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# ORIGINAL ARTICLE

# Anthropological Significance of dental calculus deposits as proxy identity signatures of the host and oral microbiota: A scoping review

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## Abstract

Dental calculus is a creamish-yellow to brownish-black hard crust deposited on teeth, and having preserved the dietary microremains, biomolecules, oral microbes and secretions deposited over a long period of time. It has been widely used as a valuable material in the anthropological, archaeological, microbiological and forensic research work. The microbial and host DNA extracted and sequenced from the dental calculus deposits (DCD) may help researchers of diverse scientific disciplines to establish identify of the unknowns, entrapment of drugs and the infectious agents in past or contemporary human populations. The archaeological dental calculus can contribute in reconstruction of dietary habits, food practices and disease status of past individuals, thus help in attributing the geographical and occupational affinity of ancient skeletal remains. Advancing dental calculus analysis through validation studies, technological innovations, interdisciplinary collaborations, longitudinal research, and ethical considerations holds promise for its robust forensic applications. The current status of anthropological, archaeological and microbial research involving dental calculus deposits and its forensic importance have been presented in a nutshell in this article.

**Keywords:** Forensic anthropology, Dental Calculus, Dietary and disease Status, Oral Microbiome, ancient DNA

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## BACKGROUND

Oral health status of past populations has been a matter of interest for anthropologists and archaeologists; particularly based on macroscopic observations (Wasterlain et al., 2011). Human life and the state of health or oral disease can be traced in dental calculus better than in other fossil substrates precisely as calcification of the diverse bacterial biofilm structurally preserves and protects the bacterial cells from multiple external factors (Willmann et al., 2018). Identification of unknown human remains and/or missing persons is the most challenging task for the global forensic experts. Teeth and bones serve as excellent reservoirs of biomaterials eligible for human identification; the former are the strongest ones, commonly found well preserved in forensic anthropological contexts (Raj et al 2013). Dental calculus is a is densely mineralized microbial biofilm preserving dietary contents (vegetal, animal or aquatic), oral microbes and oral secretions (like saliva and gingival crevicular fluid), deposited over a long period of time (Hardy et al 2009, Buckly et al 2014, Gismondi et al 2018). It is a creamish-yellow to brownish-black hard crust deposited on inner surface of tooth or the dental prosthesis which can be collected non-invasively from the jaw fragments. The host’s oral microbiome and the micro-debris circulating in oral cavity buildup and get preserved as a tightly adhered calcified dental plaque called dental calculus deposits (DCD) during the routine lifetime of an individual; particularly due to the poor oral hygiene, periodontitis or dental crowing (Singh and Goel, 2017, Fons-Badals et al 2020). Dental calculus undergoes periodic but continuous mineralization to incorporate diverse, stable and well-preserved microbial, toxic and dietary (fibers, phytoliths, pollen, fat) microremains in it (Putrino et al 2024). DCD harbors past human oral microbiota that can provide deep insights into the decedents' lifestyle, diet, health, disease, possible geolocation, ecology and even socio-cultural affinity of an individual; thus presenting a more holistic ante- mortem profile of the unidentified individuals, even from its very minute quantities (Mackie et al 2017, Blatt et al 2022). Dental calculus serves as vital bio-material in situations where excessively decomposed or badly degraded, damaged and fragmented human remains of disturbed skeletal conformity are retrieved from forensic anthropological contexts. Dental calculus stands as a compelling biological material with significant forensic implications, especially in identification of severely degraded human remains (Figure 1).


**Figure 1: Anthropological applications of dental calculus**

The genomic and forensic experts are well acquainted with the benefits of analyzing calculus, conveniently collected from the living or recently dead individuals or even skeletonized archaeological human remains (Singh and Goel, 2017). Studying molecular markers from the dental calculus is crucially needed for profiling human remains of historical, museum, or forensic importance as it contains DNA in sufficient quantity of identification purposes (Lisman et al., 2023). Anthropological and molecular examinations of calculus can help in attributing biological, cultural, geographical identity of unknown human remains, in addition to revealing the microbial and host-DNA diversity. Calculus can complement or supplement the traditional forensic anthropological findings, particularly in instances where preservation of other tissues is compromised. Dental calculus may contain up to 1000 times more DNA than the bone of the same person, thus it has huge potential to revolutionize the ancient biomedical research, to be use as an alternative source material of ancient human DNA when other skeletal tissues don't yield (Warinner et al 2014, Forshaw, 2022).Dental calculus serves as an adjuvant and non- invasive evidence in forensic human identifications by establishing human-host DNA profile, living conditions, behaviour, lifestyle, dietary status etc. of the decedent (Blatt et al 2022, Bonczarowska et al 2022).

Calculus provides a clean, stable, durable and hospitable environment for the microbial communities, food and non-food particles and thus, serves as a viable source of forensic identifications based on autosomal and Y-STR markers extracted from it (Sawafuji et al 2020, Lisman et al., 2023). The gingival secretions, shed epithelial cells, macrophages and oral inflammatory processes are the probable sources of DNA incorporation into dental calculus deposits (Warinner et al 2015, Mann et al 2018); having relatively low quantity and fragmented DNA (highest concentration found in subgingival plaque). The highly degraded and challenged samples generally contain low amounts of DNA (with PCR inhibitors), which presents a very tough and challenging task for their forensic identifications. Majority of available dental calculus research is primarily focused on estimating dietary habits, oral microbiota and mtDNA haplogroup affiliations of archaeological human remains (Black et al 2011, Damle 2016). The quality of DNA isolated from calculus significantly differ from that obtained from the teeth; dentine DNA is more degraded and damaged than the fragmented and shorter length but more stable and less challenged DNA obtained from dental calculus. The comparison of DNA yields from the paired dental calculus and dentine samples from the same tooth of an individual has identified dental calculus as the richest known source of ancient biomolecules in human hard tissues (Willmann et al 2018, Li et al 2022). As calculus DNA can provide only preliminary identity profile of the contaminated samples (Lisman et al., 2023); it can be more useful than the bones in identification strategies of such human remains. Human DNA embedded in calculus hydroxyapatite is comparatively well preserved, to be used as a potential investigative tool for not only for forensic purposes but also as a valuable tool in microbiological research (Higgins and Austin, 2013). The quantity of dental calculus collected from dentition (ideally 20 mg) is crucial as comparatively higher amounts of calculus provide better DNA amplification results (Lisman et al., 2023). Very limited published research work is available about the extraction and analysis of human DNA content from dental calculus for forensic anthropological purposes.

Accumulation of dental calculus is a considered a serious oral health problem by the periodontists, so calculus deposits are generally removed and disposed of as a clinical waste material. Dental calculus deposition below the cervical line may be the indicative of periodontitis or other non-infectious diseases which, in turn, may have diverse forensic or bio-archaeological interpretations (Muro and Cucina, 2024). The rough and porous surface of the calculus deposits promote bacterial deposition and bone degeneration, which makes it beyond control even with standard hygiene measures. Building-up of calculus deposits usually compromises oral hygiene by promoting increments in pathogenic plaque, resulting into excessive destruction of periodontal tissues to further enhance the risks of systemic diseases like diabetes (Preshaw et al 2012, Mealey and Klokkevold, 2019). The formation and accumulation of dental calculus varies between teeth and among individuals.; maxillary molars and mandibular incisors being more prone to supra-lingual calculus (White, 1997).The food and non-food micro-particles like starch granules, pathogens, parasites, pollens, diatoms, seeds, vegetal fibers, cereals, phytoliths, the accidently entrapped small insects, etc., found preserved in dental calculus matrix can be examined with the help of optical microscopy (OM), scanning electron microscopy (SEM) or energy-dispersive x-ray spectroscopy (EDXR) to provide information about different identity components of an individual (Dobney and Brothwell 1986, 1988, Lieverse 1999, Henry et al 2011, De la Fuente et al 2012, Radini et al., 2017; Strömberg et al., 2018, Fotakis et al 2020, Godoy Allende and Samplonius 2022). Quantifying traces of nicotine alkaloids in calculus using Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC- MS) can help in better understanding the consumption of such intoxicants by smokers (Eerkens et al 2018). For forensic identification purposes, the supragingival calculus is preferred to the sublingual one as the latter is heavily mineralized, contaminated with gingival haemorrhigal components and is tightly anchored to the teeth.

The microscopic examination of calculus deposits is highly useful in providing decisive data for the provenance and establishing their forensic or medico-legal identity. Dental calculus may be taken as an ectopic growth which can replace the destructive analysis of human skeletal remains. The cultural identity, habits (smoking/drugs/tooth-brushing), occupation hazards (painting, mining, farming), disease or pollution exposures, COVID-19 infections etc., of an individual can be objectively assessed from the trace elemental composition analysis of dental calculus (Bergstrom 1999, Radini et al 2017, Yaprak et al 2017, Eerkens et al 2018, Sørensen et al 2021, Berton et al 2021, Li et al 2022). The phytoliths and plant fibers entrapped in dental calculus can help in identification of eaten plant sources, season or environment in which they grew, travel routes and burial geolocation of unknown individuals (Blatt et al 2011). It can also serve as possible geographical and occupational marker of identity of an individual. The pollens, hair and plant debris are the frequent inclusions in human dental calculus (Dobney and Brothwell 1986, 1988). The particles embedded in calculus can help in identifying occupationists like metal-workers, carpenters, and other artisans. Heavy metals in calculus of production workers has been identified as occupational exposure marker (Abdazimov, 1991). Researches have mentioned that drug residues are better entrapped in the interior of the calculus material than in the blood; few drugs are found in comparatively higher concentrations in dental calculus (Sørensen et al, 2021). Thus, the manner of death, work-related intoxication or individualized habits can be reconstructed from toxicological analysis of calculus (Charlier et al, 2010). It is the rough and porous structure and unique mineralized status of calculus that strongly embodied DNA within it and limits the acidic or exogenous microbial attacks on DNA preserved in hardened matrix of calculus (Warinner et al 2014; Mann et al 2018). Among the typical sources of endogenous DNA, the saliva/oral fluids, proteins and mucosal/epithelial cells are found commonly embedded in dental calculus, endorsing the possibilities of isolating mtDNA from the dental calculus.

**Forensic Anthropological Significance of Dental Calculus**

Calculus is routinely removed to maintain sound dental health, it has been recently recognized as an informative material to understand ancient diet and health in forensic archaeological and anthropological sciences (Forshaw 2022). Dentition of all known (ancient or contemporary) populations have calculus and its prevalence varies widely depending upon the age, sex, ethnicity, systemic health, dietary and dental hygiene status of the individuals. Developing dental calculus is an oral health concern but its scientific analyses have significant repercussions in anthropology and forensics. The quantum of information that can be decoded from the material and molecular evidence found entrapped and preserved in calculus is vast and ever expanding. The entrapped cellular or tissue fragments can help in identification of ancient human remains, reconstitution of dietary and occupational habits, idiosyncratic nutritional status and sometimes, the manner of death/pathologies and for this, the classical optical microscope (OM), scanning electron microscopy (SEM) or elemental surface analysis are best technique for such examinations (Dobney and Brothwell, 1988). Dental calculus obtained from archaeological human samples has been found a rich reservoir of ancient host biomolecules and human DNA (mtDNA and nuclear DNA) (Warinner et al 2015, Singh and Goel 2017, Mann et al 2018, Lisman et al., 2023).The host mt-DNA and bacterial DNA, along with micro-particles entrapped in calculus matrix, can be successfully extracted and analyzed using advanced bio-molecular, spectroscopic or microscopic methods which, in turn, have revolutionized our understanding about the identity, diets, ancestry, occupations, migration patterns, disease, pollutant/metallic exposures, health and environmental conditions of past individuals and the populations. The molecular and chemical analysis of dental calculus can help understand the intricate dynamic relationship existing between humans and their microbes (Warinner et al 2015). The recent improvements in genome sequencing strategies, bioinformatics armamentarium, laboratory workflow designs and contamination controls have significantly enhanced the potential of ancient microbial research (Gilbert et al 2005, MacLean et al 2009, Kaczynski et al 2012, Weyrich et al 2015).

The consumption of plant-based pharmaceutical or psychoactive substances/products and other stimulants is common in modern as well as ancient times, particularly for treatment of some medical conditions (Fiorin et al 2018, Gismondi et al 2018, Godoy Allende and Samplonius 2022). Sørensen et al (2021) reported that such inclusions, entrapped and preserved in dental calculus, can be detected with the help of a new, sensitive and ultra-high performance LC-TMS (Liquid Chromatography-Tandem Mass Spectrometry) technique from the modern as well ancient forensic toxicological samples. Dental calculus, as a non-invasive biological material, can also be helpful for monitoring betel-nut chewing or tobacco smoking (containing numerous heavy metal) exposure to the human oral cavity (Yaprak et al 2017, Zhang et al 2019). Many instances have been reported in literature when the craft and trade related bye-products like stone crystals, cellulose fibers or ceramics have been reportedly found preserved in dental calculus to provide information about craft and trade practices in ancient populations worldwide (Blatt et al 2011, Coccato et al 2017). Supragingival dental calculus can be used for monitoring heavy metal (like cadmium, mercury) exposures to oral cavity and thus assessing the risks of heavy metal poisoning in oral cancers (Zhang et al 2019; Charlier 2013) with the application of various analytical techniques like TEM (McDougall 1985).

Calculus is a calcified form of oral microbial plaque biofilm and is a rich source of host and oral microbiome DNA (Brealey et al 2020). Reconstructing life-histories of our recent past ancestors is of prime importance in anthropological research work. As human oral microbiota is both culture and geography specific, so the genetic mutations in ancient microbial DNA help in tracking human migrations that happened in past populations, highlighting the potential ability of microbiota DNA as genetic signal of cultural affinity of pre-historic populations (Dominguez-Bello and Blaser 2011, Eisenhofer et al 2019). The changes in human oral microbiome signify changes in dietary patterns over time, indicating a marked increase/decrease in oral pathologies and dental calculus. Recovery of plant food debris from archaeological dental calculus can help in reconstruction ofpast food practices.

**Deciphering Oral Microbiota from Dental Calculus**

Dental Calculus has been widely used as a tool in microbial forensics to estimate microbial health and the oral hygiene of the deceased. The comparative analysis of DCD from ancient and modern dentition can help understand the issues related to the abundance and diffusion of different microorganisms in the environments of the past or contemporary biospheres (Brundin et al., 2013, Weiß et al., 2020). The microbial cells constitute about 70% of total dry weight of calculus, highlighting that majority of calculus DNA is microbial in nature (Human Microbiome Project Consortium 2012, Aas et al 2015, Warinner et al 2014, Ottoni et al 2021). The constantly evolving microbes have helped in laboratory studies of bacterial evolution which, in turn, has increased our information about the dynamics of evolution, adaptive changes, and respond to queries impacting human health (Bonczarowska et al 2022). About 70% dry weight of dental calculus is constituted by microbial cells representing the host’s plaque containing his/her lifetime oral microflora (Aas et al 2005, Dewhirst et al 2010, Velsko et al 2019, Kazarina et al 2021). The dental calculus harbors past human oral microbiota that can provide deep insights into decedents' lifestyle, diet, health, disease, possible geolocation, environmental conditions and the cultural affiliation etc., of an individual; thus presenting a more comprehensive ante-mortem profile of the unknown ones (Blatt et al 2022), even from its minute quantities (Mackie et al 2017). Ancient oral microbiota is considerably more heterogeneous than the modern one and this heterogeneity of ancient oral microbiome composition can offer more insights into human evolution (Jersie-Christensen et al 2018) as good oral hygiene discourages calculus formation (2022). Application of advanced genetic techniques to detect microbial communities in ancient skeletal remains has revolutionized the paleopathological research towards pathogenic evolution and identification from profiling of human oral microbiome (Bos et al 2019, Arning and Wilson, 2020, Li et al 2022, Naud et al 2022). The extraction and sequencing of ancient microbial DNA and its comparison with contemporary microbial strains has significant relevance to practice of modern medicine and dentistry for understanding host-microbiome interactions (Akcalı and Lang, 2018).

Human oral microbiome research based on dental calculus and transitions in oral microbiota have significantly contributed in understanding the functional diversity of microbial ecologies, oral diseases, interactions between humans, microbes and the abiotic factors etc., during life-time of an individual (Warinner et al 2015). It is the mineralization process of calculus happening during life that makes it resistant to environmental contaminations and let the microbial community get preserved in it (Jin and Yip, 2002). The temporal and spatial variations in oral microbiota can provide crucial information not only to the forensic anthropologists and archaeologists, but also to medical researchers like microbiologists, and the evolutionary biologists. Microbial evolution provided deep insights into major changes in human condition like diseases, epidemiological transitions, biogeographic range expansions, colonialism and industrialization (Warinner et al 2015, Blatt et al 2022). Studying DNA from prehistoric bacteria or viruses may lead to the discovery of techniques and approaches to fight contemporary strains/forms of such infectious agents. Recent studies have necessitated that for ancient DNA analysis of current microbial diseases, the information about the past genotypic and phenotypic signatures of the responsible bacteria needs to be collected. Thus, the crucial knowledge about the past oral microbiome may also help medical professionals and physicians use ancient microbial DNA signatures to track/identify such infectious agents in modern day populations.

**Archaeological Reconstructions from Dental Calculus**

Dental calculus collected from ancient human remains can be analyzed from anthropological, molecular, elemental, toxicological/chemical and forensic perspectives, to assess its potential utility for forensic anthropological purposes in identification of the host and the oral microbiota with the following objectives:

- To assess the microbial health and oral hygiene of an individual from the microbiota DNA extracted and sequenced from dental calculus
- To assess the utility of dental calculus for screening dietary status, food diversity, heavy metal and drug of abuse exposures in ancient and modern northwest Indian individuals from its micro-particle, elemental and stable isotope analyses.
- The importance of dental calculus as potential source of geographical and occupational identity of anindividual.
- Dental calculus as supplemental source of human DNA
- Assessment of changes in oral microbiota, dietary constituents and environmental exposures by comparative analyses of dental calculus collected from ancient and contemporary samples.

**Detection of drugs and related metabolites in calculus**

Though dietary reconstructions from calculus have been widely studied, relatively fewer studies have considered the deposition of pharmaceutical or phototherapeutic residues in DCD (Hardy et al 2012, 2018, Gismondi et al 2020). Direct oral ingestion of substances, inhalation of smoke/vapor, and/or the release of cellular or serum residuals into the saliva and gingival crevicular fluid may facilitate entrapment and preservation of a large variety of pharmaceutical and psychoactive drugs in dental calculus. The variations in entrapment and deposition of these things in calculus from different individuals may depend upon differences in the diet, health, local pH, salivary flow etc. and lastly the stability of the substances entrapped in the calculus (Sorensen et al 2021). Use of medicinal plants in ancient times has been deciphered from chemical indicators in archaeological dental calculus samples (Hardy et al 2009, Gismondi et al 2018). Smoking or chewing of tobacco (nicotine) or any other product having skewed concentrations of toxic heavy metals like arsenic, mercury, silicon, lead or cadmium have been revealed from archaeological calculus (Yaprak et al 2017, Eerkens et al 2018, Zhang et al 2018, 2019); ICP-MS analysis of dental calculus being a novel non-invasive technique for analysis of environmental exposure to heavy metals (Zhang et al 2018). Drug residues of cocaine, heroin, 6-monoacetylmorphine (6-MAM) and tetra-hydrocannabinolic acid A (THCA-A) have been found trapped in the interior of the modern calculus material (Sorensen et al 2021). Revealing smoking/chewing habits, use of pharmaceutical products, illicit psychoactive drugs/stimulants and their metabolites in dental calculus is a crucial forensic archaeological inquiry. In post-mortem cases, calculus may serve as a valuable adjunct to the existing analyses where traditional investigations don't provide any leads. Saliva have higher affinity to accumulate basic drugs in higher concentrations than the blood owing to generally lower pH of the former.

**Methodologies commonly used for sampling and preparation of DCD**

Dental calculus can be collected from ancient and modern teeth/jaw samples from archaeological samples and individuals coming to dental clinics for their routine dental check-up, cleansing and screening. The detailed nutritional, demographic and disease history of an individuals can be recorded by following the dietary-recall method. The collection, sampling, preparation and analysis of the dental calculus is done according to the standardized suggested protocols.

**(i) Sample collection and Preparation**

Each subject having will be looked for presence of dental calculus, particularly supra-gingival calculus on the lingual or buccal surfaces of mandibular as well as maxillary teeth. Sufficient quantity of dental calculus can be easily removed from teeth without scratching or scraping tooth surfaces (Scout and Poulson, 2012). Collecting calculus samples from different teeth is preferred for obtaining sufficient representative data for testing and comparisons. The location and severity of calculus is to be photographed prior to collection. Two 3-5 mm long fragments of supra-lingual calculus are generally removed (particularly from 2 to 3 maxillary molars or mandibular incisors) using sterile or decontaminated hand or ultrasonic scaler (White 1997, Velsko et al 2019). Then the fragments are stored in aluminum foil or sterile box without any conservative agent. Now the collected samples are cleaned with non-contaminating plastic fine scrapers and a delicate vaporization of air pressured is maintained for about 10 minutes. A very fine fragmentation of each sample is done before transferring the calculus to a 1.5mL sterile tube for further analyses. Dental calculus has no architecture of its own, so fragmentation doesn't cause loss of any information to be gleaned from it as it allows better penetration of the fixative substances. In order to rehydrate and fix the samples, the calculus fragments are immersed in 20% diluted acetic acid for 48 hours for slow decalcification of the DCD fragments. The scaler needs to be cleansed in-between samples collection using 5% NaOCl and rinsing with distilled water. Use of gold covering must be avoided as it contaminates elemental analysis of the calculus.

**(ii) Dental Calculus Examination Protocols**

Various morphoscopical, optical, chemical/elemental/biochemical and molecular methods have been suggestedto examine dental calculus for forensic or osteo-archaeological or anthropological purposes.

**a. Micro-particle examination**

The entrapping and preservation of the cellular fragments, starch granules and other inclusions in dental calculus are examined by its SEM and OM analysis using the standardized protocols like those of Henry and Piperano (2008), Charlier et al (2010) and Power et (2015) which involve the microscopic examinations coupled with surface elemental analyzer. Optical microscope will also be used for micro-particle analysis of some calculus samples. The prepared DCD samples mounted on microscope slides, and added with half drop of glycerol are examined under optical or a standardized and cross-polarized light microscope to examine all the micro-remains embedded in calculus samples. Scanning Electron Microscope (SEM) examination of each sample directly performed on the sample under an energy-dispersive X-ray spectroscope and the images are stored using a standard resolution digital frame store.

**b. Cytological Analysis**

The cytological analysis of the DCD in suspension starts with the sampling of 200 lL from the supernatant. This liquid is then centrifuged to obtain two spots per slide. Four slides are produced by sample: two slides are colored by the technique of Papanicolaou after a fixing of the spots with a lacquer; the two other slides are colored by the technique of the May–Grünwald–Giemsa (MGG) after fixing of the spots to the air.

The study of the remaining solid phase (base of centrifugation) is carried out after a new centrifugation (3500 turns per minute for 10 min). The supernatant is kept in reserve (for a later possible cytological study) while the base is recuperated then fixed 24 h in the AFA (acetic acid, formaldehyde and alcohol). Soon after fixation and decalcification, the sample is put in cassette on a foam, followed by the traditional circuit of inclusion (dehydration in xylene and increasing alcohol baths, then inclusion in liquid paraffin, cooling, section with the microtome (from 6 to 10 lm), deposit on an albumenized slide, air-drying free, dewaxing and rehydration by immersion in xylene then in increasing alcohol baths to distilled water). Four colorations are carried out for each sample: periodic acid Schiff (PAS), Gram, toluidine blue and hematoxylin–eosin-saffron (HES) (Henry and Piperano 2008, Charlier et al., 2010, Power et al., 2015).

**c. Elemental concentrations and Stable isotope composition**

Dosseto et al (2024) reported that measuring Sr isotopes in fossil calculus using (Thermo-Fisher Neptune Plus MC ICP-MS) helped in correctly identifying the composition of soil where remains are buried, and not that at the time of calculus formation, thus cannot help in estimating the geographical origin of human remains found in forensic contexts. For elemental and isotopic analysis, firstly, the calculus fragments are rinsed with the distilled and de-ionized water, dried at 50°C and then powdered in a steel mortar and pestle. Following the protocol outlined in Scott and Poulson (2012) for sample preparation and experimentation, stable isotope analysis of calculus is done. The elemental and isotopic concentrations are measured using elemental analyser and stable isotope ratio mass spectrometer (IRMS), respectively. The measured isotopic concentrations are reported in units of ‰ in the usual notation vs. VPDB for carbon, and vs. air for nitrogen. NIST and IAEA standards are used as a standard for 13C, 15N, weight % C, and 0.02% for weight % N analyses. The samples having C: N ranging from -2.8 to 3.6 are considered for dietary analyses as the isotopic composition of dental calculus is considered to be the isotopic composition of the diet (Cheung et al., 2017)

**d. Ancient DNA extraction, sequencing and reconstruction of mitochondrial genomes**

The DNA extraction, its purification, quantification amplification, DNA Library preparation, target enrichment, sequencing (amplicon sequencing and next generation sequencing) from dental calculus in accordance with the standard/established contamination control protocols, precautions and workflows is crucially important. Prior to processing for DNA extraction, all calculus fragments are to be decontaminated with UV irradiation for 1-2 minutes. The crushed calculus powder is washed in 1 ml 0.5 M EDTA for 15 minutes to remove loosely-bound contaminants. This step is repeated before incubation overnight at room temperature. Then add a 100 µl proteinase K solution to the samples and incubate at about 400° C for 5-6 hours, followed by continued digestion under agitation at room temperature until decalcification is complete. Refresh the digestion buffer solution for dentine samples after 48 h and combine the two supernatants for subsequent analyses. After digestion, extract DNA by previously established phenol-chloroform separation protocol and purify by silica adsorption. Elute the DNA into 30 µl of EB buffer and quantify 1 µl of each extract using a Qubit High Sensitivity dsDNA assay. The extracted DNA is indexed into libraries using the NEBNext DNA Library Prep Master set for 454. The blunt end adapters are prepared and used for ligation at a concentration of 0.5 lM in a final volume of 50 ml. The Bst Polymerase fill-in reaction is inactivated after incubation by heating to 808C for 20 min and then freezing overnight

The Next Generation Sequencing (NGS) strategies like Amplicon and Shotgun Metagenomics are used to characterize oral microbiome from the collected dental calculus deposits. Amplicon sequencing of microbial DNA are done to focus on one or more of the nine variable regions of the 16S rRNA gene to characterize the taxonomic structure and diversity of the extracted microbiome. Shotgun metagenomics is used amplify and sequence a subset of total DNA in the sample as it is free from primer bias and is potentially the most informative genetic approach to microbiome identification (Cappellini et al 2014). Precautions regarding primer bias, shorter base-pair length are considered while interpreting and comparing amplicon sequencing data of oral microbiome. The sequence data is analyzed using bioinformatics tools to reconstruct mitochondrial genomes and study the composition of oral microbiome community.

## CURRENT RESEARCH STATUS

Until recently, the research potential of calculus was not recognized as it is usually considered as a discarded material. Dental calculus has been, archeologically and forensically, overlooked by forensic genetics community. However, it is now fully understood that dental calculus has ample anthropological, medical and forensic significance as it stores valuable information in the form of micro-particles and biomolecules embedded within its hardened rough matrix. The presence of dental calculus in the human remains has remained the subject matter of recent forensic anthropological and osteo-archeological research work (Charlier 2013) as it serves as a rich source of host-associated biomolecules; ingested/inhaled (intentionally or accidentally) during routine daily life of an individual (Radini et al 2017). Most of calculus research is restricted to document its quantity and location on teeth, preservation of microorganisms in it or its clinical and pathological significance in archaeological skeletons (Brothwell 1981, Dobney and Brothwell, 1988). It has immensely helped in revealing human prehistory, dietary variations and shifts, and disease signatures from ancient oral microbiome analysis of past populations (Hansen et al 1991, Dobney 1994). The dental calculus gleaned from ancient and modern dental remains have been widely used for studying the composition and profiling of oral microbiota, remnant food debris, disease microbes entrapped within calcium phosphate mineral salts of saliva and dental plaque (Henry and Piperno, 2008; Henry et al., 2011; Warinner et al., 2014). The entrapped red blood cells, vegetal fibers, crystals, mineral salts in calculus deposits can be analyzed with multiple scientific (cytological, histological, chemical, molecular and optical) techniques. Sophisticated analytical and biochemical methods have been used to understand the composition of dental calculus and it is the porosity capacity of DCD which makes it ideal for variety of research.

It is a sturdy and rigid material due to mineralization that occurs on supra- and/or sub-lingual surfaces of teeth throughout the life time. Supra-gingival calculus is primarily anchored to the mandibular lingual sides of anterior teeth and also to the buccal surfaces of maxillary molars whereas the sub-gingival calculus can be found in entire dentition, specifically on proximal tooth surfaces. Calculus is formed due to the assimilation of mineral salts from the salivary and gingival fluids in the plaque and restricted both between and on the teeth surfaces. In addition to mineral components, DCD houses a variety of inorganic and organic remnants of bacterial, salivary and dietary origin, incorporated either during mineralization or post-calcification the quite porous calculus. The plant remnants in the form of phytoliths and starch grains have potential utility in estimating ancient dietary habits as well as population-level food variations. The starchy foods constitute 50-70% of energy intake in modern as well as pre-agriculturist human diets (Hardy et al 2009). The sophisticated dietary regime of Neanderthals, primarily based on plant-based foods, has been revealed through dental calculus (Henry et al 2011).

Formation and presence of dental calculus is highly population-specific; prevalence frequency being largely determined by the oral hygiene status (Anerud et al. 1991, Blank et al 1994, MacPherson et al 1995), age (Beiswanger et al 1989, Anerud et al. 1991, MacPherson et al 1995), sex (Beiswanger et al 1989, Anerud et al. 1991), ethnicity, diet (Schroeder 1969, Bhat 1991), dental cares (Beiswanger et al 1989, Anerud et al. 1991, Blank et al 1994, MacPherson et al 1995), systemic diseases (Emrich et al 1991), medicine intakes (Turesky et al 1992, Breuer et al 1996), education level etc., of the individual. In non-westernized groups, calculus formation starts soon after tooth eruption and continues to a maximum at age of 30 years (White, 1997) but no such thresholds are available for the westernized world.

Mann et al., (2018) found that DNA obtained from dental calculus was consistently more abundant and less contaminated than DNA extracted from the dentin. Most of DNA obtained from calculus is of microbial in origin, derived from the entrapped oral microbial communities in it. proposed the identification of The bonafide microbial taxa can be profiled and compared from identification of microbiomes in the ancient or historical dental calculus samples (Weiß et al., 2020). Dagli et al., (2015) identified dental calculus as one of the rich sources of ancient microbial DNA from the microbial colonies entrapped in it and thus, highlighted the implications of ancient DNA research in palaeo-microbiology. Similarly, it was found an alternate source of mtDNA for biological profiling of unknown human skeletal remains older as 1,000 years; focusing on some benefits and limitations of DNA research from calculus (Black et al., 2011). Adler et al, (2013) sequenced and amplified 16S rRNA gene of oral microbiota from 5500 BCE–1600 CE old dental calculus samples, using the next-generation sequencing technique. Charlier et al (2010) investigated the past pathologies, individualistic food habits, environmental and work-related exposure to pollutants from examination of the dental calculus collected from different archaeological dental samples and stressed that such information can help in identification and ascertaining cause and manner of death of an individual.

Singh and Goel (2017) collected dental calculus from lingual surfaces of mandibular incisors from 20 dental students of Moradabad (UP) and found that majority of dental calculus samples yielded DNA ranging from 21 to 37 μg/ml (mean quantity of 23.5 μg/ml). No other Indian study could be retrieved from the accessible literature that described the use of dental calculus for forensic or anthropological purposes.

**Systematic Review of Available literature**

To scrutinize the current status of dental calculus research for forensic anthropological purposes, the scientific databases were searched using search engines like PubMed, ScienceDirect, SAGE, Springerlink, Clinical Key, WoS and Google-Scholar, using the terms like 'Dental calculus, Forensic identification', Microbial forensics and oral microbiome, Ancient DNA and stable isotope analysis, 'Occupation and dietary status from calculus, Oral microbiota and forensic odontology etc.(Figure 2) Snowball sampling technique of cross-referencing was used to identify the related articles and, the PRISMA guidelines were followed to include the eligible research articles. The full-text articles published till 2022 and having information relevant to scope of present systematic review were further analyzed in-depth. A total of 89 research articles were identified, out of which only 31 studies were found within the ambit of the aim and objectives of present study. The inclusion criteria were to consider the studies related to forensic identifications based on dental calculus and those published till 2022; the studies not satisfying the inclusion criterion were neglected for further analyses.

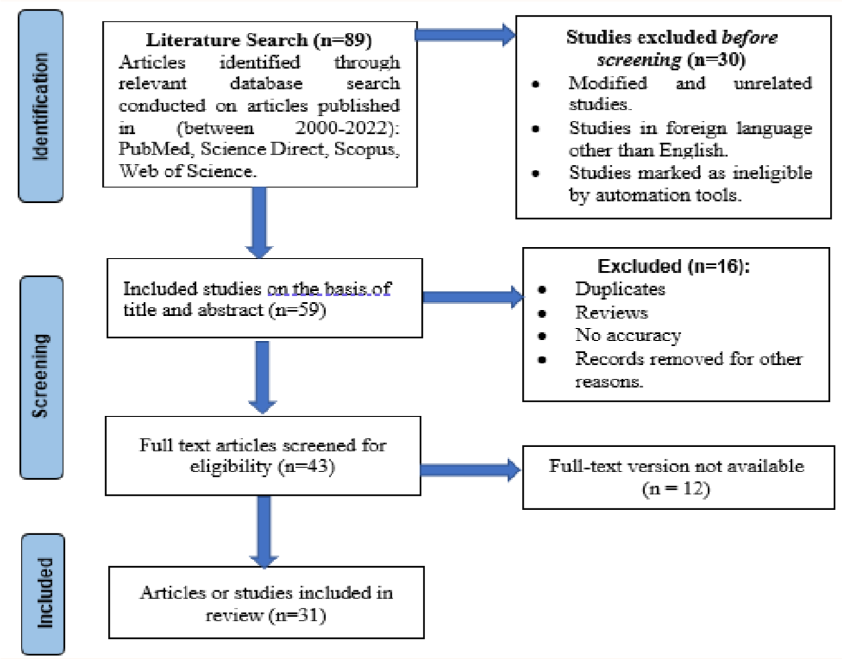


**Figure 2: Flowchart showing stages of selection of articles for systematic review**

The systematic review revealed that dental calculus serves as a rich source of host-associated biomolecules, ingested or inhaled during routine daily life of an individual. Dental calculus was reported as a promising biological material for establishing identity, assessing health and dietary status, estimating geo-affinity, exposure to heavy metals or drugs of abuse and establishing other identity credentials of an unknown individual. Present systematic review revealed that majority of dental calculus studies were reported from the Europe and dental remains from archaeological sites and cemeteries were most commonly used for the purpose. Most of the studies were restricted to some European countries and only one pilot study (Singh and Goel 2017) was reported from India. The type of teeth or the site (supra-/sub-lingual) from where calculus was collected wasn't mentioned in most of the studies. Almost all populations have dental calculus, though its prevalence varies with the age, sex, population-affinity, systemic health, dietary and dental hygiene status of an individual (Warriner et al 2015, Mann et al 2018). Till very recently, the scientific importance of dental calculus for forensic anthropological casework was not recognized as it couldn't attract the desired attention of the scientific community. However, it is now being appreciated as a significant population and individuality marker due to the abundance of host-associated routinely preserved micro-particles and biomolecules in it (Radini et al 2018).

**Prevalence of Dental calculus in Ajnala teeth and its Significance**

Ajnala skeletal remains reportedly belong to 282 Indian soldiers killed in 1857, and their corpses were disposed of in an abandoned well and a religious structure was built over its periphery (Cooper 1858, EIP 1859, Bates and Carter, 2017). It was only in early 2014 that these remains were exhumed non-scientifically by amateur archaeologists after getting information from the written records. Thousands of teeth, jaw fragments, cranial fragments, damaged bones and numerous items of contextual and personalized identity have been retrieved from the Ajnala skeletal collection (Sehrawat et al 2016) (Figure 3). The Ajnala jaw fragments were examined for prevalence of dental calculus to help in provenance of these badly damaged and fragmented human remains to estimate the dental health and hygiene, dietary habits, habits of tooth-brushing, tobacco smoking, betel-nut chewing etc. of the deceased individuals. Negligible number of jaw fragments had dental calculus deposits, signifying the sound dental health and hygienic status of Ajnala individuals. This observation endorsed the previous observations about their dental health status estimated on the basis of prevalence of dental pathologies

(Sehrawat and Singh, 2018). As the standardization, reproducibility and accuracy analysis of results of anthropological and archaeological research with DCD is still in nascent stage, it needs to be further investigated in different population groups for fruitful research avenues. Ajnala calculus samples will be studied both molecularly and chemically and compared with the calculus from contemporary Indian samples/studies to estimate their geolocation, epidemiological status, exposure to heavy metals or pollutants, tooth-brushing, tobacco smoking, betel-nut chewing and drug abuse. Though the prospect of dental calculus as a biomaterial has yet to be rigorously and systematically scrutinized (Mann et al., 2018), it is expected to help forensic anthropologists in India to use dental calculus for provenance of unknown human remains retrieved from forensic scenarios, even when only some tooth/teeth are retrieved from such contexts.

**Figure 3: Badly damaged Ajnala skeletal remains and calculus deposits in jaw fragments**

**Challenges and Probabilities of Dental Calculus Research**

In most forensic situations, highly degraded and contaminated/challenged samples (containing PCR inhibitors)containing low amounts of DNA are retrieved for forensic identification purposes which presents a very toughand challenging task for extraction of DNA; dental calculus is one of such materials. The concentration of DNAisolated from calculus does not significantly differ from that obtained from the teeth, though teeth DNA is moredegraded than the undamaged DNA extracted from calculus, thus calculus DNA is more stable and less challenged than that obtained from the dentin. Though calculus DNA is more fragmented and shorter in length,it is merely sufficient to obtain a putative profile of an individual; suitable enough for preliminary forensic identification strategies

(Lisman et al., 2023). The biggest challenge for geographic identification of vegetal orfaunal inclusions in calculus is the lack of comprehensive reference libraries, and alteration of geolocation signatures due to travel, illness and diet.

Calculus has been widely used as a valuable tool in anthropological, archaeological, microbiological and forensic research. Majority of available dental calculus research is primarily aimed at estimating dietary habits, oral microbiota and mtDNA haplogroup affiliation of archaeological human remains (Black et al 2011, Damle 2016); and limited published research work is available about the quantification of human DNA content in dental calculus for human identification purposes. Dental calculus can prove more useful than the bones in the identification of fragmented or burnt human remains. Human DNA bound in calculus hydroxyapatite is comparatively well preserved, thus it can be taken as potential investigative tool for forensic purposes (Higgins and Austin, 2013). The quantity of collected dental calculus (ideally 20 mg) is crucial as significantly higher amounts of calculus yield better DNA amplification results (Lisman et al., 2023). Research involving higher numbers of jaw fragments is certainly needed to authenticate the forensic utility of dental calculus for forensic identification purposes. The pioneer research work utilizing to-be-thrown' dental calculus will certainly help future forensic, anthropology, public health, and dentistry experts as an important biological material for establishing identity, estimating health and dietary status, exposure to heavy metals, drug abuse and other credentials of an unknown person. Forensic anthropologists and geneticists are expected to engage in collaborative research to explore its possible role in the shared objective of human identification. Scientific scrutiny of larger numbers of jaw fragments is certainly required to corroborate the utility of dental calculus deposits for forensic identification purposes. The pioneer research work utilizing 'to-be-thrown' dental calculus will certainly help future forensic, anthropology, public health, and dentistry experts as an important biological material for establishing identity, estimating health and dietary status, exposure to heavy metals, drug abuse and establishing other unknown credentials of an individual. Forensic anthropologists and geneticists should explore collaborative research possibilities in the field to explore the fullest potential of dental calculus as an adjuvant and non-invasive evidentiary material towards forensic human identifications.

## CONCLUSION

Microbial forensics has significantly contributed as a useful tool in medicine, bioterrorism, biosecurity, food trade and human identifications (Castro and de Ungria 2022). Dental calculus can prove more useful than the bones in the identification of badly damaged, fragmented or burnt human remains like Ajnala skeletal remains. The dental calculus entraps cellular or tissue fragments, past human oral microbiota that can provide deep insights into decedents' lifestyle, diet, health, disease status, occupational habits, possible geolocation, environmental conditions and the cultural affiliation etc., of an individual; thus presenting a more comprehensive ante-mortem profile of the unknown individuals (past or contemporary). Though the anthropological and archaeological research involving dental calculus is still in its nascent stage, comprehensive research in different population groups is need of the hour for fruitful research avenues and utilization of DCDas an alternative material for ancient DNA research.

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