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Chronique / Kroniek

Developments in biological anthropology and proteomics at University of Ghent, Belgium

In 2020, the ArcheOs research laboratory for biological anthropology was established in the Department of Archaeology at the University of Ghent (hereafter UGent). Research on all aspects of biological anthropology is being conducted in collaboration with national and international partners. The presence of isotope and proteome laboratories at UGent has created the opportunity to establish an in-house workflow for their application in bioarchaeological contexts. Here we focus on the development of palaeoproteomics at UGent. Palaeoproteomics is the analysis of ancient proteins and can be described as the study of proteins from archaeological, historical, and paleontological remains and materials. These ancient proteins are studied to help us understand past subsistence strategies, past lifeways, biometrics, and material culture.

1. Diet

ZooMS (Zooarcheology by MS), the most well-known of the proteomic methods, has been widely used to identify faunal material from animal husbandry practices (e.g., it allows for the distinction of morphologically similar sheep and goat remains (Birch *et al.*, 2019), as well as the identification of butchered animal remains or animal bones used for tools or personal adornments. In anthropology, it is particularly useful for its application to fragmented or non-morphologically nondiagnostic hominin remains (McGrath *et al.*, 2019) ahead of radiocarbon dating or aDNA (ancient DNA) analyses. Beyond these applications, LC-MS/MS based (see below) proteomics has been applied to ancient dental calculus to identify subsistence directly from the human remains. Although it may not capture the diversity of the foods consumed, and it is biased towards certain food groups (Hendy *et al.*, 2018b), it has been particularly informative in furthering our understanding of dairy consumption. Ceramics containing food remnants have also been successfully analysed and shed light on human subsistence patterns (Dunne *et al.*, 2012; Roffet-Salque *et al.*, 2015; Lucquin *et al.*, 2016; Spiteri *et al.*, 2016).

2. Palaeopathology

Recently, age and sex, key facets of the osteological identification, have been successfully studied using ancient protein analysis. Sawafuji *et al.* (2017) showed a negative relationship between alpha-2-HS-glycoprotein abundance and age. More recently MS techniques on dental enamel samples, have examined the preservation of amelogenin X and Y, with Y being present in males only, making it possible to use it as a marker of sex (Sawafuji *et al.*, 2017).

Bone proteomics have also been applied in the study of ancient disease processes with varying levels of success. Although it is possible to detect leukocyte-derived proteins (Sawafuji *et al.*, 2017), their accuracy at detecting specific inflammatory processes is being challenged. Samples taken at the sites of palaeopathological lesions have yielded indications of their cause, such as osteogenic sarcoma (Buckley, 2019), and pathogenic bacterial proteins from lesions have also demonstrated the presence of tuberculosis (Boros-Major *et al.*, 2011) although there are still some methodological considerations for this type of application (Hendy *et al.*, 2016). The task now lies in developing methods to reliably detect disease in the skeletons even when there are no visual signs on the skeleton.

Dental calculus preserves not only food remains but also bacterial taxa (FAGERNÄS *et al.*, 2020). Metaproteomic analysis has demonstrated intra-populational differences in the oral microbiome. Metaproteomics may in future be applied to other sites, such as the soil samples collected in the pelvis during excavation, and lead to insights into diet and disease through the detection of plant, animal, bacterial, and viral proteins (HENDY, 2021).

3. HUMAN EVOLUTION STUDIES

In the study of palaeoanthropology, ancient proteins have offered an alternative to ancient DNA (aDNA) for studying hominin phylogeny, especially when DNA preservation is proble-matic. Distinct proteins of hominin species have allowed the identification of non-diagnostic Neanderthal (WELKER *et al.*, 2016) and Denisovan specimens (CHEN *et al.*, 2019). Welker et al. used enamel protein sequences to reveal that Gigantopithecus, an extinct giant ape, was a sister taxon to Pongo (WELKER *et al.*, 2019). These deep time studies demonstrate that the longevity of proteins may yield phylogenetically important data that would have seemed impossible to discern with DNA degradation.

4. METHODOLOGICAL DEVELOPMENTS IN PALAEOPROTEOMICS

Until recently, ZooMS involved MALDI-MS, wherein peptide mass fingerprinting (PMF) is used to identify the species origin of peptide residues, mostly in bone (BUCKLEY, 2018). Indeed, digesting a pure protein mixture yields a set of unique peptide precursor masses that can be used to identify the origin of certain proteins (BUCKLEY, 2018; DESMOND *et al.*, 2018; MCGRATH *et al.*, 2019). However, nowadays, more complex mixtures of proteins can be digested into peptides and are readily analysed using LC-MS/MS (HENDY, 2020, 2021). This advance is comparable to the extension of aDNA studies from mtDNA to nuclear DNA sequencing in ancient samples. In LC-MS/MS, the peptides are separated by liquid chromatography (LC), then ionized using electrospray ionization (ESI), followed by fragmentation of individual peptides to allow for identification of each peptide separately. This is referred to as bottom-up LC-MS/MS (see HENDY, 2021).

Future optimizations of LC-MS/MS based palaeoproteomics will focus on the validation of identifications, often derived from low abundant peptides in the samples. The need for extra validation is illustrated by the current scepticism around *e.g.* the robustness of the claim for the presence of tuberculosis (BOROS-MAJOR *et al.*, 2011; HENDY *et al.*, 2016). In addition, sample preparation protocols – *e.g.* for the analysis of food crusts or lithic tools (KOOYMAN *et al.*, 2001;HENDY *et al.*, 2018a) – can be optimized by creating replicas that will allow to assess the impact of any adjustments to the protocol on bulk sample without losing ancient material. Besides the optimization of sample preparation protocols, improving data acquisition and data analysis is paramount.

For acquisition, an initial discovery phase will first be extended on the new generation high resolution QTOF instruments and their novel acquisition strategies, which are currently being beta tested at ProGenTomics. The resulting shortlist of detectable proteins will enable the development of more targeted and thus sensitive assays like Multiple Reaction Monitoring (MRM) on tandem quadrupole instruments. These measure the tell-tale peptides that have been identified during the discovery phase and aid in archaeological interpretation. These techniques allow to detect peptides down to attomole quantities as also recently shown by ProGenTomics in the context of Sars-Cov-2 detection in patient samples (VAN PUYVELDE *et al.*, 2021). MRM approaches also allow to scale up the throughput of samples. This way, large collections of bone samples can be screened for the presence of human remains at a rate of 10-20 samples per hour, i.e. up to 500 per day.

The final challenge is the lack of false discovery rate (FDR) control for low amounts

of peptide identifications when using regular database searching during data analysis. Therefore, ProGenTomics will introduce spectral prediction tools from the Compomics group (UGent) to predict fragment intensities (MS^2PIP) and retention times (DeepLC) of the annotated peptides (BOUWMEESTER *et al.*, 2021, GABRIELS *et al.*, 2019). These predicted spectra can be used to confirm the identifications and increase confidence of the scientific claims.

5. CURRENT PROJECTS AND PRELIMINARY DATA

Presently, ArcheOs and ProGentomics are engaged in several ongoing projects, as well as setting up large-scale collaborative research for the years to come.

One avenue of current research is into method and protocol optimization, assessing both the amount of sample material necessary and the most applicable sample processing protocol. The focus now is predominantly on human bone, tooth, and calculus, alongside prehistoric pottery food crusts. The goal is to establish the variation in yield and results between different existing protocols (*e.g.*, using HCL vs. EDTA as a demineralization agent) and to create an optimal sampling and processing method for archaeological samples. As the various existing protocols are delivering slightly different results, it is crucial to take the effects of the lab process into account in result interpretation. From an archaeological perspective, winnowing down necessary sample size to the lowest successful weight is essential, especially in unique and precious samples.

In tandem with this methodological research, several archaeological questions are being explored using palaeoproteomic analyses. One current project is focusing on identifying markers of suboptimal bodily health using palaeoproteomics in historic Northwest-European collections. The focus is both on potential pathogen peptides and peptides which are part of the physiological response to disease and physical stress. Preliminary data from one rib shaft fragment of a non-adult skeleton from post-medieval Aalst (Belgium) run using two processing protocols found 116 peptides covering 16 proteins. In addition, a pathogen peptide and histone peptide were identified. These results are as yet too anecdotal for any solid conclusion but are mentioned here to illustrate the range of information to be gained from palaeoproteomic analyses. In addition to this historic research, Neolithic and human palaeoanthropological projects are ongoing as well, in which palaeoproteomics are being applied to a host of questions from basic sex estimation of commingled remains to larger lacunae in prehistoric knowledge. An emphasis is placed on maximally embedding the palaeoproteomic data within the framework of other available biological anthropological data, both macroscopic and through other biomolecular methods such as stable isotopes and aDNA. In this way palaeoproteomics is coming into its own as a unique tool to study life in the past within a cohesive framework.

6. CHALLENGES

From the summary above it is clear that palaeoproteomics is an extremely promising approach for the field of biological anthropology. It is still in its infancy, however, so careful consideration of the results and their implications is necessary. Poorly understood are aspects of sample contamination by taphonomic and laboratory processes, as are authentication markers, to ensure the distinction between endogenous and modern sources of proteins. Additionally, differential preservation rates of peptides in dry human remains need further assessment. Nevertheless, the opportunity to develop a workflow for palaeoproteomic analyses of human remains and archaeological objects is both exciting and promising and only possible through the close collaboration between anthropologists and archaeologists and the proteomic specialists from ProGentomics.

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