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SYMPOSIUM IN MEMORIAM. ARTHUR C. AUFDERHEIDE
THE SCIENTIST, THE FRIEND (1922-2013)

PALEOGENETICS AND MUMMIES

Albert Zink

Eurac Research - Institute for Mummy Studies
Viale Druso 1, 39100 Bolzano, Italy
albert.zink@eurac.edu

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Abstract. The molecular analysis of ancient DNA represents a unique opportunity for the study of human evolution, population dynamics, and disease evolution in mummified human remains. The investigation of ancient pathogen DNA has led to the detection of a wide range of bacterial, protozoal and viral infections in ancient tissue samples. In the 1990s and 2000s, Arthur C. Aufderheide, together with his colleagues, significantly contributed to the development of this field with his groundbreaking work on the molecular identification of tuberculosis and Chagas disease in South American mummies.
More recently, the introduction of next generation sequencing (NGS) technologies and DNA capture techniques, has further improved the opportunity to study ancient human remains. One of the first mummies for which whole genome reconstruction was attempted successfully, is the 5,300-year-old Tyrolean Iceman. The sequencing revealed detailed information on his ancestry, his physical appearance, physiological parameters and the presence of pathogens and disease susceptibility.

Keywords. Ancient DNA. Mummies. Paleogenetics. Iceman. Pathogens.

1. INTRODUCTION

In addition to the achievements and groundbreaking work of Art Aufderheide (Fig. 1) in the area of mummy studies, he contributed greatly to the development

Fig. 1. Arthur Aufderheide during his public lecture at the 1st Bolzano mummy congress in 2009.

and onset of the field of paleogenetics. In particular, he was interested in the molecular detection of ancient pathogen DNA in mummies from South America and, together with his colleagues, he published some of the first articles dealing with the molecular identification of infectious diseases in mummified remains, such as tuberculosis and Chagas disease (Salo *et al*. 1994; Arriaza *et al*. 1995; Guhl *et al*. 1999). From the beginning, he realized the great potential of ancient DNA research in mummy studies, but was also aware of the limitations and possible risks with regard to contamination issues and degradation processes. As in all aspects of his scientific work on mummies, Art Aufderheide was a pioneer and advocate for applying modern methods to mummy research. This contribution is dedicated to the outstanding work of Arthur Aufderheide with a particular focus on paleogenetic research in mummies.

1.1. Paleogenetics

The field of ancient DNA or paleogenetics emerged about 30 years ago, when the first studies on the retrieval of DNA from ancient specimens have been published (Higuchi *et al*. 1984; Pääbo, 1985). Since then, the field has significantly changed from the detection of small DNA fragments from single specimens to large-scale genome wide studies of past populations (Skoglund *et al*. 2012). By

using modern sequencing technologies, it became possible to perform wide-ranging research in human evolution (Prüfer *et al*. 2014), population dynamics and past migration patterns (Brandt *et al*. 2013; Sankararaman *et al*. 2014), and gain insights into the phenotypes of our ancestors, such as skin and eye color (Rasmussen *et al*. 2010). Although the detection of ancient DNA is still limited by factors that lead to the degradation of DNA, such as the activity of enzymes and microorganisms, hydrolytic and oxidative processes, high temperature and humidity, soil DNA may survive up to 800,000 years in the case of Pleistocene fauna (Orlando *et al*. 2013) or 400,000 years for hominin fossils from the Iberian Peninsula (Meyer *et al*. 2014). The risk of amplifying exogenous contaminants and the highly fragmented endogenous DNA used to be a major limitation and challenge in the era of PCR-based approaches (Pääbo *et al*. 2004). This was mainly overcome by the application of high-throughput sequencing technologies and targeted enrichment strategies. This approach can be used to identify endogenous ancient sequences by looking for DNA degradation patterns that accumulate over time and which are typical of ancient DNA (Krause *et al.* 2010).

1.2. Ancient pathogen DNA

As in other studies in the field of paleogenetic research, the retrieval of ancient pathogens was initially mainly based on the PCR amplification of small and specific DNA fragments in human remains to prove a supposed infectious disease. First successful studies were performed in the 1990s on skeletal and mummified remains, including the work of Arthur Aufderheide, where *Mycobacterium tuberculosis* DNA was detected leading to the confirmation of a tuberculosis or leprosy infection in the investigated specimens (Spigelman & Lemma, 1993; Salo *et al*. 1994). In the following years the diagnostic abilities of this approach were further demonstrated by the detection of several other pathogens such as *Mycobacterium leprae* (Rafi *et al*. 1994), *Plasmodium falciparum* (Taylor *et al*. 1997), *Escherichia coli* (Fricker *et al*. 1997), *Yersinia pestis* (Drancourt *et al*. 1998), *Trypanosoma cruzi* (Guhl *et al*. 1999), *Treponema pallidum* (Kolman *et al*., 1999), *Corynebacterium spp.* (Zink *et al*. 2001) and *Leishmania donovani* (Zink *et al*. 2006). Most of these studies were designed to detect ancient pathogen DNA in single cases or small series of selected samples. In a few, more recent studies on the molecular detection of *M. tuberculosis* complex DNA, larger series were analyzed including specimens with nonspecific or even without morphological alterations probably due to an infection with tuberculosis (Faerman *et al*. 1997; Zink *et al*. 2001; Mays *et al*. 2002; Fusegawa *et al*.

2003; Zink *et al*. 2003a). These attempts demonstrated the high capability of palaeomicrobiology. The fact that tuberculosis can be detected in morphologically conspicuous or even unremarkable specimens, underlined the unique diagnostic possibilities. It was no longer just a tool to confirm a previous diagnosis based on morphological or radiological results, but became an important new method in palaeopathological research. The molecular investigation of larger sample series further allowed an evaluation of the frequency of tuberculosis in historic populations.

In more recent studies, advances in the application of DNA array capture and next-generation sequencing (NGS) technologies enabled full genome investigations of ancient pathogens that have led to new insights into disease evolution. As an example, the combination of DNA array capture and next-generation sequencing (NGS) technologies allowed the reconstruction of complete bacterial genomes of *Yersinia pestis* and *Mycobacterium leprae* strains from Medieval Europe, showing a remarkable genetic conservation of both pathogens throughout the last 1000 years (Schuenemann *et al*. 2013; Bos *et al*. 2011; Wagner *et al*. 2014). Over the last years, an increasing number of ancient bacterial, viral and eukaryotic pathogens have been successfully sequenced that has led to the newly established branch of ancient pathogen genomics (Spyrou *et al*. 2019).

1.3. Ancient DNA and mummies

The first studies dealing with the detection of ancient DNA in mummies did mainly represent single case studies, such as the first mitochondrial DNA analysis of the Tyrolean Iceman (Handt *et al*. 1994) or the PCR-based detection of *M. tuberculosis* DNA in a South American mummy (Salo *et al*. 1994) and Egyptian mummy (Nerlich *et al*. 1997). As described above, in just a few studies and mainly focusing on ancient pathogens, a wider approach was applied to mummy studies, by analyzing a higher number of samples using molecular techniques (e.g. Zink *et al*. 2003a). Again, Art Aufderheide made an important contribution at that time with his work on Chagas disease in South American mummies, dating back up to 9000 years (Aufderheide *et al*. 2004). The early paleogenetic work in mummies was accompanied by skepticism on the survival of DNA in mummies, in particular those deriving from hot and dry climates, such as from Egypt (e.g. Marota *et al*. 2002). This has led to a long-lasting controversy in the field that tended to develop into a fundamental debate and away from a scientific discourse (Zink & Nerlich, 2003; Gilbert *et al*. 2003). The controversy was finally put to rest with the onset of next-generation sequencing in the field of mummy studies. In 2012, Keller and

colleagues published the first work on a complete genome sequence of an ancient mummy, the 5300-year-old Tyrolean Iceman (Keller *et al*. 2012). In the following years, the application of next-generation sequencing technologies has led to an increasing number of genomic information on mummies (Gomez-Carballa *et al*. 2015) and the reconstruction of pathogen genomes, such as those of *M. tuberculosis* (Bos *et al*. 2014; Kay *et al*. 2015), hepatitis B virus (Kahila Bar-Gal *et al*. 2012; Patterson Ross *et al*. 2018) and *H. pylori* (Maixner *et al*. 2016). The publication of genomic DNA from ancient Egyptian mummies by Schuenemann and co-workers (2017) finally demonstrated that DNA may survive in mummies from hot climates and thereby proved the early skeptics wrong.

2. ANCIENT DNA AND THE ICEMAN

The application of modern sequencing technologies opened new ways for the study of the 5300-year-old glacier mummy, commonly known as Ötzi or Iceman. The paleogenetic studies of the Iceman revealed new and important information on his ancestry, disease predisposition and the presence of pathogens. In the following, an overview and the latest findings in the Iceman will be given and summarized.

2.1. The Iceman's genome

The paleogenetic study of the Iceman started with the analysis of the hypervariable region (HVS1) of his mitochondrial DNA (Handt *et al*. 1994) and later, the entire mitochondrial genome was sequenced (Ermini *et al*. 2008; Rollo *et al*. 2006). In 2012, with the improvements in modern sequencing technologies together with the excellent preservation of his biomolecules as demonstrated in other studies (e.g. Janko *et al*. 2010), it became possible to perform a whole-genome sequencing analysis of the Iceman. Therefore, DNA was extracted from a small bone sample taken from the Iceman's left ilium under sterile conditions in the Iceman's preservation cell using established protocols. Subsequently, a sequencing library was generated and high-throughput sequencing was performed on a SOLiD 4 platform (Life Technologies facilities, Foster City, CA, USA). The next-generation sequencing approach revealed about 40% reads that mapped unambiguously to the human reference genome. Thereby, an overall coverage of the human genome of 96% was retrieved. A comparison with the previously published mitochondrial DNA showed a full concordance and thereby confirmed the authenticity of the ancient Iceman DNA (Keller *et al*. 2012).

The sequencing results revealed many new insights into the presence of diseases, physiological parameters and the ancestry of the glacier mummy. It could be shown that he had likely brown, eyes, blood group 0 and that the he was lactose intolerant. The Iceman genome underwent a further detailed analysis of genetic risk factors, specifically for DNA sequence variations, so called SNPs (single nucleotide polymorphisms) that are linked with diseases. The most intriguing finding was that the Iceman showed a strong genetic pre-disposition for increased risk for coronary heart disease (CHD). This was of particular interest as the CT scans of the Iceman computer tomography scans of the Iceman revealed major calcification in carotid arteries, distal aorta, right iliac artery (Fig. 2) and coronary arteries, as strong signs for a generalized atherosclerotic disease (Murphy *et al*. 2003; Gostner *et al*. 2018). The genetic pre-disposition could have significantly contributed to the development of the arterial calcifications.

Other cardiac risk factors, such as overweight, tobacco smoking, lack of physical activity and a high fat diet, can generally be ruled out in the glacier mummy. Based on the previous studies mentioned above, the Iceman was walking intensively in the mountain area, he had a slim and well-trained body, and his nutrition was well-balanced with low amounts of proteins and saturated fats. Tobacco was not avail-

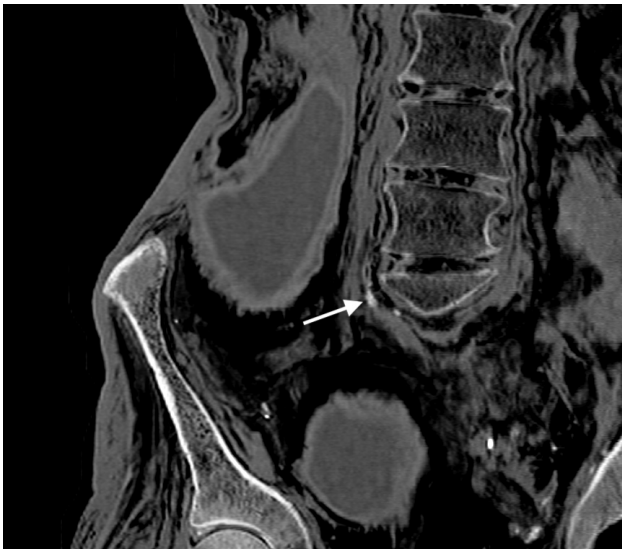

Fig. 2. Coronal CT reconstruction showing calcifications in the right iliac artery (arrow).

able in that time period, although dark staining in his lungs indicate that he inhaled smoke during his life, most likely from open fires (Zink *et al*. 2014).

2.2 Ancestry of the Iceman

The genome sequence analysis of the Iceman further enabled a detailed analysis of his mtDNA and Y chromosome for a better understanding of his ancestry and his relation to early European populations. The Iceman belonged to a rare Y haplogroup (G2a2b), which is present today at a low frequency in Europe. Only on the islands of Corsica and Sardinia this Y haplogroup is still represented relatively frequently. This has led to the conclusion that the Iceman and the population on Sardinia and Corsica had common ancestors who immigrated to Europe during the Neolithic period. In wide parts of the European mainland, the representatives of this group underwent several admixture events in the course of time and only in remote regions such as the Mediterranean islands they likely survive in greater numbers until today (Sikora *et al*. 2014). In contrast, it was shown that his mitochondrial DNA (mtDNA) belongs to a novel lineage of haplogroup K1 (K1f) that seems to be not present in extant populations. In a detailed analysis, haplogroup K DNA samples were analyzed from more than 800 individuals analyzed in previous studies and collected new modern samples from South Tyrol. The results were compared to more than 1000 complete K1 mtDNA sequences from modern populations. As a result, it was shown that the K1f haplogroup is most probably absent in present-day populations and therefore, it was suggested that mtDNA Iceman´s lineage could have disappeared during demographic events starting in Europe from c. 5,000 years BP (Coia *et al*. 2016).

2.3 *H. pylori* in the Iceman's stomach

In a systematic re-evaluation of the radiological examinations carried out on the Iceman, the completely filled stomach could be identified (Gostner *et al*. 2013). This has led to a thorough investigation of the Iceman's stomach content. Thereby, samples of the Iceman's stomach were subjected to next-generation sequencing and targeted enrichment that allowed the detection and full genome reconstruction of the stomach pathogen *Helicobacter pylori* (Maixner *et al*. 2016). The pathogen is of particular interest for clinicians as well as evolutionary biologists, as it is found in approximately half the world's human population and about 10% of the carriers develop stomach diseases, such as gastritis, stomach ulcers or gastric carcinoma.

By the application of a thorough bioinformatics sequence analysis, the ancient *H. pylori* of the Iceman was classified as virulent strain that is today associated with inflammation of the gastric mucosa. In addition, using a paleo-proteomics approach, proteins in the Iceman's stomach were observed that are known to be involved in the inflammatory host response. These results supported the presence of a possible stomach disease in the Iceman, but as the stomach mucosa was not preserved, it remained impossible to obtain a definite diagnosis and reveal whether the Iceman could have suffered from gastritis, stomach ulcer or even gastric carcinoma.

A surprising result was obtained by the phylogeographic analysis of the ancient *H. pylori* strain. Instead of hpEurope, the modern *H. pylori* strain found in most Europeans, the 5,300-year-old bacterium matched to the modern population hpAsia2, commonly found in Central and South Asia (Fig. 3). As it can be clearly ruled out that the Iceman is not of Asian origin, but grew up and lived at the southern side of the Alps, the results had to be interpreted differently. A possible answer was found by considering the evolution history of the stomach pathogen that is closely linked to its human host. The *H. pylori* strains found in most Europeans today (hpEurope) have putatively originated from recombination of the two ancestral populations Ancestral Europe 1 and 2 (AE1 and AE2). It was further assumed that the admixture of the two ancestral strains has already happened in the Middle East or Western Asia between 10,000 and 52,000 years ago and hpEurope was introduced into Europe as the first Early Neolithic farmers arrived in Europe (Moodley *et al*. 2012). In contrast to the evolutionary model based on the study of modern *H. pylori* strains, the Iceman's strain is a nearly pure representative of the bacterial population of Asian origin (AE1) that existed in Europe before hybridization (Maixner *et al*. 2016). This has led to the suggestion that the African population (AE2) arrived in Europe within the past few thousand years, which is

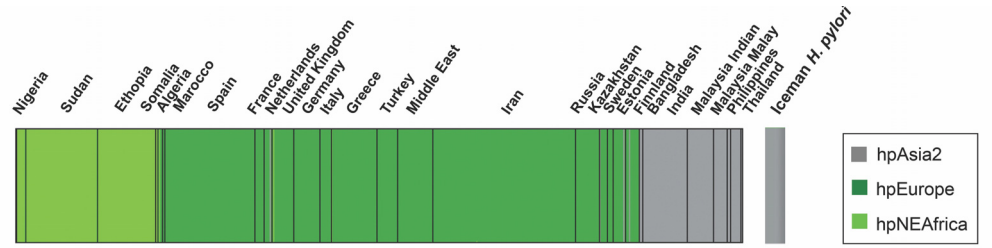

Fig. 3. Phylogeographic assignment of the Iceman's H. pylori strain (Bayesian cluster analysis performed in STRUCTURE) showed a match to the modern population hpAsia2.

much more recent than previously hypothesized. In summary, the metagenomic approach and genome reconstruction provided indications that the ancient *Helicobacter pylori* strain was a potentially virulent strain that is today strongly associated with gastric disease. Moreover, the study allowed new interesting insights into the ancestry and evolution of the pathogen and underlined the high complexity of ancient European population history.

2.4 The Iceman's last meal

In addition to the detection of a stomach pathogen, the molecular investigation of the completely filled stomach of the Iceman also allowed a detailed reconstruction of his last meal (Maixner *et al*. 2018). Therefore, a combined approach, including classical microscopy as well as modern molecular analyses using the whole spectrum of available biomolecules (ancient DNA, proteins, metabolites, lipids), has been applied to samples taken from the stomach content. The initial macro- and microscopic analysis of the samples showed an extraordinarily well preservation of the material with compact pieces of plant and animal food remains that display a hydrophobic "fatty-like" character (Fig. 4). The animal macro fossil remains

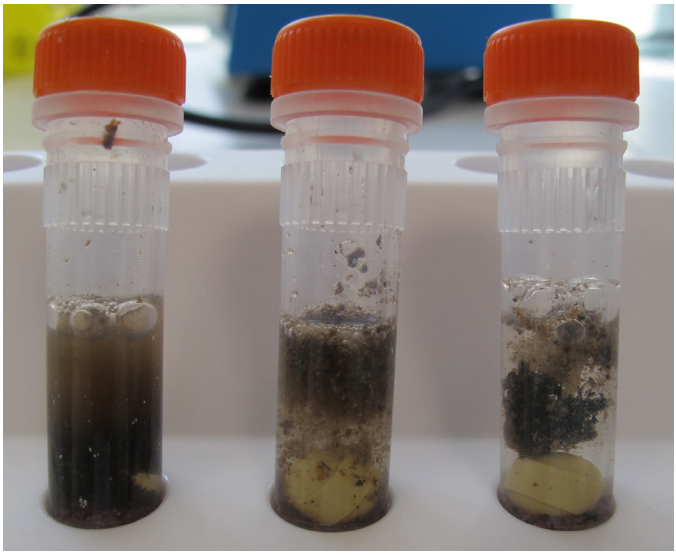

Fig. 4. Samples of the colon (left), duodenum (center), and stomach (right) rehydrated in phosphate buffer. The stomach content shows a hydrophobic "fatty-like" character.

consisted primarily of adipose tissue and muscle fibers. By using a multi-omics approach (metagenomics, proteomics and lipidomics) the specimens could be clearly assigned to the wild mountain goat ibex and, in addition, traces of red deer DNA were detected. A microscopic and spectroscopic analysis of the ibex meat fibers provided insights how the Copper Age meat has been prepared. The well-preserved meat fibers still show striated fiber structures that disappear as soon as meat gets cooked or fried and therefore, indicated that the Iceman consumed smoked or air-dried meat. Lipid analysis of the high proportion of fat in the Iceman's stomach revealed that the triglycerides distribution patterns are consistent with the consumption of ibex muscle fat and subcutaneous adipose tissues, but not with the consumption of dairy products. Therefore, it was concluded that the Iceman used ibex as a food source for both the meat and the fat in his last meal. The major part of plant macro remains in the Iceman's stomach were assigned to cereal bran. The microscopic and molecular analysis indicated that the bran derives from the early domesticated wheat species einkorn (*Triticum monococcum*). Surprisingly, a continuous presence of the toxic bracken fern (*Pteridium aquilinum*) was detected in the analysed content material. As fern species have long been used as for the expulsion of intestinal parasites, such as tapeworms, it could be a possible explanation, that the Iceman used the fern as a medicine against his gastrointestinal issues (Zink *et al*. 2019). However, other explanations, such as the usage of fern as food wrappings or unintentional swallowing has to be considered as well.

3. OUTLOOK

The recent developments in the field of paleogenetics offers a huge potential for the study of mummies. It is now possible to have a detail look into the ancestry of mummies, population developments, the detection of diseases, as well as the investigation of intestinal contents. Moreover, in combination with other methodologies, including the analysis of all different biomolecules, such as proteins, lipids, glycans, elements and metabolites, cutting-edge molecular research will provide more important insights into disease predispositions and evolution of pathogens, as shown in the studies on atherosclerosis (Keller *et al*. 2012) and *H. pylori* (Maixner *et al*. 2016). A great potential for further studies lies in the analysis of mummy microbiomes, as indicated in a recent study on the Iceman. Thereby, we have contributed to the investigation of *Prevotella copri,* an important member of the human gut microbiome (Tett *et al*. 2019). The comprehensive analysis of modern and ancient gut

microbiomes provided new insights into the genetic diversity and evolutionary history of *P. copri,* suggesting a loss of strain diversity due to Westernization and changes in diet. This work provided first evidences for a decline in gut microbiota diversity within the last millennia which is a possible underlying factor linked to the rise of modern diseases such as obesity, asthma, or food allergies (Tett *et al*. 2019).

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