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=== REVIEW ===

MOLECULAR DIAGNOSIS OF PATHOLOGIES IN ANCIENT HUMAN REMAINS. A CASE STUDY: THE BIOARCHAEOLOGICAL STUDY OF A NEOLITHIC SKELETON DISPLAYING SYMPTOMS OF DIABETES

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SUMMARY. Diagnosing diabetes in archaeological human remains is a difficult task. Molecular methods can provide additional information that can help the paleopathologists come closer to a correct diagnostic. A set of skeletal remains from the Suplacu de Barcău (Romania) archaeological site, dated to the Neolithic period, displays pathological changes that indicate diabetes.This would become the oldest documented case of diabetes. This paper describes the strategy we designed for further ancient DNA analysis.

Keywords: ancient DNA, diabetes, Neolithic, paleopathology.

Introduction

One of the goals of bioarchaeology is to understand how the health status of past populations is affected by cultural, socioeconomic and demographic changes.

Determining how disease affected past populations is a laborious task and the methods used for this type of study have rapidly evolved over the past decades. The first step in the analysis of archaeological remains is the visual examination and recording of specific measurements used for assessing the age and sex of an individual, and to discover any signs of pathology. Since the soft tissues are usually absent, for a disease to be diagnosed in ancient remains it should produce changes

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that affect the bones and teeth. Diseases that impact the bones perturb the normal turnover balance, and they can be either proliferative or erosive (Waldron, 2009). Diseased bone is relatively easy to recognise. Determining what caused the abnormality is the more challenging task. There may be a number of conditions that cause pathological changes in bones; there are cases where distinguishing between them is difficult and misdiagnosis is quite frequent. Further scientific analysis can be used to increase the certainty of the diagnostic in these cases.

Diagnosis in paleopathology differs greatly from the one in clinical practice. The formal approach for clinical diagnosis is based on knowing the medical history and examining the signs and symptoms of the disease; with this information the clinician will arrange for a series of investigations (e.g. blood tests, biopsies, biochemistry, ultrasound etc.) by which the initial diagnostic can be confirmed. The purpose of a diagnostic is to indicate the proper treatment. In contrast, when it comes to examining ancient remains, the paleopathologist can obtain the information about his 'patient' based on the visual examination of the bones, radiography, histology and ancient DNA analyses.

Examination of individuals affected by various conditions can provide answers to a number of questions. Diseases affecting the bone are more often chronic than acute, which means that the individual lived with the disease enough time to allow the changes in the bone to develop. Therefore, examination of these cases can indicate how the quality of lifeof the individual was affected by the symptoms during his lifetime and how the disease reflected the social status of the individual (is there a difference in the burial rituals between these individuals and the rest of the population?). Also, it can hint at the level of medical care and compassion in populations that leave no written evidence.

Infectious diseases in ancient populations have been a major preoccupation for those studying the history of medicine. Molecular methods to detect human pathogens (*Mycobacterium, Treponema, Yersinia,* etc.) have been developed and are continuously expanding. Some notable examples are presented below.

Tuberculosis is a disease that has been well known throughout human history. Pott's disease or spinal tuberculosis is a complication of tuberculosis that is easily identified in skeletons. Tuberculosis is caused by a bacterium in the genus *Mycobacterium*. There are two species that can infect humans: *M. bovis* and *M. tuberculosis*. The appearance of the infected bones is identical; the only way to distinguish between the two is by detection of specific molecular markers: a series of genes with specific mutations for each of the species (Bachmann *et al*., 2008). Detection of mycolic acids using mass spectrometry is another way to confirm the presence of *Mycobacterium* in archaeological human remains.

Tertiary syphilis is another infectious disease that affects bones. Syphilis is caused by bacteria of the species *Treponema pallidum.* Diagnosing syphilis can sometimes present problems and ancient DNA techniques have been used to confirm the initial diagnostic. Isolating and amplifying genes specific to treponemal DNA can confirm the presence of the pathogen (von Hunnius *et al*., 2007).

Besides infectious diseases, there are many other conditions that affect the bone formation and its remodelling such as vitamin deficiencies (lack of vitamin C causes scurvy, lack of vitamin D causes rickets), osteoporosis, starvation etc. When studying such pathological modifications of metabolism, age, sex, ancestry, and lifestyle are factors that must be taken into consideration, before reaching a conclusion about the health status of the individual.

Ancient DNA studies can be targeted at diseases with a genetic component. The study of these genetic factors in past individuals can help the understanding of how such conditions evolved.

The need for an interdisciplinary approach arises in such cases. The archaeological context of the grave gives information about the lifestyle and the cultural practices of a population and the socioeconomic status of an individual in a population.

Paleobotanical and zooarchaeological data offers an image of the environment where the individual lived.

Stable isotope analysis of carbon and nitrogen in bone collagen is used to reconstruct diet/nutrition. It is possible to determine whether the source of protein in the diet was from animal, plant or a combination of the two. The amount of ^{13}C relative to ^{12}C is a measure of the dietary dependence on C3 or C4 plants. The proportion of ^{15}N to ^{14}N isotopes indicates the trophic level of an organism. (Leatherdale, 2013)

Approaching all these aspects together paints a clearer image and helps to better understand the health of individuals or populations.

When developing a strategy for the study of an ancient pathological case it is crucial to relate to modern studies of clinical characteristics of that particular disease. The investigations rely on modern medical data and medical studies are focused mostly on cohorts from the developed countries of the world. There are differences in lifestyle, diet and habitat between the subjects of modern studies and past populations and this must be taken into account.

Case study: Suplacu de Barcău

The subject of this study are the skeletal remains of an adult male from the Suplacu de Barcău archaeological site dated to the Neolithic period.

Suplacu de Barcău (Bihor, Romania) is an archaeological site that contains a Neolithic settlement specialized in producing polished stone tools for use in the community and for exchange with other settlements (Ignat, 1998).

Radiocarbon dating was used to confirm the age of the remains. They were dated to 3970-3910 B.C.

The physical anthropology analysis was performed using the protocols described in *Standards for data collection from human skeletal remains* (Buikstra and Ubelaker, 1994).

Sex (male) was determined based on morphological features of the skull (the supraorbital margin, glabella and mental eminence) and pelvis (subpubic concavity, ventral arc, greater sciatic notch, preauricular sulcus).

Age at death (33-45 years) was determined based on the morphological changes of the pelvis (pubic symphisis, auricular surface) and the sternal end of the ribs.

A number of dental pathologies have been noted: 4 dental caries, 5 abscesses and 11 teeth lost ante mortem (Fig. 1).

Joint degeneration processes are observed in the right humeral head and right hand phalanges.

Four of the left-side ribs present remodeled fractures.

Cribra orbitalia (Fig. 2) presents itself as macroscopic porosity on the orbital roofs and is a sign of anemia caused by lack of iron or malnutrition. The presence of *cribra obitalia* generally suggests great metabolic stress.

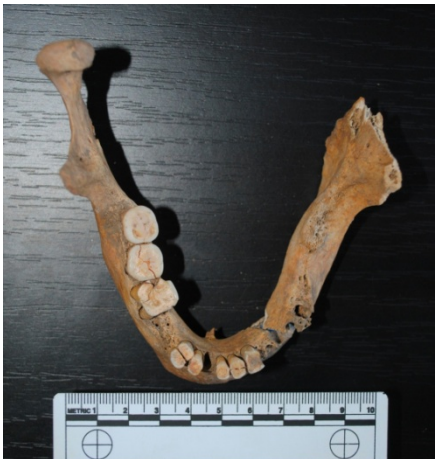

Figure 1. Dental caries, ante mortem tooth loss

Figure 2. *Cribra orbitalia*

L4 and L5 vertebrae are fused with S1 by large exostoses specific for DISH (Diffuse Idiopathic Skeletal Hyperostosis) (Fig. 3). These are formed by the ossification and calcification of the longitudinal ligaments of the spine. DISH is more common in men than in women and rarely occurs below the age of 40. In modern populations, DISH is found in association with obesity and type II diabetes; an association with abnormal vitamin A metabolism has also been noted (Rogers and Waldron, 2001).

On the right calcaneus a major lesion (38.70x19.80 x116.56 mm) can be observed. There is remodeling around the lesion consistent with a bacterial infection affecting both the compact and trabecular bone. On the left calcaneus there are four smaller lesions affecting the compact bone and reaching the trabecular bone (Fig. 4).

DISH, the infection on the calcanei (as part of a diabetic foot complex) and *cribra orbitalia* (as a sign of nutritional stress) considered together are strong indicators of chronic diabetes. Carries and ante mortem tooth loss have also been associated with diabetes. As such, this would become the oldest documented case of diabetes, surpassing an ancient Egyptian case dated to 2055-19110 B.C. *(*Dupras *et al.*, 2010).

Figure 3. DISH (Diffuse Idiopathic Skeletal Hyperostosis)

Figure 4. Lesions on both calcanei

Diabetes is known since ancient times. The Ebers papyrus is one of the oldest preserved medical documents. It was written in Egypt around 1550 B.C. and it is a compendium of incantations and remedies for various mental and physical disorders. It also contains the first known medical reference to diabetes, more precisely to a typical symptom, polyuria: '…to eliminate urine which is too plentiful' (Loriaux, 2006). Indian physicians around the same time identified the same symptom, polyuria, but added the notion of *madhumeha* or "honey urine", noting the urine would attract ants. However, there are few cases of skeletal remains that have been diagnosed with diabetes with any degree of certainty (Dupras *et al*., 2010, Rogers and Waldron, 2001, Bruintjes, 1987).

A notable example is the case of the skeleton from the archaeological site of Dayr al-Barsha, Egypt dated between 2055- 1911 B.C. This skeleton displayed similar pathological changes to the one at Suplacu: DISH, ante mortem tooth loss, abscesses, and degenerative processes in the shoulder joint. However, this individual suffered amputation of the forefoot bilaterally. This could have been a treatment for a case of diabetic foot, treatment that requires certain surgical skills.

In order to obtain more information and increase the certainty of the diagnostic, we proceeded with developing a strategy for further investigations. This is a very challenging task, because diabetes is a complex disease, which is caused by genetic factors as well as lifestyle and environmental factors.

An important factor to consider was the individual's age at the time of death, since different forms of diabetes arise either during childhood or adulthood. Because the individual was a 33-45 years old adult, type 1diabetes is unlikely. At the same time, this is a case of chronic diabetes, so the individual lived with his condition for years before death. This places the debut of the disease somewhere between the ages of 20 and 40.

Other important aspects to consider are the environmental factors: diet, lifestyle, habitat etc.

Archaeological research in the Suplacu de Barcău area provides valuable information about the environment and the lifestyle of the Neolithic population in the area. During the middle Neolithic, Suplacu de Barcău was a permanent settlement of an agricultural community as suggested by the presence of storing pots, grinding stones and traces of wheat. An important part of the economy was stockbreeding. Big cattle (cow and buffalo), small cattle (sheep and goat) and birds (chicken, pheasant, and partridge) were raised. Hunting and fishing were largely practiced in the area. Bones from stags, hares and wild boars were uncovered (Ignat, 1998). The vegetal sources of food in typical European Neolithic populations were cereals (wheat, barley, rye, etc.), lentils and peas (Rottli and Castigliano, 2009).

We believe the presence of diabetes in this population is related to the lifestyle and diet changes during the Neolithic period (i.e. the transformation from hunter-gatherer based to agriculture based). A hunter-gatherer style of nutrition is based on wild game, fruits and roots. With the introduction of agriculture the proportion of carbohydrates in the diet increased. New sources of carbohydrates were the starch in cereals and the lactose in cattle milk.

C/N stable isotope analysis is used to confirm a typical diet for Neolithic agricultural populations: a mixture of food sources based on plants and meat (Richards *et al*. 2003).

Diabetes has also a genetic component. Ancient DNA analysis can be used for detection of certain mutations associated with diabetes by modern studies. There are numerous studies on various groups across Europe. To narrow down the number of mutations to test for, age was the primary factor to take into consideration.

We decided on detecting SNPs in genes involved in glucose metabolism and insulin production associated with Maturity Onset Diabetes of the Young (MODY), insulin resistance and Maternally Inherited Diabetes and Deafness (MIDD).

The term MODY is used to describe a group of clinically heterogeneous, non-insulin-dependent forms of diabetes. All show dominant inheritance and are defects that affect pancreas development/differentiation or normal b-cell physiology. The variations include the age at onset, severity of the hyperglycaemia (and hence risk of complications) and associated clinical features (Ellard and Hattersley, 2008).

Heterozygous loss-of-function mutations in the glucokinase (GCK) and hepatocyte nuclear factor-1 alpha (HNF1A) genes are the most common cause of

monogenic diabetes in the majority of populations studied and account for approximately 80% of UK patients with a genetic diagnosis of monogenic diabetes (Ellard *et al*., 2007).

Glucokinase (GCK) phosphorylates glucose, preparing it for glycolysis and further energy production. Glucokinase is often referred to as the beta cell glucose sensor because its activity level directly reflects the glucose concentration in the cell (Molven and Njølstad, 2011). This glycolytic enzyme plays a key role in maintaining blood glucose homeostasis. In the pancreatic beta cells, GCK controls insulin secretion and biosynthesis. Mutations in this gene raise the glycaemia threshold for insulin release, meaning the glucose levels necessary for stimulation of insulin production are higher. In the liver, GCK regulates glycogen synthesis and gluconeogenesis (Am *et al*., 2009).

Genes that encode the transcription factors HNF1A and HNF4A were also strongly associated with MODY. The hepatocyte nuclear factors (HNFs) constitute a family of transcription factors that are important for the correct development and function of the liver (Molven and Njølstad, 2011).

HNF4A encodes an orphan hormone nuclear receptor that, together with HNF1A, HNF1B, and HNF3B, constitutes part of a network of transcription factors required for gene expression in pancreatic beta cells, liver, and other tissues. In beta cells, these transcription factors regulate expression of the insulin gene as well as genes encoding proteins involved in glucose transport and metabolism, and in mitochondrial metabolism, all of which are linked to insulin secretion. The fact that heterozygous nonsense and missense mutations in HNF4A lead to an insulinopaenic form of MODY strongly suggests that beta cell function is sensitive to the amount of HNF4A and that haploinsufficiency is the likely mode of molecular pathogenesis in that condition (Barroso *et al*., 2003).

Another gene studied in correlation with type 2 diabetes was ABCC8 (ATP-binding cassette transporter sub-family C member 8). This gene encodes an ATP-sensitive potassium channel present in the membrane of beta cells (Florez, 2008). This channel controls the secretion of insulin out of beta cells and into the bloodstream.

There is a type of diabetes caused by mutations in the mitochondrial genome. Proper mitochondrial function is central to maintaining glucose homeostasis. Characteristic for this type of diabetes is strictly maternal inheritance. The patients also tend to suffer from impaired hearing. Mitochondrial diabetes is therefore often denoted Maternally Inherited Diabetes and Deafness (MIDD) syndrome (Molven and Njølstad, 2011). A SNP in MT-TL1 (Mitochondrially encoded tRNALeucine 1) was linked to premature aging of beta cells and a decrease of glucose induced insulin production (Janssen *et al*., 2007).

Polymorphisms in the CAPN10 gene have also been associated with diabetes and insulin resistance by studies made on populations from Germany, Finland,

Denmark, The United Kingdom, Poland, Czech Republic and France (Tsuchiya *et al*., 2006). CAPN10 encodes a protease expressed in the pancreas, liver and muscle. It is considered to have a role in insulin secretion, insulin action and production of glucose by the liver.

Table 1 summarizes the gene polymorphisms that best fit the age of onset and the symptoms in the Suplacu de Barcău case.

Table 1.

Genes of interest and SNPs associated with diabetes

Gene	SNP	Encoded element	Function	Associated with
GCK	rs1799884 C→A C→G	Glucokinase	Phosphorilates glucose to glucose-6-phosphate	MODY
MT-TL1	3243 A→G	Mitochondrially encoded tRNALeucine 1	tRNA for protein sythesis	Maternally Inherited Diabetes and Deafness
HNF4A	rs2144908 A→G	Hepatic nuclear factor 4α	Transcription factor	MODY
HNF1A	rs1169288 G→T	Hepatic nuclear factor1α	Transcription factor	MODY
CAPN10	*rs3792267* A→G	Calpain 10	Protease active in pancreas	Insulin resistance

Considering the age of the skeleton and the probable degradation of DNA sequencing the genes in their entirety would not be feasible. It is safe to expect tofind intact regions of 100-150 base pairs around the mutations of interest. For this a pair of primers was designed for each polymorphism allowing amplification of the neighboring regionby PCR. PCR products are then cloned and sequenced, in order to detect the presence of the SNPs.

Conclusions

Diagnosis of metabolic paleopathologies is a challenging process. There are a number of molecular methods that can be used to increase the certainty of the diagnostic. There is no standard method; it is rather a question of finding the right approach for the given situation.

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