Plant inclusions in dental calculus and their visibility in micro-CT scans. An examination of three individuals from the Sint Jansbeek site in Arnhem, NL.

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19th of January 2022, Leiden

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Acknowledgements

First and foremost, I would like to thank Dr Amanda G. Henry for all her support during this project. You were always quick to help and happy to share your knowledge whenever I had a question. I am very grateful for all the things I have learned from you, and I will miss working together!

Thank you to Dr Dominique Ngan-Tillard and Ellen Meijvogel-de Koning for bringing our micro-CT ideas to fruition and taking great care of the samples. It was a pleasure visiting your faculty and learning so much about the method.

For the help with identifying microscopy finds, I would like to thank Dr L. Shumilovskikh (Department of Palynology and Climate Dynamics, Albrecht-von-Haller-Institute for Plant Sciences, Georg-August-University Göttingen), Dr Carla J. Dove (Feather Identification Lab, National Museum of Natural History (Smithsonian)), and Walter F. Rowe (Department of Forensic Sciences, The George Washington University).

Finally, I would like to thank my family and friends for their support during this second corona-influenced writing process.

It could not have been done without you!

1 Introduction

This thesis presents the results of archaeological dental calculus analysis of several individuals from the site of Jansbeek/Eusebiuskerk in Arnhem, The Netherlands, using light microscopy and micro-computed tomography (micro-CT) scan images.
Dental calculus is mineralised dental plaque, which adheres to teeth. It is of interest to researchers because it traps informative biomolecules and preserves well in archaeological contexts. The aim of this research is two-fold: To compare micro-CT and light microscopy as detection and examination methods for plant micro-remains in dental calculus, and to gain further information about diet in late medieval to early modern Arnhem.

While in the present-day dental calculus deposits are usually removed during dental hygiene routines and treatments, such deposits contain a wealth of information about health, including diet and (ancient)DNA of not only humans but also microbes. In addition, the unique structure and composition of dental calculus deposits allows for entrapment of small particles ranging from pollen grains to charcoal particles from smoke inhalation. By examining such inclusions in dental calculus, it is possible to make inferences about which kinds of materials came into contact with the oral cavity, either via consumption, inhalation, or through the use of teeth as tools. When focusing on plant remains (such as phytoliths and starch granules) it is possible to find out more about the types of foods people in the past consumed, and under certain circumstances how those foods were prepared (for example by boiling, grilling, etc.) (Hardy et al., 2009; Henry et al., 2009b).
Unfortunately, most methods used to extract plant materials lead to the destruction of the dental calculus and reduce possibilities for other analyses. Weighing the potential knowledge gain against the destructiveness of a given analysis is taken seriously in the field of archaeology, since destructive analysis can only be performed once. Especially in contexts where not a lot of archaeological material is available, this can be a concern. Therefore, this project tests the use of micro-CT as a non-destructive method to assess and potentially identify plant material in dental calculus. Ideally, thanks to micro-CT enabling us to investigate the inner structure of the calculus, it would be possible to make more informed decisions on the potential knowledge gain achievable.

1.1 Thesis outline

After this brief introduction into the topic of the thesis, Chapter 2 will provide more information on dental calculus, including its formation and role in archaeological research. Chapter 0 outlines the approach to dental calculus research taken in this thesis project, giving an overview of the research aims and specific research questions to be addressed. The fourth chapter describes what materials were used, and the fifth chapter explains the methods used to analyse the materials in order to answer the research questions. The sixth chapter then provides the results of the analyses, which are put into context and discussed int chapter seven. The final chapter provides a conclusion of the results and their possible interpretations in addition to reviewing the suitability of the chosen methods to reach the research goals.

2 Dental calculus and dietary reconstruction

This research project focuses on how human dental calculus from archaeological contexts can serve as a record of past diets and behaviours. Clinical studies of dental calculus or calculus in other species will not be explored, except for how these inform our understanding of past human samples.

Since the research focus of this thesis lies on human diet in the past, this chapter mainly concerns human dental calculus from archaeological contexts, rather than discussing clinical studies or dental calculus in other species. Before exploring the relevance of archaeological dental calculus and its potential for dietary reconstruction, it is important to understand how calculus forms during the life of an individual, and how this might influence the archaeological record. Afterwards, the plant micro-remains involved in dietary reconstruction based on dental calculus, mainly starch grains and phytoliths, are introduced.

2.1 Dental calculus formation

In order to understand the implications of dental calculus presence and composition in an archaeological context, it is necessary to discuss the processes that lead to its formation.

2.1.1 Dental plaque

The (potential) precursor to dental calculus is dental plaque, the white, soft layer of accumulated micro-organisms adhering to the teeth. These micro-organisms inhabit the oral cavity, and teeth offer a smooth surface for them to accumulate on (Lieverse, 1999). The exact make-up of the plaque deposit depends not just on the makeup of an individual's overall oral microbiome but may also vary between different areas of the oral cavity depending on factors such as oxygen availability, amount of salivary flow, pH, and temperature (Hillson, 2005; Lieverse, 1999). In addition, these factors are not constant and may fluctuate throughout the day and result in changes of local microbiome within hours, for example after eating, drinking, or while asleep (Hillson, 2005; Lieverse, 1999). Dental plaque deposits may accumulate more easily in some locations rather than others due to the cleaning effect of friction against the tongue, lips, and cheeks (Cooper, 2017; Hillson, 2005).

A plaque deposit is made up of a polymer matrix produced by micro-organisms, within which microparticles from outside sources (like foodstuffs or inhaled dust particles) may

get trapped. Plaque may also influence salivary pH when sucralose and amino acids from food or drink is metabolised by bacteria. Depending on the process, this can result in more alkaline or more acidic saliva, the latter of which is associated with tooth de-mineralisation and dental caries (Hillson, 2005).

2.1.2 Dental calculus formation and composition

Dental plaque can mineralise by incorporating calcium phosphate from saliva, conserving the plaque matrix, and forming dental calculus (Lieverse, 1999). Mineralisation may take place in different events throughout an individual's life, albeit with no specified frequency. In fact, the rate and extent of dental calculus formation is different between individuals and within an individual oral cavity (Fons-Badal et al., 2020; Hillson, 2005; Kinaston et al., 2019; Salazar-García et al., 2014). New plaque accumulates on top of dental calculus, and the same processes apply as if it had adhered to directly to the tooth surface. It may be removed or change composition depending on the circumstances, or it may mineralise into dental calculus at a given time (Hillson, 2005). When given the chance, these mineralisation events can lead to the build-up of considerable dental calculus deposits, consisting of a calculus layer of differing thickness or properties per mineralisation event. Dental calculus primarily consists of calcium, phosphate, and oxygen with smaller amounts of carbon, sulphur, iron, silicon, sodium, aluminium, magnesium, chlorine, and fluoride (Hillson, 2005; Lieverse, 1999). Next to the inorganic components allowing for the exceptional preservation of dental calculus in archaeological contexts, there are organic microparticles such as plant residue, proteins, and DNA (Kinaston et al., 2019; Salazar-García et al., 2014).

However, the mechanisms causing the mineralisation of dental plaque are not well understood (Christina Warinner et al., 2015). Some potential influencing factors such as diet, genetics, the composition of the oral microbiome, saliva properties, and dental hygiene have been identified (Hillson, 2005; Lieverse, 1999; Radini et al., 2017). Research into the process is ongoing, and new potential factors, like dental crowding, have been identified (Fons-Badal et al., 2020). For living, modern populations, it has been noted that affliction rates are also influenced by age, sex, ethnic background, prescribed medication, non-insulin dependent diabetes, and the regularity at which professional dental cleanings are performed (White, 1997). Sufficient flow of relatively alkaline saliva containing calcium and phosphate ions has been linked to increased dental calculus formation, as have been diets high in protein and sugars, or carbohydrates (Cooper, 2017; Hillson, 2005; Kinaston et al., 2019; Radini et al., 2017). In addition to calcium and phosphorus, higher levels of urea in the saliva may also play a

role (Fons-Badal et al., 2020).

Since saliva is a significant factor in supplying minerals, the salivary gland ducts can influence which areas of the dentition are more prone to developing dental calculus (Jin et al., 2002). The parotid salivary gland empties towards the back of the upper jaw, close to the maxillary molars, and the submandibular and sublingual glands supply saliva to the underside of the tongue. As a consequence of the higher saliva accessibility in those areas, dental calculus deposits tend to be more pronounced on lingual tooth surfaces of incisors and canines (the tooth surfaces facing the tongue), whereas it is the buccal surface for maxillary molars (the tooth surfaces facing the cheek) (Hillson, 2005; Radini et al., 2017). Despite this observed formation likeliness at these sites, it should be kept in mind that dental calculus has the potential to form wherever there is a plaque deposit.

2.1.3 Research interest

Dental calculus is increasingly of interest for archaeologists for several reasons. The highly mineralised nature of dental calculus facilitates excellent preservation of any of its components in archaeological contexts, and its structure envelopes and protects inclusions (Hardy et al., 2009; Kinaston et al., 2019). In addition, dental calculus is relatively abundant, being nearly omnipresent in humans regardless of period or geographic location (although the overall amount of dental calculus may differ depending on dental hygiene practices). The contents of dental calculus, such as the microparticle inclusions, proteins, lipids, and aDNA can provide fascinating information about life in the past.

Researched aspects of dental calculus also include its chemical and mineral composition, physical structure, and the various kinds of inclusions contained within it. It has been studied employing various methods, and is a useful source of information on diet, health, immune system, and pathogen evolution. Analysis of dental calculus has provided information about the diets of two 2-Ma *Australopithecus sediba* fossils (Henry et al., 2012), the first direct evidence of milk consumption (Warinner et al., 2014), the use of medicinal plants (Gismondi et al., 2018; Hardy et al., 2018), and oral microbiome composition shifts during major developments in human history such as the Neolithic and Industrial Revolutions (Adler et al., 2013). Recently, it has been hypothesised that dental calculus may be useful in assessing past infections with Sars-Cov-2 in asymptomatic patients that did not develop detectable antibodies, although research is ongoing (Berton et al., 2021).

Within archaeology, a lot of research is aimed at gaining insight into the health and diet of past individuals and populations, as well as the environment they lived in. Both dietary and environmental reconstruction commonly rely on plant micro-particles, which may be recovered from a variety of contexts including sediments, artefacts (like cooking implements and vessels), or dental calculus (O'Regan et al., 2016; Zhang et al., 2017).

2.2 Dietary reconstruction from plant micro-remains in dental calculus

Plant micro-remains are microscopic parts of plants that may retain taxon-specific morphologies. Common micro-remains include pollen grains, starch grains, and phytoliths. Their specific shapes can be identified when compared to modern reference material, which provides information about the types of plants that were present in archaeological contexts (Henry et al., 2009b).. Often, plant micro-remains are small enough to be incorporated into dental calculus deposits.
This research focuses on starch grains and phytoliths, since they are a more direct record of intentional consumption of food items and are less likely to be included due to accidental inhalation. Depending on the time period in question and the plant species represented in the dental calculus deposit, it may be possible to make inferences about social and economic status as well (De Cupere et al., 2021).

2.2.1 Starch granules

Starch granules are an end-product of carbon fixation by photosynthesis and act as an energy reserve in most green plants. Varying quantities of starch can be found in every type of plant tissue such as leaves, stems, roots, seeds, tubers, and even pollen grains (Hardy et al., 2009; Zhang et al., 2017). It is most abundantly found in plant tissues that require long-term energy storage, such as seeds, fruits, and roots or tubers (Henry et al., 2009b).

Starch granule formation begins at a central point (one hilum, or several hila) and continues in alternating layers (lamellae) of two glucose polymers, amylose and amylopectin. This results in starch granules having a discrete, concentric, and semi-crystalline structure which causes an extinction cross under cross-polarized light. Most starch granules range from 1µm to around 100µm in size (Zhang et al., 2017). There are morphological differences which make starch grains taxonomically distinct, including shape, size, polarization cross, surface fissures, lamellae, and hila. Starch from

archaeological contexts is identified based on these characteristics as well, commonly using transmitted cross-polarised light microscopy with 200-400 times magnification (Hardy et al., 2009; Henry et al., 2009b; Zhang et al., 2017).

In addition to indicating the kinds of plants that were consumed, starch grains can also record how those plants were processed. Thanks to its semi-crystalline structure, starch can be stored for a relatively long time and is insoluble in cold water. However, it can be broken down into water-soluble sugars by digestive enzymes or undergo changes during food processing, for example by grinding, boiling, baking, or fermenting (Henry et al., 2009a). It can be assumed that most of the starchy foods consumed by humans have undergone a cooking process, although, depending on the process foodstuffs may not have been cooked through entirely which could result in the presence of both modified and unmodified starches (Hardy et al., 2009, p.250). One of the modifications starches undergo during cooking is gelatinisation, which happens when starch is combined with water and heated beyond ca. 60° (see Figure 2-1).

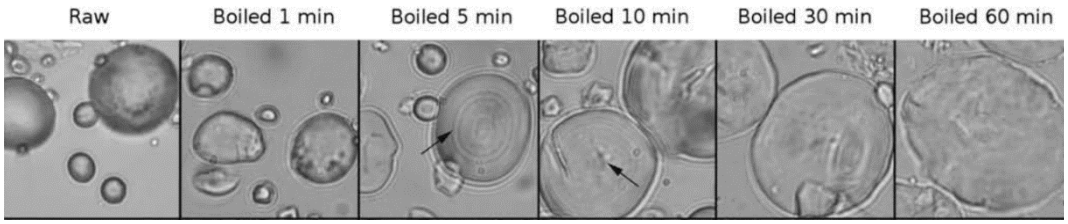


Figure 2-1 Changes to wheat starches during boiling (Henry et al. 2009, p.919)

Gelatinisation leads to swelling, which affects the refractive properties and structure of the starch granule. As a gelatinised starch cools down it undergoes retrogradation, which may make it more resistant to enzyme breakdown. When consumed, the digestion process of starch starts with the enzyme amylase, of which ample amounts can be found in saliva. If a starch granule is incorporated into the dental plaque matrix, however, it is protected from amylase and has a chance of preserving (Hardy et al., 2009, p.250; Henry et al., 2009b).

An example of the changes to starch granules as a result of different food preparation methods can be seen in Figure 2-2 .

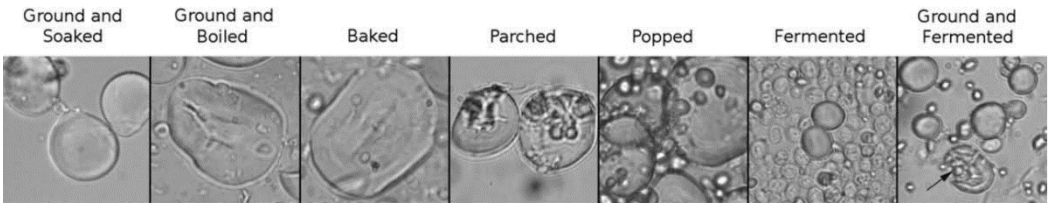


Figure 2-2 Modified wheat starch (Henry et al., 2009,p.920)

While archaeological starch can also be retrieved from artefacts and sediment samples, dental calculus offers certain advantages for dietary reconstruction. For one, the inclusion of starch granules within the dental calculus matrix may decrease diagenetic changes and chances of contamination. In addition, dental calculus provides a direct link to food consumption, as opposed to starch recovered from an artefact which relies on the interpretations of its use (Hardy et al., 2009, p.254). However, the persistence of starch granules in archaeological contexts is not well understood yet and appears to contrast the biodegradability of starch and its use in biodegradable plastic (Hardy et al., 2009, p.249).

2.2.2 Phytoliths

Phytoliths are microscopic inorganic inclusions occurring in plants, consisting of silica sourced from the groundwater as monosilicic acid (Rosen, 2015). As it accumulates in the plant tissue, bodies of opaline silica are formed, with many (but not all) plant taxa cells shaping distinctively cell- or tissue-shaped phytoliths. The shape depends on whether the phytolith was produced in a specialised silica-accumulating cell (idioblast), or in an (intra-)cellular space of the plant. The former mechanism results in phytoliths with little resemblance of the parent cell, whereas the latter typically take on the shape of the parent cell and may be more diagnostic as a result (Rosen, 2015, p.254). Broadly speaking, phytoliths range in size from 10µm to 30µm, occasionally with diameters of over 1000µm (Rashid et al., 2019, p.236). Many plant families produce distinctive phytoliths, commonly allowing genus-level diagnostics and even species-level for some taxa (see Figure 2-3).

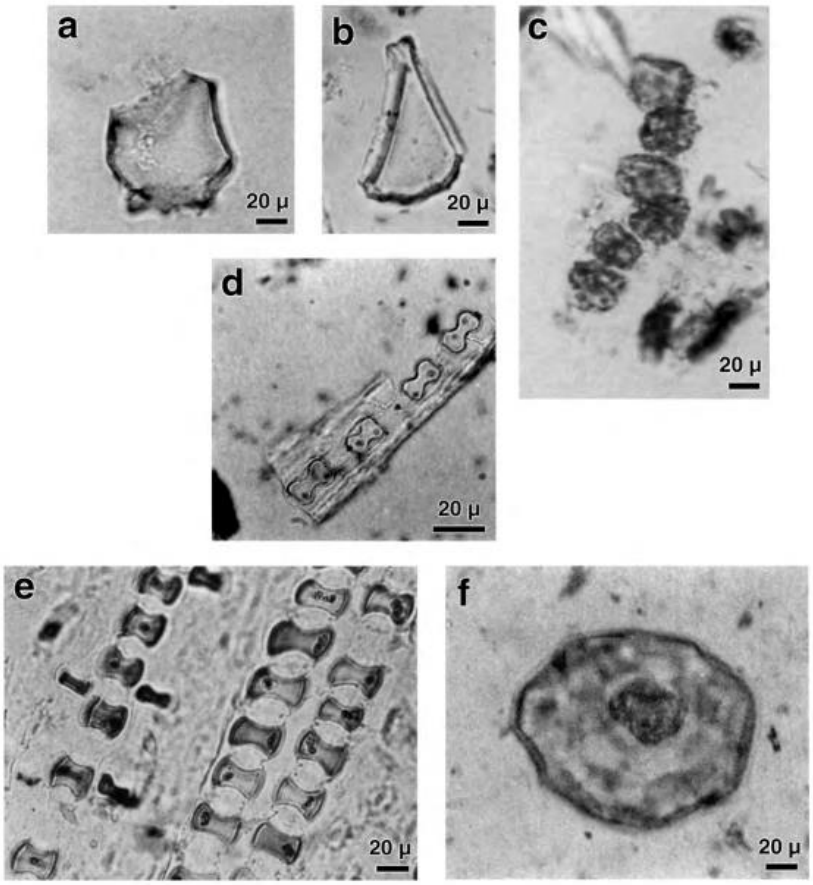


Figure 5.1 Examples of distinctive phytoliths: (a) keystone bulliform from a raised field soil sample; (b) keystone bulliform from Oryza sativa*; bulliforms are diagnostic of grasses; (c) angled and folded spheres from* Canna edulis*; (d) bilobate short cells from* Phragmites australis*; bilobates are diagnostic of panicoid grasses; (e) saddle-shaped short cells from* Bambusa australe*; saddles are diagnostic of bamboos and chloridoid grasses; (f) scalloped sphere from* Lagenaria siceraria*. Photographed at 400 X.*

Figure 2-3 Examples of phytoliths (Rosen 2015, p.255)

Silica accumulations have different benefits for a plant, such as forming a structural support to retain seeds, stabilising air-canals, or reducing evaporation in leaf tissue (Rosen, 2015). After a plant's death, the contained phytoliths may either dissolve or be preserved in soil or archaeological sediments. They may also preserve as inclusions in dental calculus or coprolites. In the soil, phytoliths preserve best in a low temperature and a low pH, although water availability and microbial activity factor in as well (Rashid et al., 2019; Rosen, 2015). To date, one of the earliest grass fossils known is a phytolith dated to the late Early Cretaceous 113-101 Ma ago, which was extracted from a duck-billed dinosaur's dentition (Wu et al., 2018).

The development of phytolith analysis in archaeology is relatively recent, and has been tied to the research foci of agricultural intensification and reconstruction of past environments followed in the 1970s and -80s (Rosen, 2015, p.265). Phytolith analysis in archaeology allows for inferences about plant-people relationships at sites where the

preservation of organic materials is poor even if charred. In addition, it requires relatively little equipment beyond a microscope with 200-400x magnification (Rosen, 2015, p.281). Phytoliths are produced in many plant families, and may be diagnostic to the genus- or species level (Rashid et al., 2019; Rosen, 2015). Even when they are not always identifiably at plant species level, they can still be diagnostic and provide insight into palaeodiets, adaptive strategies, origin and dispersal of domesticated plants, agricultural practices, and plant processing. Quantitative analysis of phytoliths from palaeosediments can shed light on the vegetation dynamics and climate conditions in the past, as well as help interpret the evolution of plants (Rashid et al., 2019, p.242). The evolution of plants in connection with human history, domestication, and climate is of specific interest to archaeologists since it allows for reconstruction of plants used as food or raw material. For instance, phytoliths have been used to explore the spread of maize consumption in the Andes (Rosen, 2015,p.327).

Phytoliths found in tool or vessel residues may shed light into their function, although it should be noted that naturally accumulating sediments also contain phytoliths (Rosen, 2015, p.253). Extracted from coprolites and dental calculus, phytoliths have been used for dietary reconstruction of not only human palaeodiets, but also that of other animal species (Rashid et al., 2019, p.243; Wu et al., 2018). For example, the consumption of dates has been evidenced by phytoliths in Neanderthal dental calculus from Shanidar Cave in Iraq (Henry et al., 2011).

2.2.3 Problems with the analysis of plant micro-remains in dental calculus

As outlined above, plant micro-remains are useful for identifying dietary components and behaviour in the past, including those extracted from dental calculus. However, there are certain issues that need to be addressed.

Methods used to extract micro-remains include the removal of calculus from the tooth, followed by chemical and/or mechanical disaggregation of the calculus (Tromp et al., 2017). These extraction methods can cause irreparable loss to the sample and prevent multiple methods (e.g., micro-remains and ancient DNA) being used on the same dental calculus deposit.

There is a need to test new methods that may help us avoid these problems in the future. One of these methods is micro-CT, which has previously been applied to archaeological dental structures (cf. O'Hara et al., 2019) and allows a view of the inner structure of objects with a low chance of causing damage. While the effect of X-rays on DNA in living organisms is well-documented, radiation from conventional micro-CT

analysis does not usually reach a high enough dose to damage ancient DNA (Immel et al., 2016). Therefore, a comparison of both the micro-CT and the traditional methods will be performed in this project, to see which provides a better understanding of diets.

3 Approach

This study seeks to obtain data which will help to address the research gaps in the applicability of micro-CT to archaeological dental calculus inclusions. In addition, with the help of dental calculus analysis, plant micro-remains will be examined to gain insight into the diet of individuals from the (post-)medieval Arnhem site.

3.1 Research aims

The aims of this research are as follows:

- Establish the appearance of organic micro-remains in micro-CT scans.
- Examine the suitability of micro-CT scanning for non-destructive identification of potential organic micro-remains in archaeological dental calculus.
    a. Examine scans of archaeological dental calculus for potential micro-remains and attempt to identify them.
    b. Extract the micro-remains and identify them, comparing results to initial scan observations.
- Analyse the extracted plant remains to gain insight on the types of plant foods consumed in (post-)medieval Arnhem.

3.2 Research hypotheses

The following hypotheses will be tested:

1. There are plant remains in the dental calculus deposits sampled from the Arnhem collection, which can be visualised using micro-CT
2. Those plant remains observed in the micro-CT scans can be linked to the plant remains yielded from extraction.
3. The extracted plant remains allow a reconstruction of diet.
4. Isolated plant micro-remains can be visualised using micro-CT.

4 Materials

For this research, several teeth with adhering dental calculus deposits were examined. All teeth stem from individuals from the same Dutch medieval and post-medieval site in Arnhem, and whose remains are partially kept at Leiden University. Of the six collected molars, three deposits were sampled and scanned in the micro-CT. Subsequently, they were partially dissolved to undergo plant microfossil analysis using light microscopy.

4.1 The site and collection of Arnhem Eusebiuskerk / Jansbeek

During building activities in the southern portion of the historic city centre of Arnhem in 2017, archaeological consultancy company RAAP (Regionaal Archeologisch Adviserings Project) was hired by the municipality of Arnhem to lead an accompanying excavation of the area under construction. Findings include the former riverbed of Sint Jansbeek, a stream running through Arnhem into the Rhine, old city walls, buildings (including the old church Broerenkerk), refuse pits, and human remains of the former inhabitants of Arnhem next to the Eusebiuskerk church. A total of 722 graves were excavated, however, during anthropological examination only 667 of them were securely identified as primary burials. In the old churchyard (Oude Kerkhof, dubbed WP10), 659 individuals were found, and another 20 were buried by the Broerenkerk churchyard. Due to the long-term use of the cemetery, the burial stratigraphy consists of several levels and grave density in parts of the area was as high as 3.9 graves per m^2.

This seemingly unplanned burial system is also illustrated by several areas with secondary, disarticulated bone deposition, where remains from older graves cut by subsequent burials were deposited. As a result of this high burial density and frequent overlapping of burials, older human remains are underrepresented in the dataset of primary graves. Graves located closer to the surface were also subjected to disturbance and destruction in the past, be it due to the World War II or levelling and construction work (Zielman & Baetsen, 2020, p.85).

Based on historic sources, deceased individuals have been buried around the church from 1361-1829 AD, which includes the “plague year” of 1636 AD. It is assumed that the cemetery stopped being used in 1829 due to a change in law, forbidding any burials within the city centre from the first of January 1829. The dating of the graves, through artefacts or in some cases radiocarbon dating of the remains, was estimated to range roughly from 1350 AD to 1829 AD, depending on their location within the churchyard

which could be divided into two phases (1350-1650 AD and 1650-1829 AD) (Zielman & Baetsen, 2020, p.96, 112).

It has been cautiously concluded in the excavation report that the people buried around the church were of average to low socio-economic status (Zielman & Baetsen, 2020, p.358).

The human remains recovered during the excavation were moved to the depot of Arnhem municipality, who later agreed to loan part of those remains found in a primary burial context to the Laboratory for Human Osteology at Leiden University (Faculty of Archaeology). Most of the bone material found in secondary contexts has since been reburied again in Arnhem (Zielman & Baetsen, 2020, p.10).
A physical anthropological analysis of part of the primary inhumation assemblage was carried out by RAAP / Willem Baetsen MSc and drs. Steffen Baetsen, following international standards and the methods recommended by the Laboratory for Human Osteoarchaeology (2017) of Leiden University, *Barge's Anthropologica,* and the Amsterdams Academisch Medisch Centrum (Zielman & Baetsen, 2020, p. 366).

4.2 Sample selection

The samples from Leiden University's collection were selected based on several criteria. Ideally, a sample would:

1. Be a first molar (irrespective of maxillary or mandibular), due to its eruption time allowing for a long period of calculus accumulation and its involvement in mastication of foods.
2. Be a loose tooth, for in-situ scanning purposes and to avoid destruction of human remains or dental calculus stratigraphy when extracting teeth.
3. Have a decently sized dental calculus deposit.

Finally, we chose samples to represent both male and female individuals, due to sex potentially having an influence on dental pathology (cf. Lukacs & Largaespada, 2006) which may also affect dental calculus formation.

With these criteria in mind, it was possible to sample five loose teeth total and chip off parts of a calculus deposit from one other. Except for one second molar, all teeth are first molars. A summary of the samples can be seen below in Table 4-1.

Table 4-1 Sample set

Find no.	Feature no.	Sex	Loose?	FDI code	Tooth
V-1752	862	F	Yes	1.6	1st molar
V-2053	1015	F	Yes	2.6	1st molar
V-2433	1245	F	Yes	1.7	2nd molar
V-1399	708	M	Yes	1.6	1st molar
V-2451	1255	M	Yes	1.6	1st molar
V-1318	669	M	No	1.6	1st molar

Find no. = Find number of excavated skeletons; Loose (Y/N) = whether tooth still within jaw or not; FDI code = international code describing which side and position a tooth is from.

All selected molars are maxillary molars, which was not a conscious selection but may have been influenced by the disproportionate preservation of maxillary dentition. The excavation report notes that for more than 35% of the primary inhumations, no mandibles were preserved (Zielman & Baetsen, 2020, p.434).

Due to time constraints with the availability of the CT scanner, we were able to scan only the calculus from three of these individuals: V-1752, V-2451, and V-1318.

5 Methods

In order to fulfil the research aims, two different methods were applied to collect data from the study group.

First, the selected dental calculus samples were scanned with a micro-CT to acquire pictures of many sections throughout each calculus deposit. This allows a look into the inner structure of the deposits, including factors such as density, but also allows recognition of irregularities in appearance which may represent inclusions of varying materials (both inorganic and organic).

To establish whether some of these inclusions are organic, the dental calculus deposits are then dissolved and any organic remains extracted. These remains are then viewed under a light microscope to classify them (mainly) into phytoliths, starch granules, etc. After classification of the micro-remains, they will be compared to potential inclusions observed in the micro-CT scans. Finally, the organic inclusions relevant to dietary reconstruction are observed to gain insight into the diet of the studied individuals.

5.1 Micro-CT

5.1.1 Basic principle of (micro-) computed tomography

X-ray computed tomography (CT) is a visualisation method that can non-destructively and non-invasively show the inner structure of materials by taking radiographs (two-dimensional projections) from different angles. This imaging method requires minimum sample preparation, unlike other methods which may require thin sectioning, slicing, or staining.

The resolution of images produced by micro-CT usually range from ~400 nm to 70µm, referring to pixel size in 2D and isotropic voxel size in 3D images. The basic principle behind micro-CT is the detection of X-rays which have passed through a sample (Orhan, 2020, 20).

Generally, micro-CT is composed of an X-ray tube, sample holder, a detector and CCD camera, and filter. The X-rays generated by the X-ray tube fly through the sample, casting a projection on the detector/camera. The scintillator in the detector converts the X-rays into visible light so that the cameras may detect them. In order to record more projections, and ultimately reveal the inner structure of the sample, either the sample itself or the detector and X-ray tube are rotated by 180° or 360°.

Each projection is acquired by the detector in a 2D grayscale, representing which X-rays were not absorbed by the sample but reached the detector. The densities of materials in

the sample are related to the absorption of the X-rays. With different reconstruction methods, the 3D image of a sample may be reconstructed once a large stack of 2D projections is collected, the higher the number of 2D projections, the better the resolution.

5.1.2 Applications of micro-CT

The use of micro-CT may be limited due to time and financial constraints, with the scanning time for a single maize kernel at 6µm resolution being reported at around two hours (Schoeman et al., 2016,p.21). Nonetheless, a comparison of X-ray micro-CT against other imaging techniques (such as MRI) has shown that X-ray are more convenient and less costly (Schoeman et al., 2016).

What makes micro-CT attractive to research is its ability to non-destructively and directly provide a 3D image of a sample, allowing precise measurements of the internal structure (Orhan, 2020, p.51). Thanks to these possibilities, research using micro-CT has become increasingly popular in the previous years, particularly in Material Sciences and Life Sciences.

Micro-CT has been used for a variety of applications, including linking material properties with microstructure, exploring the formation process of holes in cheese, and assisting with a wide variety of medical interventions (Cnudde & Boone, 2013; Peyrin et al., 2014; Schoeman et al., 2016).

Among early adopters of laboratory micro-CT systems were bone biologists and dentists, exploring the architecture and mechanical properties of bone and dental tissues. Common clinical research interests include osteoporosis and mechanical properties of bone (Djomehri et al., 2015; Orhan, 2020; Peyrin et al., 2014). Micro-CT has also been used in archaeology, for example to examine artefacts such as a Bronze Age sword (Mödlinger, 2008) or to assess vitamin D deficiency based on mineralisation defects in dentine (Veselka et al., 2019). It has not commonly been applied to dental calculus, however, despite the potential for revealing information both about the microscopic structure of this material, and potentially enabling the identification of inclusions without destruction of samples.

5.1.3 Sample preparation for micro-CT

Sample treatment varied slightly between the teeth based on whether they were loose or attached to the jaw in-situ. One of the individuals' skulls (V-1318) was taken to the laboratory to remove part of the dental calculus deposit with a dental descaler, which was then deposited into an Eppendorf tube. The loose teeth were simply bagged whole, and taken to TU Delft, Faculty of Civil Engineering and Geosciences (TU Delft Faculteit Civiele Techniek en Geowetenschappen) to discuss further requirements with Dr. Ir. Ngan-Tillard and Ing. Meijvogel-de Koning, who were very familiar with both the micro-CT and the related software. The Phoenix Nanotom M micro-CT scanner allows samples of up to 1kg weight with a maximum sample diameter of 120mm and can achieve scan resolutions of 0.5-1 micron. Running with 180kV and 0.5mA, it was possible to achieve scan resolutions of up to 3.5µm for the study samples.

While mounting a complete loose tooth into the micro-CT would be possible, it was not recommended since it would result in lower resolution of the dental calculus scans. Instead, the calculus would be removed from the teeth (like for V-1318) and mounted on beeswax attached to a glass stick (see Figure 5-1).

Figure 5-1 V-2451 mounted on a glass stick

5.2 Light microscopy

5.2.1 Sample preparation

In order to be able to examine the organic remains in the samples under the microscope, they were extracted from the mineralised calculus matrix.

The sub-samples of the dental calculus deposits were each placed in an Eppendorf tube, to which 500µL EDTA (Ethylenediaminetetraacetic acid) were added to demineralise the calculus.

During the dissolution process, samples were placed on a vortex. Once the deposits were completely demineralised, the sample tubes were centrifuged for 5-10 minutes at 3000rpm and 450µL of supernatant EDTA were removed. The remaining 50µL were extracted in 10µL aliquots to be mounted on microscopy slides with an equal amount of glycerol. Between analyses, the samples were refrigerated at approximately 4-6°C.

Each slide was examined under an AxioScope microscope at 400x magnification, using both brightfield and cross-polarised light. Photographs of the micro-remains were taken using the AxioVision software. A minimum of five photos were taken per object, with the goal of capturing multiple focal planes and the appearance under cross-polarised light. When possible, the micro-remains were turned in order to accurately describe their morphology in 3D. Additionally, a written description of each object was created. Micro-remains were identified based on their morphology in comparison to published material (e.g. Luo et al., 2019; Rosen, 2015), comparison to modern material from Dr Henry's reference collection, and by expert identification (requesting help from various researchers, as noted in the results below).

5.3 Limitations

With a small sample size of three dental calculus deposits, caution must be applied, as the findings may not be generalisable. The small sample size also makes it impossible to perform meaningful statistical analysis. In addition, while inclusions were recovered from the samples, the majority could not be used for the dietary reconstruction.

The scope of this study was limited due to the time required for scanning and microscopy. In addition, the deposits had to be scanned first before moving on to destructive analysis, which resulted in waiting times.

6 Results

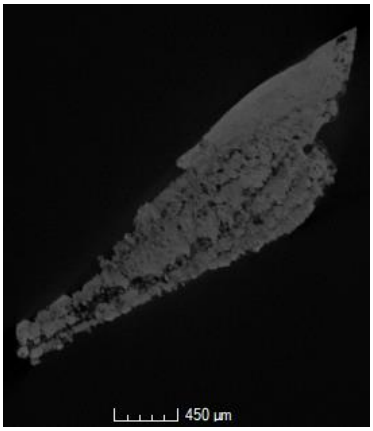
6.1 Micro-CT scan results

The resolutions achieved for the scanned dental calculus samples from Arnhem can be seen in Table 6-1. Despite issues with noise, and at times with mounting the samples securely, the scans successfully showed the inner structure of the dental calculus deposits in high resolution.

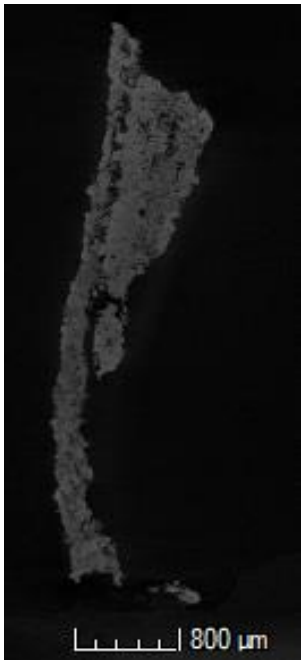
Table 6-1 Scan resolutions

Sample	Resolution
V-1318	5µm
V-2451 - 1	8µm and 4µm
V-2451 -1.1	3.5µm
V-1752	6µm

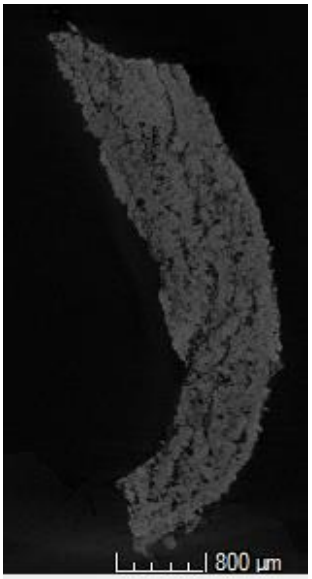
Generally, density differences and layering were observed in all scanned samples. As can be seen in the scans of V-1318, density differences did not only illustrate layering of dental calculus and organic or other material not detectable by micro-CT, but also density differences throughout the calculus itself (see Figure 6-1).



xy



zy



zx

Figure 6-1 Visible layering and density differences in calculus deposit. V-1318.

On this sample, the deposit had chipped away from the tooth cleanly, and the surface previously attached to the tooth was clearly identifiable (see Figure 6-2).

Figure 6-2 Mounted dental calculus deposit (V-1318)

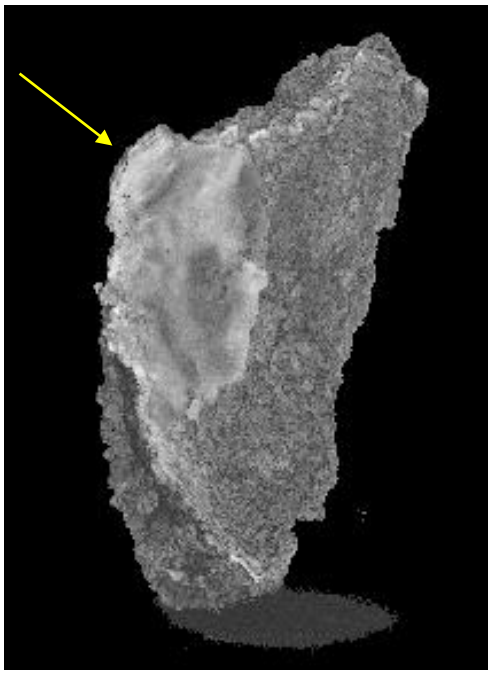


Figure 6-3 3D model of calculus (V-1318)

No evidence of plant microfossils was found in the scans. Following the results from the light microscopy analysis outlined below, none of the inclusions extracted from the samples could be recognised or retraced to their in-situ location within the calculus. Nonetheless, the scans shed light into the structure of each dental calculus deposit, allowing comparisons between them and highlighting the variability of calculus between individuals.

The scans are available at 10.17026.

6.2 Light microscopy/phytolith/starch analysis results

The samples examined under the microscope can be seen in Table 6-2. It should be noted that not all samples examined under the microscope were scanned using micro-CT.

Table 6-2: Samples examined under light microscopy

Find no.	Label	Slides	Volume	µCT scan?	Starch?	Other?
V-1752	N1	3	18mg	unscanned	Yes	Fibres
V-1752	N2	3	27mg	scanned	No	Phytolith, Fibres
V-2451	N3	2	8mg	unscanned	No	Phytolith
V-2451	N4	2	14mg	scanned	Yes	Phytolith, insect hair, mite remains

In all microscopy samples, only two starch granules were identified. Several different types of possible phytoliths were recorded, in addition to the other types of inclusions such as fungal remains or fibres. Each of these categories is described in the sub-sections below.

6.2.1 Starch

Two starches were found in the study samples, both of which were somewhat damaged and may have undergone modification.

The first starch is roughly circular in outline from plan view, with an area of distinct damage or gelatinisation along one margin (see Figure 6-4). The hilum is centric and refractive, and the extinction cross widens towards the margin. The starch is roughly 15µm in maximum diameter, although the undamaged outline is closer to 10µm in diameter. Unfortunately, this starch could not be turned, so it was not possible to confirm its 3D shape. Nevertheless, in shape, appearance of the cross and hilum, it most closely resembles starches from wheat.

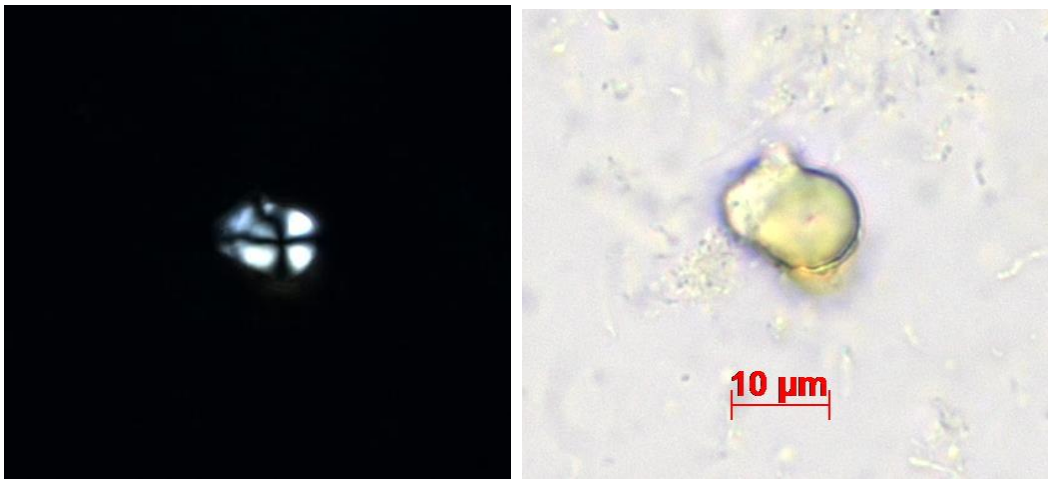


Figure 6-4 Starch with damage, N1 slide 3-31.

The second starch could be examined more easily in terms of its three-dimensional shape, which appeared to be discoidal but rounded-off, with a slight recurve. The hilum was obscured by a fissure but appears to be centric based on the shape of the extinction cross. The extinction cross appeared differently depending on the perspective, from one perspective it looked like a typical Maltese cross (four straight arms), while from the other it appeared to be damaged (see Figure 6-5).

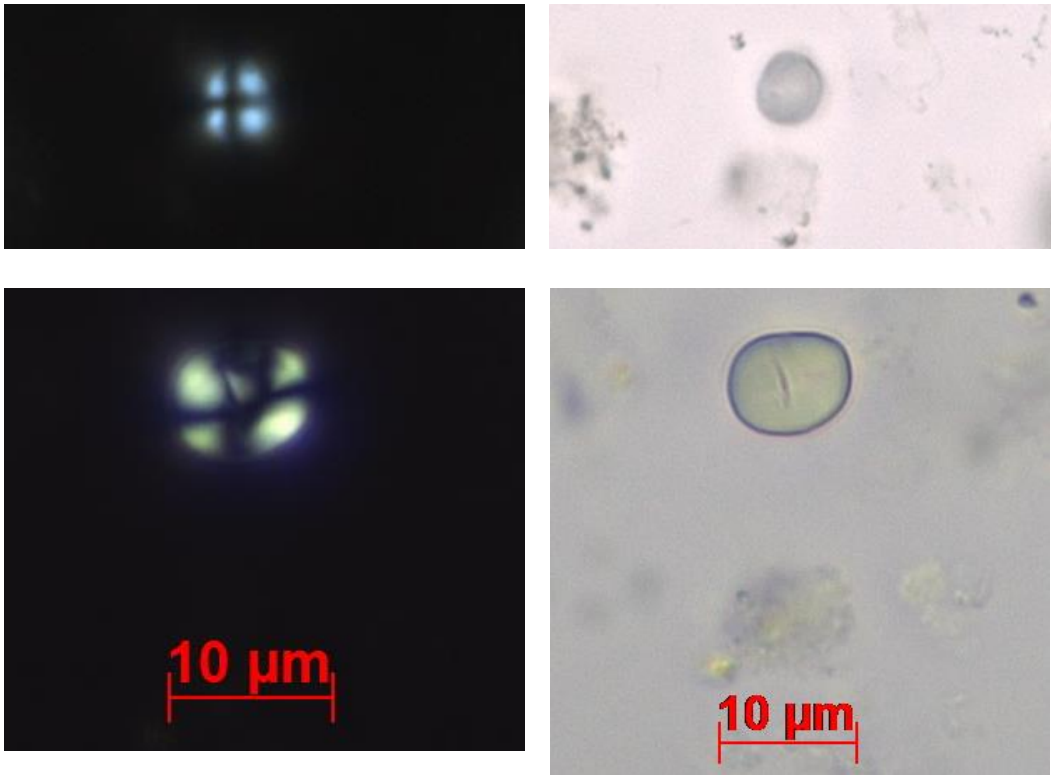


Figure 6-5 Potential wheat starch with damage. N4, slide 1-1.

While these starches are not distinctive enough to allow identification to species level, they share affinities to starches from wheat or other members of the Triticeae (which also include barley and other relatives).

6.2.2 Phytoliths

Two potential phytoliths were found in the samples, of which one may be a grass phytolith. One was similar to a 'bilobate' which is a shape found frequently in grass species. The other was more amorphous or blocky. It was not possible to make further identification of this particle (Rosen, 2015,).

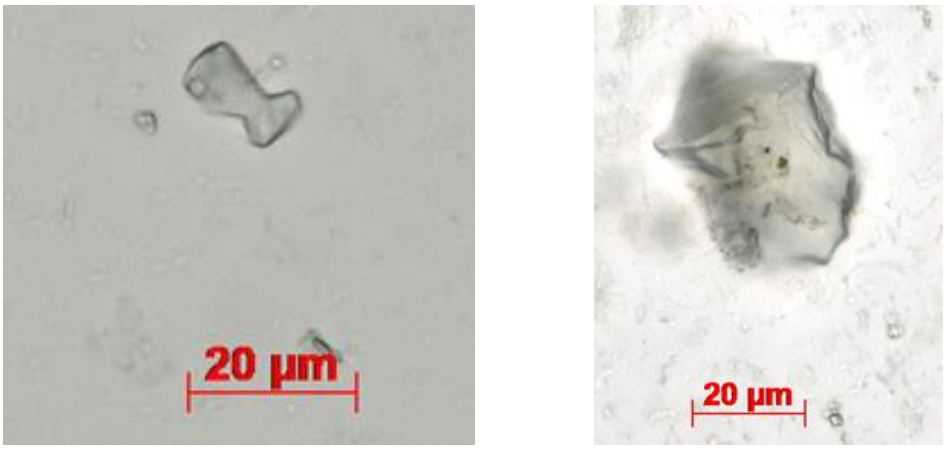


Figure 6-6 Possible grass phytolith (left), unidentified phytolith (right)

6.2.3 Fungal remains

Fungal remains, such as spores and hyphae, were present in all samples.

Some of the fungal spores exhibited morphologies that allowed a closer identification. With the help of Dr L. Shumilovskikh, it was possible to further identify one of the fungal spores, *Brachysporium obovatum,* from sample N4, slide 1 (10) (see Figure 6-7). Many of the *Brachysorium* species are associated with saprobic conditions and decaying wood of different substrates (Réblová & Seifert, 2004).

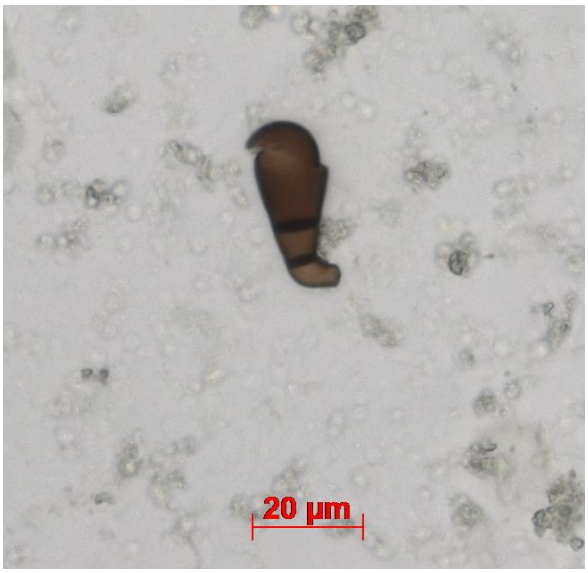


Figure 6-7: Brachysporium obovatum (Réblová & Seifert, 2004) from N4 slide 1- 10b

Its appearance is similar to another spore from the same sample (Na, slide 1, 14), which could not be identified as belonging to a specific fungus (see Figure 6-8).

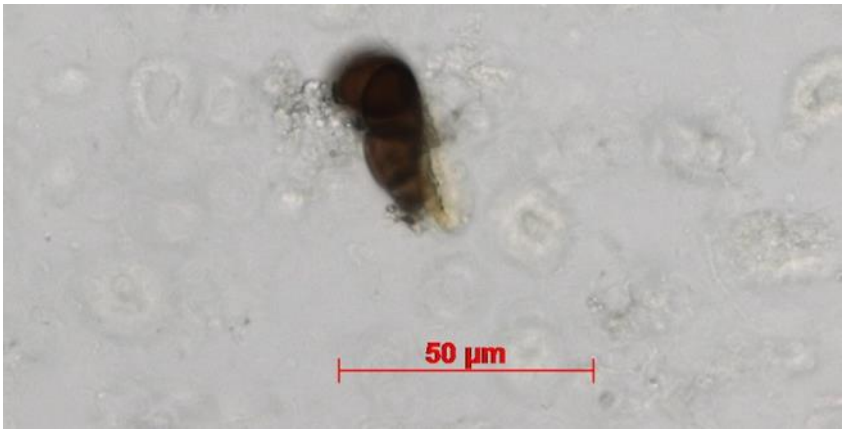


Figure 6-8 Conidial spore, unclear which fungus. N4 slide 1–14b.

Other, less clearly identified fungal remains include a possible ascospore (see Figure 6-9).

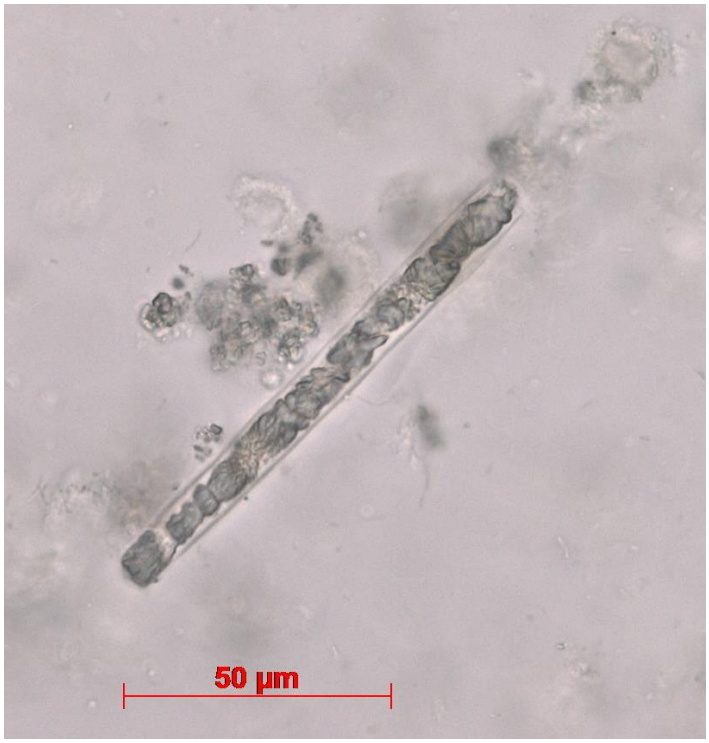
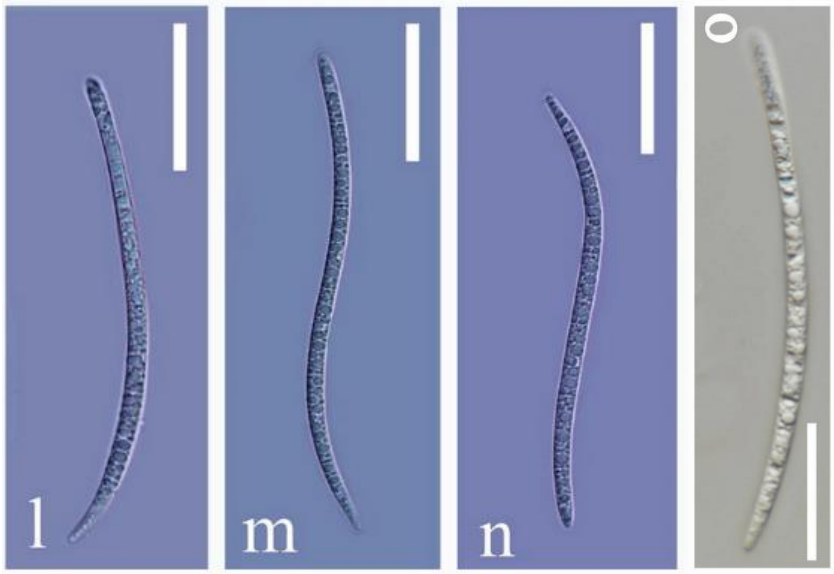


Figure 6-9 Possible ascospore. N4, slide 1-3.

This assessment is based on the ascospore morphologies of *Ceratosphaeria aquatica* (top) or *Phaeoisaria filiformis* (bottom) as described by Luo et al. (2019):



Adapted from Fig.42 and Fig. 11 by Luo et al. (2019): l-n: *Phaeoisaria filiformis* ascospores, o: *Ceratospaeria aquatica* ascospore. Scale bar: 20µm

6.2.4 Fibres

Thanks to the help of Professor Walter F. Rowe from the Department of Forensic Sciences, The George Washington University, it was possible to narrow down the identification of two bundles

of fibres. Potentially, the fibres in Figure 6-10 could be fragments of yarn, made from a seed fibre such as cotton (Walter F. Rowe, personal communication, December 15, 2021).

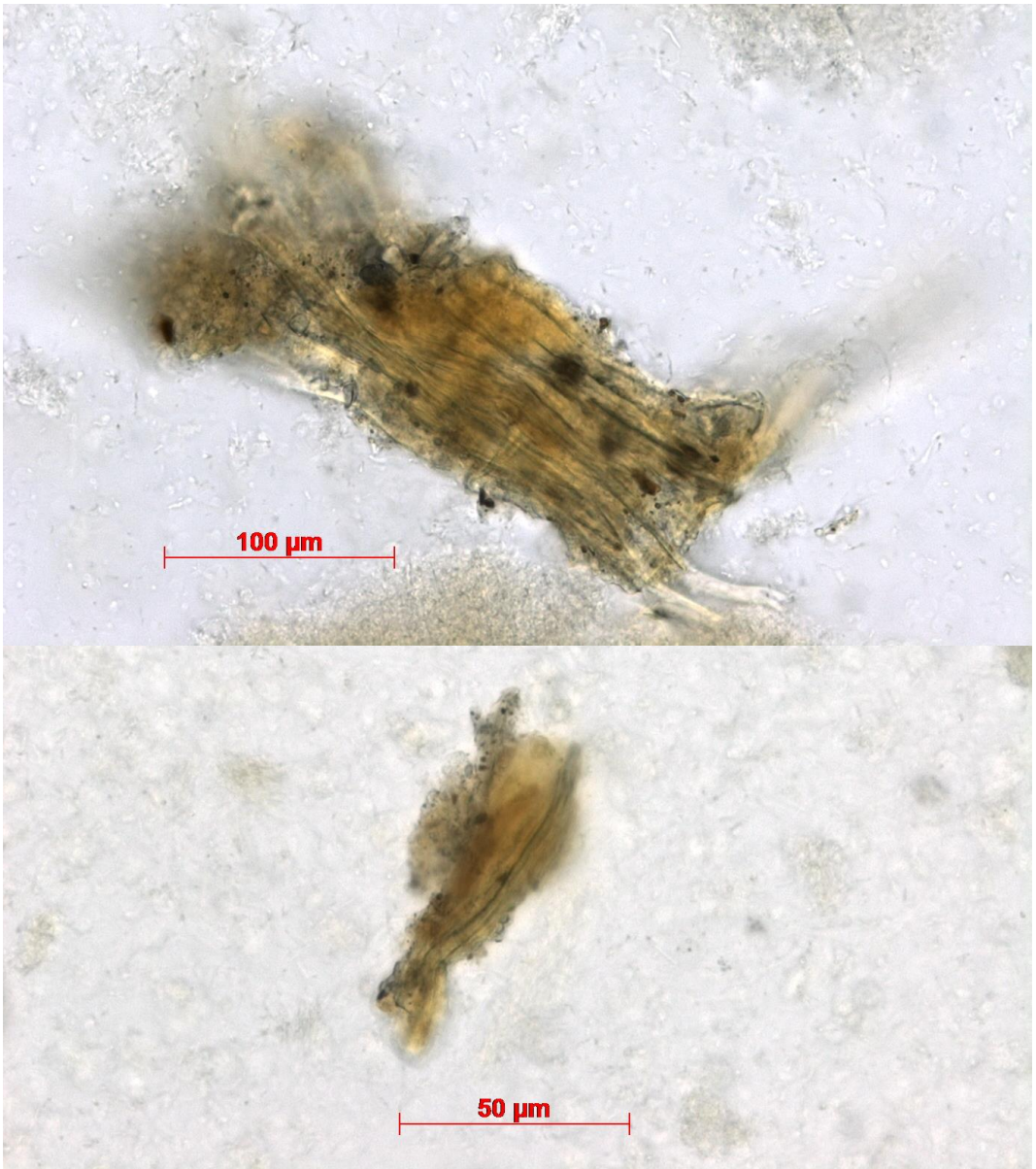


Figure 6-10 Potential cotton fibre yarn. N1 slide 3-18e (top) and N2 slide3-9b (bottom).

A more secure identification of cotton fibres would have required polarised images.

6.2.5 Other

Two potential insect hair fragments were found (see Figure 6-11), although it is unclear which animal they originate from.

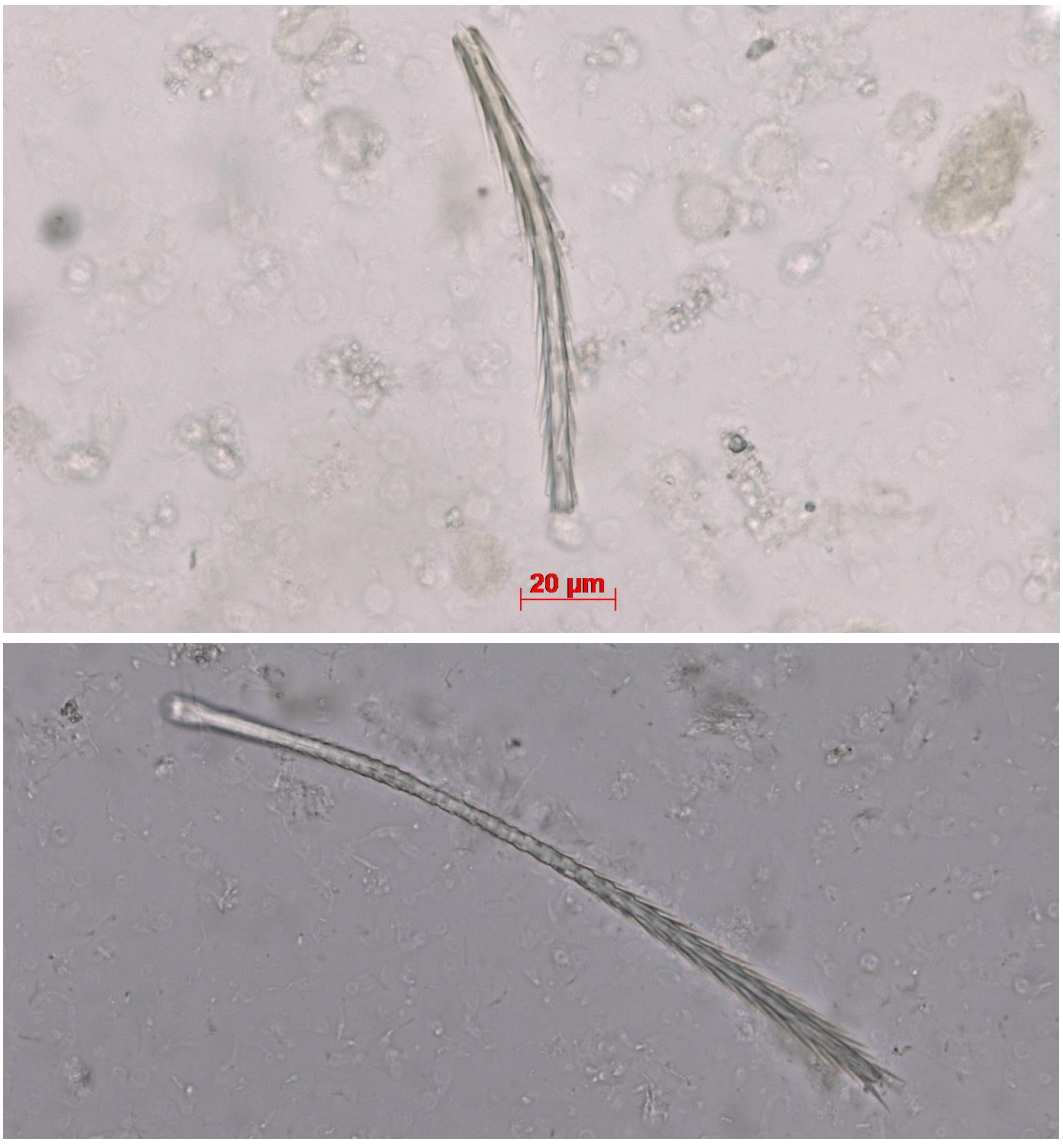


Figure 6-11 Insect hair

N4 slide-22b (top) and N1 slide2-7f (bottom) are most likely fragments of insect hairs.

Dr Carla J. Dove, from the Feather Identification Lab at the Smithsonian, was able to confirm that these remains are not feather barbules as originally suspected. This assessment is based on the presence of a root (bottom picture), as well as the morphology of the smaller hairs.

A different type of animal remain was found in sample N4, slide 2. Dr L. Shumilovskikh was able to identify this as mite fragment (type HdV-36, see Figure 6-12).

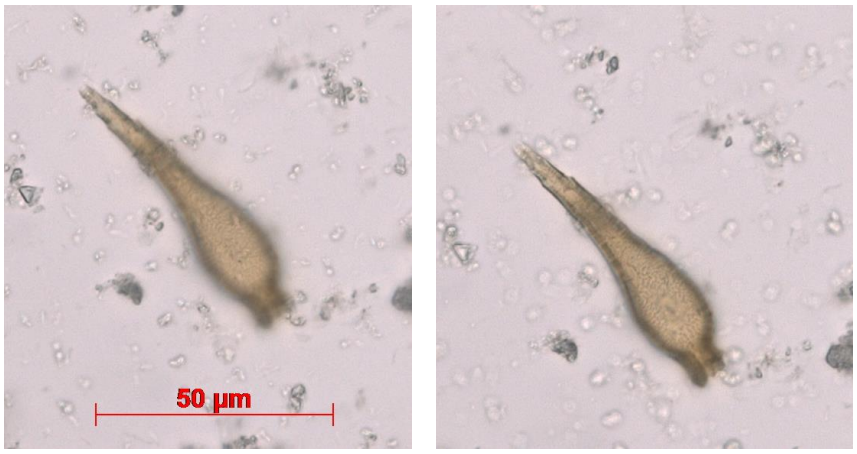


Figure 6-12 Mite fragment (HdV-36)

The HdV-No. system was introduced in 2010, and identifies the lab in which a non-pollen palynomorph has been described (Miola, 2012). The first report of this mite type was published by van Geel (1978, p.76).

7 Discussion

This chapter discusses the results of the m-CT scan and dental calculus analysis.

The results in the previous chapter indicate that micro-CT is not a suitable method for the research questions posed in this study. Nonetheless, organic inclusions were identified thanks to traditional destructive dental calculus analysis. This chapter, therefore, moves on to discuss the applicability of micro-CT and the identified organic microfossils in relation with their occurrence in archaeological dental calculus.

7.1 Micro-CT results and use of the method

The most striking result to emerge from the micro-CT data is that none of the inclusions identified under the microscope could be recognised in the scans. While this outcome was not what we had initially hoped for, it is an important contribution to our understanding of micro-CT in archaeology, nonetheless.

For one, we have learned that plant micro-remains are not incorporated into the dental calculus matrix in a way that allows them to be recognised in the micro-CT scans. They do not appear to be trapped individually, with their outline being visible as a result of being surrounded by calculus from all sides. Rather, it appears the plant micro-remains that were identified under the microscope may rather be deposited in thin layers or clusters of material, obscuring their potentially diagnostic silhouettes. In future research, this observation may help shed light into different dental calculus maturation stages in connection with mineralisation status and incorporation of organic inclusions (cf. Cooper (2017) on calculus maturation stages). Additionally, further investigations into the physical structure of these dental calculus scans are still possible. For example, void volume analysis may further our understanding of internal structure and the processes by which micro remains are incorporated into dental calculus.

No published studies attempting a similar application of micro-CT and organic dental calculus inclusions were found. While this legitimises the contribution of this study to methodological trial and error, this also means there is no other data for comparison. Since the application of micro-CT is still limited due to time and financial constraints (Schoeman et al., 2016), it is an important part of method testing to establish what the method can and cannot achieve.
The micro-CT data has provided valuable insight into the structure of each dental calculus deposit and highlighted the possibilities of differences in deposits between

individuals. Luckily, the scan data of each dental calculus deposit continue to be available despite the original deposits' subsequent destruction for analysis. As such, regardless of not reaching the research aims set for this particular study, the loss of information by a subsequent destructive analysis method was minimised and further research could be conducted on the samples in the future.

The scans are available for other researchers on DANS Easy (Data Archiving and Networked Services, https://doi.org/10.17026/dans-2av-8agb).

7.2 Interpreting the diet from Arnhem based on calculus inclusions

An initial objective of the project was to reconstruct the diet of several individuals from Arnhem based on the plant microfossils extracted from dental calculus.

It was hypothesised that the calculus deposits would contain sufficient amounts of identifiable starch granules and diagnostic phytoliths to allow for inferences about plant consumption. However, that was not the case.

The most important staple foods in the late to post-medieval period in the Lowlands are assumed to be cereal-based products such as bread and porridge, consisting of rye, barley, oats, and naked wheat (De Cupere et al., 2021). Buckwheat is also mentioned in this category due to its similar use and preparation, despite not being a cereal. Finally, during the 16th and 17th centuries, rice became available as well (De Cupere et al., 2021). Other consumed foodstuffs rich in starch include peas (De Cupere et al., 2021). From isotope analysis performed on other individuals from the Arnhem site, it is known that the typical diet included both terrestrial foods of low trophic level (vegetables, certain meats) as well as marine foods of higher trophic levels (Zielman & Baetsen, 2020, p.638). Between the 13th and 17th century, individuals in Arnhem would have seen the emergence of various foodstuffs, including sugar, coffee, tea, and tobacco (Zielman & Baetsen, 2020, p.431).

The starch granule extracted from N1 may stem from wheat and appears to have suffered some damage. However, it is unclear whether this damage is a result of taphonomic processes or food preparation procedures.

The potential phytoliths identified in this study were not morphologically distinct and thus could not contribute to the dietary reconstruction.

Relatively few findings relevant to dietary reconstruction were made in this analysis. This could be a result of the small sample size in addition to an element of chance.

7.3 Other inclusions

Fungal remains from dental calculus have been interpreted as evidence for mushroom consumption when identification allowed the narrowing down of fungus type (Gismondi et al., 2018,p.563; Power et al., 2015). However, the few more closely identified fungal remains from this study are associated with soil and decaying wood (Réblová & Seifert, 2004), which would suggest they were unlikely present due to consumption. They may have found their way into cracks in the dental calculus post-deposition, rather than having been incorporated via consumption during the individuals' lifetime. Another possible inclusion pathway is accidental inhalation of airborne spores from the surrounding air (Hardy et al., 2017,p.3).

Cotton fibres have been observed in dental calculus in previous studies, such as by Blatt et al. (2011), who identified cotton fibres in calculus from Ohio dated AD900-1100.
In the Netherlands, cotton garments only became common after cotton spinning was widely mechanised after the 1860s (de Groot & Schrover, 1995). Fibres can become entrapped in dental calculus via different pathways, such as inhalation or the use of teeth as tools (Radini et al., 2017).

Insect remains are commonly found on archaeological materials and may be used for environmental reconstruction. However, not all remains are identifiable and thus useful for inferring information about past conditions. In addition, insect remains in samples may be the result of post-excavation contamination (Henry, 2020). Studies of mite communities can shed light into environmental changes. Mites are known to be good indicators of anthropogenic changes, such as trampling or soil fertilisation (Seniczak et al., 2014). While the mite remains identified in this study is too little to confidently infer information about the environment, generally speaking, mites have been associated with wet conditions, with brackish or marine influences (Bas van Geel et al., 2003).

The excavation report explains that intestinal parasite eggs of *Trichuris*, *Ascaris, Eimeria* and *Sordaria* were found in the soil, but not with the graves (Zielman & Baetsen, 2020, p.641). While parasite eggs may be preserved in dental calculus and provide a clearer indication for contact with the mouth (Juhola et al., 2019), no eggs could be identified in the samples.

8 Conclusions

This project has been one of the first attempts to examine organic inclusions in archaeological dental calculus using micro-computed tomography. The purpose of this study was to compare micro-CT and light microscopy as detection and examination methods for plant micro-remains in dental calculus, and to gain further information about diet in late medieval to early modern Arnhem.

One research aim was to establish the appearance of organic micro-remains in the micro-CT scans. This endeavour was unsuccessful, since plant micro-remains appear to not only be too small, but also deposited in a manner which does not allow assessment of diagnostic morphological features. Therefore, this study was able to conclude that micro-CT generally is not a suitable method for this specific purpose. However, thanks to the non-destructiveness of micro-CT scanning, future research could be conducted on the samples by analysing the scans. This serves to strengthen one of the main advantages of the method, as well as the argument for development and testing of non-destructive analysis methods.

Another aim was to utilise the extracted plant microfossils to gain insight into the plant food consumption in Arnhem from the late medieval to early modern period. However, the low number of identifiable plant microfossils with significance for dietary reconstruction made it difficult to infer information. In addition to the uncontrollable factor of plant microfossil presence in a given calculus deposit, this study was limited by a small sample size. Therefore, findings may not be generalisable.

Despite its limitations, this work offers valuable contributions to the development of non-destructive methodologies in archaeology. Prior to this study it was difficult to make predictions about the contribution of high-resolution micro-CT to dental calculus analysis and dietary reconstruction. These findings have a number of practical implications which will hopefully influence future research planning.

Abstract

Dental calculus, which is mineralised dental plaque that preserves well in archaeological contexts, is increasingly of interest for researchers for several reasons. One of them is its potential to preserve plant micro-remains which can be used to infer information about diets in the past. These microscopic plant parts may exhibit taxon-specific morphologies, and their integration into dental calculus provides strong evidence in support of food consumption. However, traditional methods used to extract plant micro-remains from dental calculus are destructive, which can be a concern depending on the availability of the material. Therefore, this study aimed to test non-destructive micro-CT scanning as a method to examine plant micro-remains by comparing the results to the findings of destructive light microscopy analysis.

Materials and Methods: Three dental calculus samples from the site of Arnhem Jansbeek/Eusebiuskerk (The Netherlands), dating from roughly 1350 to 1829 AD, were scanned using a Phoenix Nanotom micro-CT scanner. The resulting scans revealed the complete structure of the samples in resolutions ranging from 3.5 to 8µm. Aliquots of the scanned samples were then dissolved using EDTA, and the extracted plant micro-remains analysed using an AxioScope microscope at 400x magnification. After identification of some of the plant micro-remains using light microscopy, an effort was made to identify them in the micro-CT scans.

Results: Light microscopy was used to identify two starch grains, two potential phytoliths, fungal remains, fibres, and fragments of a mite. None of the inclusions identified under light microscopy could be recognised in the micro-CT scans.

Discussion: The two starch grains share affinities with wheat starch, whereas the two phytoliths could not be identified further. This severely limited possibilities for a dietary reconstruction. The fact that none of the organic inclusions from the calculus could be identified in the micro-CT scans suggests that plant micro-remains are either too small to be recognised or are incorporated into the dental calculus matrix in a way that does not reflect their diagnostic morphologies. While the results did not provide material for a dietary reconstruction, and the plant micro-remains could not be identified in the micro-CT scans, testing new applications of non-destructive methods is still important. The micro-CT scans are available for future research on DANS EASY (https://doi.org/10.17026/dans-2av-8agb).

Conclusion: This study contributed to the development of non-destructive analysis methods by showing that micro-CT is not a suitable non-destructive method for the

identification of plant micro-remains in dental calculus deposits. The fact that, while the research aims were not all fulfilled as expected, the micro-CT scans will be available for other research, underlines the value of non-destructive methods.

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