Genomic Correlates of Atherosclerosis in Ancient Humans☆

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ABSTRACT

Paleogenetics offers a unique opportunity to study human evolution, population dynamics, and disease evolution in situ. Although histologic and computed x-ray tomographic investigations of ancient mummies have clearly shown that atherosclerosis has been present in humans for more than 5,000 years, limited data are available on the presence of genetic predisposition for cardiovascular disease in ancient human populations. In a previous whole-genome study of the Tyrolean Iceman, a 5,300-year-old glacier mummy from the Alps, an increased risk for coronary heart disease was detected. The Iceman's genome revealed several single nucleotide polymorphisms that are linked with cardiovascular disease in genome-wide association studies. Future genetic studies of ancient humans from various geographic origins and time periods have the potential to provide more insights into the presence and possible changes of genetic risk factors in our ancestors. The study of ancient humans and a better understanding of the interaction between environmental and genetic influences on the development of heart diseases may lead to a more effective prevention and treatment of the most common cause of death in the modern world.

Soon after Watson and Crick were awarded the Nobel Prize in 1962 for describing the structure of deoxyribonucleic acid (DNA), another Nobel Prize winner, Linus Pauling, coined the term "paleogenetics," proposing that polypeptide sequences of ancient and extinct creatures might be reconstructed by sequencing their DNA [1]. Michael Crichton popularized this concept in his 1990 novel *Jurassic Park*, purporting to recreate dinosaurs from DNA extracted from dinosaur blood ingested by million-year-old mosquitos preserved in amber, a preposterous but intriguing application of technology that, in real life, has led to the decoding of DNA from ancient humans, including extinct forms such as Neanderthals and Denisovans and ancient modern humans including Egyptian and other mummies.

Since the first ancient DNA studies were performed about 30 years ago [2,3], the field has significantly changed from the retrieval of small DNA fragments from single specimens to large-scale genome-wide studies of past populations [4]. These studies enable wide-ranging research in human evolution and ancestry [5], population dynamics including past migration patterns [6,7], and insights into phenotype, such as skin and eye color [8]. Paleogenetic analysis of ancient DNA samples can further detect the presence of bacteria and parasites, documenting infections such as tuberculosis, malaria, and Lyme disease in ancient human populations [9—11]. Moreover, advances in DNA recovery and sequencing allow full genome investigation of ancient pathogens that lead to new insights into disease evolution. The combination of DNA array capture and next-generation sequencing 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 1,000 years [12—14].

Work with ancient DNA is limited by factors that reduce the possibility of detecting ancient genetic material. One main factor is the degradation of DNA that initiates immediately after the death of an organism. The activity of enzymes and microorganisms lead to a significant fragmentation of the DNA molecules and a reduction of the overall DNA amount. This process is further enhanced by hydrolytic and oxidative processes that can also result in destabilization and miscoding of the DNA by deamination and depurination. These factors can severely disturb the polymerase chain reaction amplification severely leading to nonspecific products or incorrect sequences [15]. Several parameters such as high temperature, ultraviolet radiation, humidity, and a low pH value significantly accelerate the DNA decay [16,17]. In contrast, a dry and cool climate or rapid desiccation of an organism due to artificial or natural mummification may slow down the degradation process, increasing the probability of ancient DNA preservation. In any case, the amount of authentic DNA in ancient tissue samples is generally very low or may have disappeared completely.

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Despite these limitations, given favorable conditions such as permafrost, soil DNA may survive up to 800,000 years in the case of Pleistocene fauna [18] or 400,000 years for hominin fossils from the Iberian Peninsula [19]. Besides the state of preservation of the specimens, the risk of contamination with modern human DNA is a major limitation in ancient DNA studies. Therefore, strict rules to avoid contamination have been introduced that are now compulsory for ancient DNA analysis. The use of specially designated laboratories with physically isolated work areas, extraction, and polymerase chain reaction control amplifications but no positive control samples, repeated amplifications of the same or several extracts, inverse correlation between amplification efficiency and length of amplification (appropriate molecular behavior), and independent replication can be regarded as basic and essential for any kind of ancient DNA study. Quantitation of the number of amplifiable DNA molecules in a sample is especially important for human DNA studies, but seems to be of minor relevance for paleomicrobiological studies [15,20,21].

Polymerase chain reaction–based approaches are still hampered by the risk of amplifying exogenous contaminants and the highly fragmented endogenous DNA [15]. In contrast, new high-throughput sequencing technologies including targeted enrichment strategies allow the reconstruction of full genomes [13], even from samples with mixed metagenomic information. In addition, DNA degradation patterns that accumulate over time are now commonly used to test the authenticity of ancient DNA [22,23].

It was widely accepted that biochemical assays of macromolecular preservation are good indicators for the presence of ancient DNA in a specimen. Usually, the total amount of amino acids, the composition of amino acids, and their extent of racemization have been used to estimate the probability of ancient DNA survival. In a recent study, Collins et al. [24] provided new evidence that the extent of aspartic acid racemization seems not to be a useful screening technique for ancient DNA from bone, as they have found no correlation between the extent of aspartic acid racemization and DNA amplification success. This finding clearly contrasts with the assumptions from previous studies underlining the necessity to critically review this authentication criterion.

Taken together, the application of strict authenticity criteria is necessary and must be the basis of any ancient DNA study. This does not, however, substitute for proper study design and background knowledge, which allow successful retrieval of ancient DNA.

Recent genomic studies in contemporary humans are revealing a growing number of single nucleotide polymorphisms (SNP) that predispose to the development of atherosclerotic cardiovascular disease [25–27]. In particular, SNP located on chromosome 9p21 show a strong association with cardiovascular disease. Modern genomics has limited capacity to study past human genetic diversity in order to understand the evolutionary history of the modern human genome. It is clear from histologic and computed x-ray tomographic investigations of ancient mummies that atherosclerotic cardiovascular diseases have been present in humans for at least 5,000 years, affecting individuals with widely differing lifestyles and environments over a broad geographic and temporal range [28–30]. Studies of DNA extracted from ancient mummies permit a better understanding of the evolution and history of atherosclerosis.

ÖTZI THE ICEMAN

On September 19, 1991, a naturally mummified body, later called Iceman, or commonly referred to as "Ötzi," was discovered in the Ötztal Alps at an altitude of 3,210 m (Fig. 1). The Iceman lived around 3,300 BC and died at an age of approximately 40 to 50 years [31]. The mummy is now housed together with his belongings at the South Tyrolean Museum of Archaeology in Bolzano, Italy (Figs. 2 and 3). The extraordinary well-preserved body and the preservation of his clothing and equipment allowed unique insights into the living circumstances of early Copper Age humans in the alpine region [32,33]. Since his discovery in 1991, this mummy has undergone extensive scientific studies using a wide range of methodologies, including radiology and computed tomography, histology, isotope analysis, paleobotany, and genetic analysis. In the last few years, several advanced contemporary technologies have been applied to study the glacier mummy. These include nanotechnological analysis of soft tissue and bone samples, spectroscopy of blood remnants in his clothing, a reevaluation of radiological data, and a detailed genetic analysis of the nuclear genome (Fig. 4) [11,34,35].

Based on stable isotope analysis, it was shown that the Iceman grew up and lived the last years before his death in different valleys in the southern region of the Alps [36]. A paleobotanical study and pollen analyses of samples removed from his intestines have provided important

FIGURE 1. The discovery site of the Iceman in the Ötztal Alps (South Tyrol, Italy). The glacier mummy was found in a 40-m–long, 2.5- to 3-m–deep and 5- to 8-m–wide rocky gully at the Tisenjoch, 3,210 m above sea level.

FIGURE 2. The well-preserved mummy housed in the South Tyrolean Museum of Archeology in Bolzano, Italy.

insights into his nutrition, his last itinerary, and the season of his death in late spring [37,38]. Computed tomography (CT) scans demonstrate the presence of healed rib fractures, degenerative arthritis, some degree of vascular calcification, and the presence of an arrowhead in his left shoulder [39,40]. A recent multislice CT examination performed in 2007 clearly demonstrated that the arrowhead had lacerated the left subclavian artery, likely leading to a rapid, deadly hemorrhagic shock (Fig. 5). It could be further shown that the Iceman suffered from a severe brain injury that could have been caused shortly after or before the deadly arrow-shot [41,42]. In a recent reevaluation of the CT scans, further details on the life and death of the Iceman were revealed, including the presence of gallbladder stones and a completely full stomach [35].

FIGURE 3. An automated refrigeration system is used for the conservation of the Iceman.

FIGURE 4. Sampling of the Iceman for genetic analyses under sterile conditions.

In the years immediately after the retrieval of the Iceman, the initial genetic studies of his mitochondrial DNA were initiated with the analysis of the so-called hypervariable region [43]. In later years, the entire mitochondrial genome was successfully analyzed [44]. In a recent study, the whole nuclear genome was analyzed from a small bone sample of the Iceman. The next-generation sequencing approach revealed about 40% reads that mapped unambiguously to the human reference genome and, thereby, an overall coverage of the human genome of more than 80% was retrieved. The authenticity of the ancient Iceman DNA was confirmed by a comparison with the previously published mitochondrial DNA that revealed full concordance.

The whole genome approach revealed many interesting insights into physiological parameters, diseases, and the ancestry of the Iceman, it could be shown that he likely had brown eyes, in contrast to the previous assumption that he had blue eyes (Fig. 6). It could be further shown that the Iceman was lactose intolerant, a characteristic that is among the most significant genetic traits connected with the beginning of agriculture in Europe. The study further provided indications for recent common ancestry between the Iceman and present-day inhabitants of the area near the Tyrrhenian Sea.

The Iceman genome underwent a further detailed analysis of genetic risk factors, specifically for DNA sequence variations, so-called SNP that are linked with diseases. The most intriguing finding was that the Iceman showed a strong genetic predisposition for increased risk for coronary heart disease (CHD). This is of particular interest as the CT scans of the Iceman already had revealed major calcification in carotid arteries, distal aorta, and right iliac artery, which are strong signs of generalized atherosclerotic disease (Fig. 6) [35,40]. The genetic predisposition could have significantly

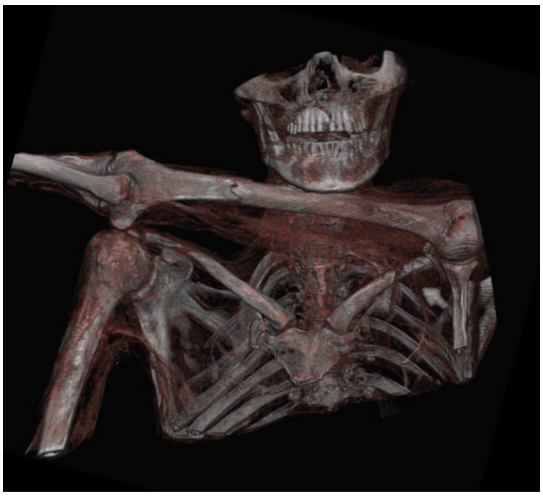

FIGURE 5. Three-dimensional CT reconstruction of the Iceman. The stone arrowhead is visible at the left shoulder region.

contributed to the development of the arterial calcifications. Other traditional 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. On the basis of the previous studies mentioned, 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 available in that time period, although dark staining in his lungs indicate that he inhaled smoke during his life, most likely from open campfires. Whether campfire smoke has a similar impact as tobacco smoke does on atherosclerotic disease is currently not known. Studies with animal models have, however, shown an interaction between wood fire exposure and the development of atherosclerosis [45]. This potential is further reviewed by Thomas et al. in this issue [46].

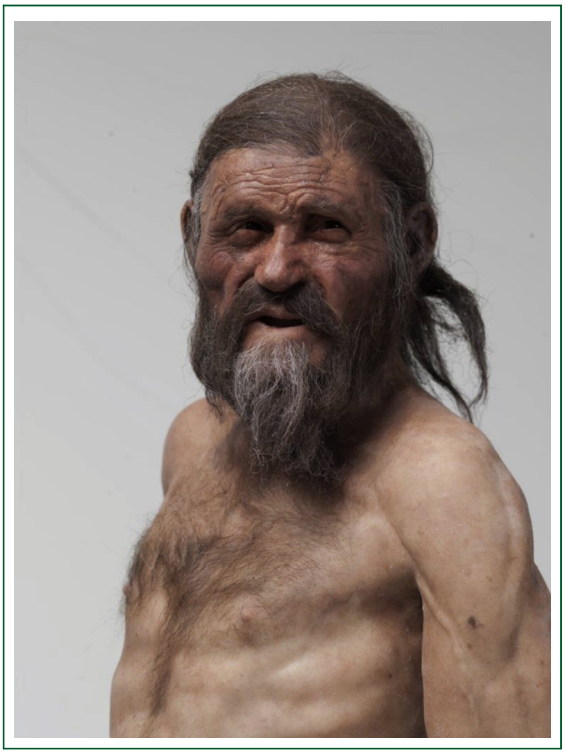

FIGURE 6. Reconstruction of the Iceman.

In the genome study of the Iceman, the investigators specifically concentrated on genetic risk factors that are linked with cardiovascular disease in genome-wide association studies (Table 1). The sequencing data demonstrates that the Iceman was homozygous for the minor allele (GG) of rs10757274, located in chromosomal region 9p21. This SNP is currently regarded as being among the strongest genetic predictors of myocardial infarction and has been confirmed in several studies as a major risk locus for CHD. In the CCHS (Copenhagen City Heart Study) and in the ARIC (Atherosclerosis Risk in Communities Study), an up to 40% increased risk for development of clinically manifested CHD in different ethnicities was found, independent of other classical risk factors [25]. Additionally, the rs10757274 SNP was described as a major risk locus for ischemic stroke and sudden cardiac death (meta-analysis of 6 independent cohort studies) [26,47,48]. The SNP analysis of the Iceman genome further revealed a homozygous minor allele of rs2383206 (GG), located in 9p21, representing another major risk factor for CHD [49]. The presence of both of the 9p21 SNP almost doubles the risk for developing CHD [50]. The Iceman's genome harbors further SNP that were related to cardiovascular disease, such as the endothelin receptor type B heterozygote variant rs5351 on chromosome 13. Yasuda et al. [51] showed that this SNP independently increases the risk for atherosclerosis in men. Additionally, the analysis revealed SNP in 3 genes that have been associated with CHD, namely *VDR, TBX5*, and *BDKRB1*. Whereas Keller et al. [11] detected the SNP rs2228570, located in a start codon of the gene *VDR*, for genes *TBX* and *BDKRB1*, novel mutations that have caused changes in the respective stop codons were found.

FUTURE STUDIES OF DNA IN ANCIENT MUMMIES

Until now, the Iceman is the only ancient human remain in which a genetic predisposition for cardiovascular disease has been detected. Although an increasing number of whole genome studies are being performed, dating back to Neanderthals and early hominins [19,52,53], no other similar detailed SNP analysis regarding these risk factors has been performed. In addition, in skeletal samples, a comparison with the phenotype, the actual presence of calcified arteries, is not possible due to the lack of soft tissue preservation. Therefore, the comparison of phenotype and genotype regarding cardiovascular disease as shown in the Iceman represents a unique opportunity for interdisciplinary study.

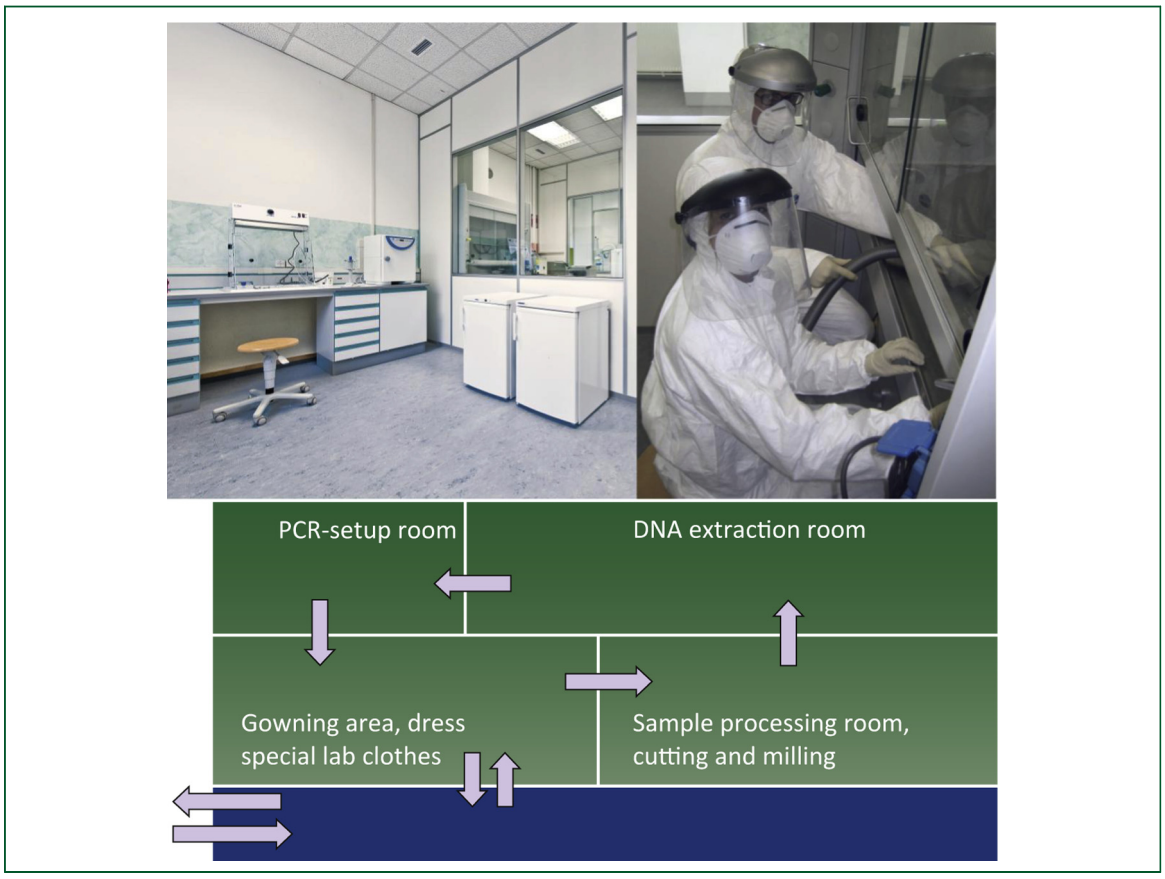

FIGURE 7. Setup of the ancient DNA laboratory at the Institute for Mummies and the Iceman in Bolzano, Italy. Samples are processed in a specific 1-way workflow following strict rules to avoid any contamination.

Several studies of the Horus Team revealed a high incidence of arterial calcifications in mummies from various geographic origins and time periods [29,30]. To evaluate the presence of genetic predispositions in these mummies, further studies are planned that will include a detailed SNP analysis of cardiovascular risk factors and possible identification of new or currently unrecognized genetic polymorphisms that are no longer present in modern-day patients. For this, full genome sequences should be obtained from the mummies (Fig. 7). However, the likelihood of success of such attempts depends heavily on the state of preservation of genomic DNA in the various mummies. The Iceman is exceptionally well preserved, probably due to the deposition in a cold and stable environment for more than 5,000 years. In other mummies, such as those from hot climates as present in Egypt or the desert areas of South America, DNA degradation may hamper potential SNP analysis. On the other hand, it has to be taken into account that the fast and efficient desiccation of the

TABLE 1. SNP related to cardiovascular disease identified in the Iceman genome

Chr.	dbSNP#	1,000 Genomes minor allele frequency	Gene	Coverage Iceman A	C	G	T	N	SNP association
chr1	rs1801133	A = 0.325	*MTHFR*	10	0	1	0	0	Cardiovascular disease
chr1	rs1764391	T = 0.354	*GJA4*	0	4	0	9	0	Atherosclerosis
chr4	rs1870377	A = 0.241	*KDR*	8	0	0	9	0	Coronary heart disease
chr9	rs10757274	G = 0.396	*CDKNBAS*	1	0	8	0	0	Ischemic stroke, sudden cardiac death
chr9	rs2383206	G = 0.459	*CDKNBAS*	0	0	8	0	0	Coronary heart disease
chr13	rs5351	T = 0.436	*EDNRB*	0	14	0	20	1	Atherosclerosis
chr19	rs1613662	G = 0.141	*GP6*	4	0	3	0	0	Myocardial infarction, age

chr, chromosome; dbSNP, single nucleotide polymorphism database; SNP, single nucleotide polymorphisms.

ancient Egyptian mummies during the mummification process, including the wrapping and embalming, may be factors that helped to slow down the natural DNA degradation process. In a few studies, it has been suggested that genomic DNA can be retrieved from Egyptian mummies [10,54]. Further studies are required to evaluate the possibility of obtaining full genomes from those mummies.

SUMMARY

Twenty-first century humans often think that much of the heart disease we suffer is the consequence of our modern lifestyle. We live sedentary, mentally stressful lives, consume large amounts of calories from ill-chosen foods, indulge sugar and salt in high quantities, and are exposed to high levels of environmental toxins, including tobacco smoke. To avoid dying of a heart attack, we are urged to engage in regular exercise; maintain an ideal body weight; avoid unsaturated fat, salt, and sugar; avoid smoke; and take medicine to keep our blood pressure, lipid levels, and blood sugar in an ideal range. Perhaps we think nostalgically of our ancestors who lived a purer life and did not have to engage in corrective actions to prevent heart disease.

Wrong! Our ancestors going back thousands of years show signs of atherosclerosis, as suggested by modern research using CT to detect evidence of calcium deposits associated with atherosclerotic plaques in the arteries of mummies as old as 5,000 years. Even though our human ancestors lived far different lives than we do, their environments and lifestyles were not protecting them against the development of atherosclerosis.

What is similar between now and then is the human genetic material, our genome, including ancient polymorphisms that were uncovered to predispose the carrier to the development of atherosclerotic cardiovascular disease. Seminal studies of genetic material obtained from 5,300-year-old Ötzi showed mutations in the 9p21 chromosomal region identical to SNP identified in contemporary humans that are strong predictors for the development of CAD. Our ancient ancestors were certainly susceptible to many other conditions, such as infectious diseases, nutritional deprivation, and trauma, which often resulted in an early age death, before atherosclerotic heart disease became clinically manifest.

Nevertheless, the study of ancient humans and the investigation of the interaction between environmental and genetic influences on the development of heart disease may provide unique pathophysiologic insights and lead to more effective prevention and treatment of the most common cause of death in the modern world.

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