology indicators. Antemortem tissue destruction likely enhances postmortem decay, which may contribute to poor preservation of pathogen DNA within infected bone, and examples of well-preserved soft tissue are relatively rare outside of a few geographic regions. Dental calculus presents a promising alternative for screening ancient skeletons for infectious pathogens. Because dental calculus calcifies during life, it does not undergo the same decomposition processes as the rest of the body, and it is nearly ubiquitous in skeletal collections[124]. DNA within dental calculus has been shown to preserve well over long time scales, and because it forms incrementally, serially entrapping microbes and debris from discrete periods of time[17], it may even be possible to retrieve pathogen DNA from individuals who survived disease events and were no longer infected at the time of death. This may make it possible to trace the evolutionary history of several diseases that have proven difficult to study using bone samples, including treponemal diseases such as venereal syphilis[125], which is of particular importance given its historical and clinical significance[125], as well as its past intractability to ancient DNA analysis[126, 127].

Although none of the above pathogens have yet been identified from archaeological dental calculus, the fact that so many infectious agents transiently inhabit the oral cavity during disease progression makes future detection of pathogens from dental calculus at least plausible. If successful, such analyses could greatly expand our understanding of human pathogen evolution.

Conclusion

The incorporation of genetic material from commensal, pathobiont, and pathogenic microorganisms into dental plaque, and later dental calculus, presents a rare opportunity to study the evolution of the human oral microbiome and associated diseases in archaeological skeletal collections spanning thousands of years. Great progress has already been made in developing the tools and technologies necessary to extract genomic and proteomic information from ancient dental calculus, and clinical research on the oral microbiome is laying the theoretical foundations for making this information relevant in today's dental practices and hospitals. Through collaborations between oral health science and ancient dental calculus research we can leverage knowledge of the ancestral oral microbiome to improve human health today.

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Figure Legends

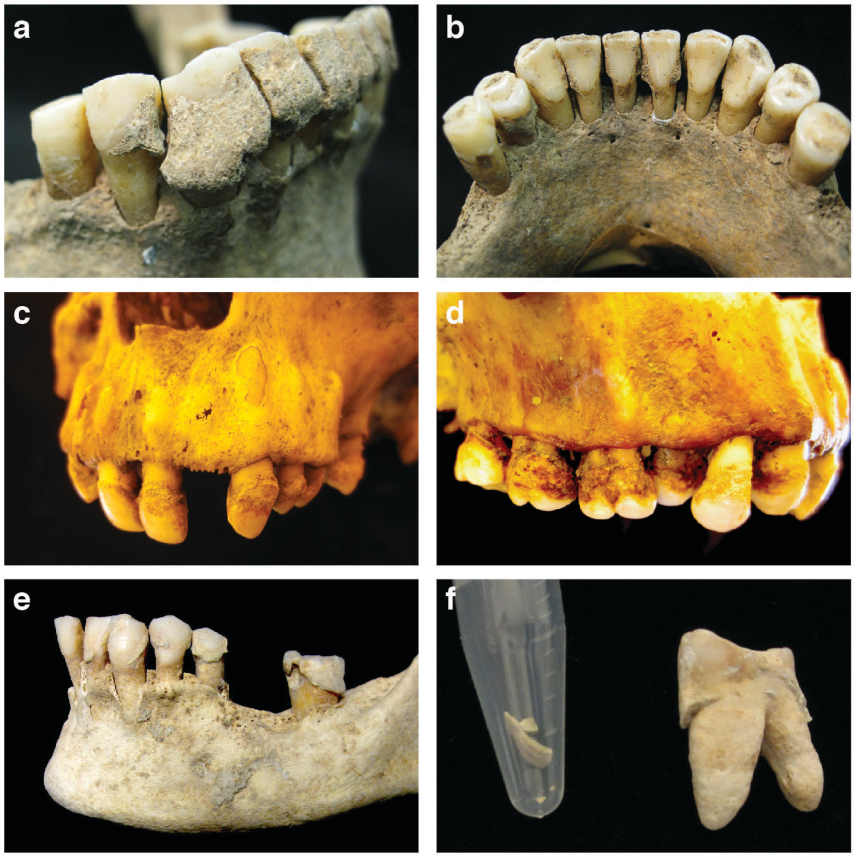

Figure 1. Archaeological dental calculus. (**a-b**) St. Petri cemetery, Dalheim, Germany, ca. 1100 CE. (**c-d**) Sky cave tomb, Samdzong, Nepal, ca. 500 CE. (**e-f**) Mortuary cave, Camino del Molino, Spain, ca. 2500 BCE. The amount of dental calculus typically required for analysis is shown in (**f**). Dental calculus from all three sites has yielded genetic data suitable for ancient microbiome analysis[20, 77].

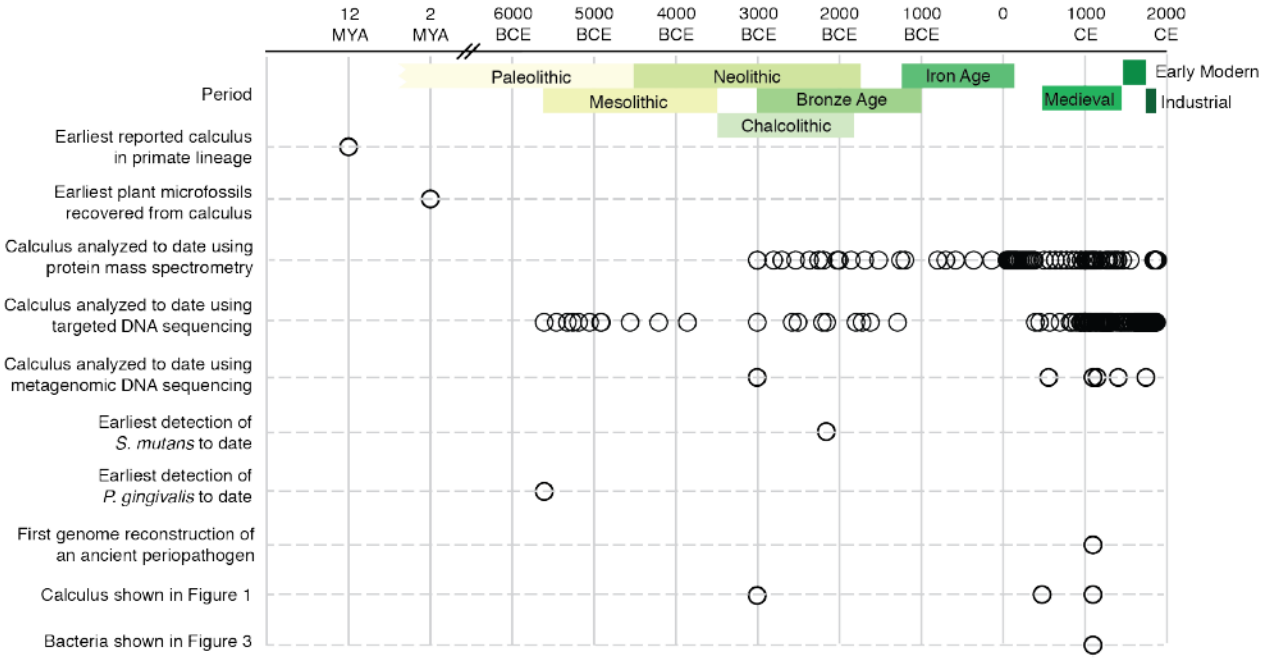

Figure 2. Idealized chronology of European prehistory and timeline of major ancient dental calculus findings to date. Includes data from Europe[18, 20, 23, 77], Asia[23, 30, 77], Africa[23, 29, 77], North America[34, 77], and South America[19]. Note that regional European chronologies differ, with major periods typically beginning earlier in southeastern Europe and later in northwestern Europe. African, Asian, and New World chronologies are not shown.

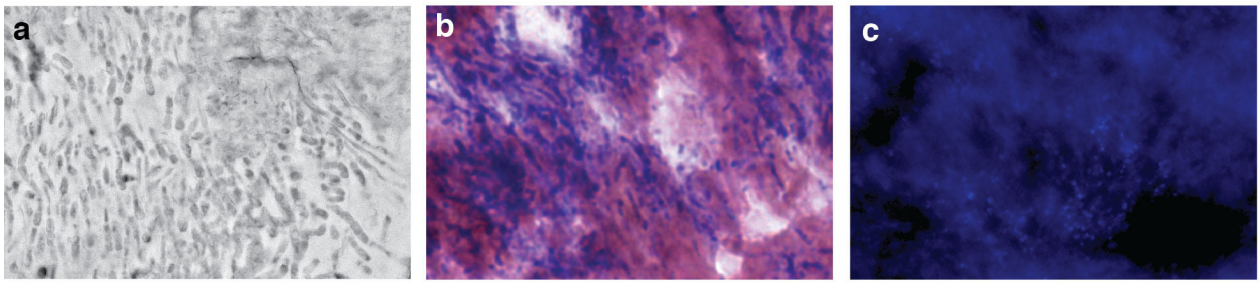

Figure 3. Micrographs of ancient bacteria embedded within archaeological dental calculus dating to the medieval period, ca. 1100 CE[20]. (**a**) Numerous calcified bacterial cells are visible within dental calculus using scanning electron microscopy (SEM) with backscattered electron imaging. (**b**) Staining of decalcified dental calculus thin sections reveals the presence of both Gram-positive and Gram-negative bacterial cells with relatively intact cell walls. (**c**) Hoechst stain, a blue fluorescent dye that binds double-stranded DNA, reveals the presence of abundant genetic material.

Figure 4. Extraction of genetic material from archaeological dental calculus requires highly specialized ancient DNA laboratories to prevent contamination from modern sources.

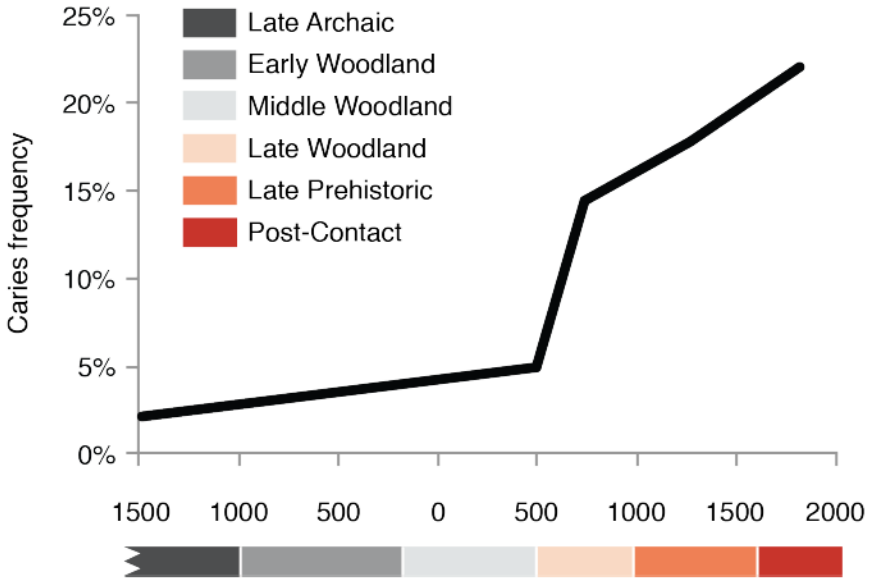

Figure 5. Dental caries frequency in prehistoric Eastern Woodland populations of North America. A dramatic increase in caries frequency is associated with the adoption of maize agriculture ca. 500 CE[12].

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