Ancient Biomolecules Unravel our History: A Technical Update with Examples from the Middle East

Sara Tomei[1]*, Ambra D'Aurelio[1], Ferhan Sakal[2], Francesca Castorina[3,4], Faisal Al Naimi[2] and Cristina Martínez Labarga[5]

[1]*Omics Core, Integrated Genomics Services, Sidra Medicine, Doha, Qatar*

[2]*Department of Archaeology, Qatar Museums, Doha, Qatar*

[3]*CNR, Istituto di Geologia Ambientale E Geoingegneria, c/o Dipartimento di Scienze Della Terra, University of Rome "La Sapienza", Rome, Italy*

[4]*Department of Earth Sciences, University of Rome "La Sapienza", Rome, Italy*

[5]*Dipartimento di Biologia, Università degli Studi di Roma "Tor Vergata", Rome, Italy*

*Corresponding author: Dr. Sara Tomei, Omics Core, Integrated Genomics Services, Sidra Medicine, Doha, Qatar

Abstract

Context: The study of ancient biomolecules represents a useful tool to address questions related to human history.

Objective: This manuscript provides an overview of the major categories of ancient biomolecules, highlighting their potentialities when applied to research.

Methods: This study gathered knowledge from recently published papers on paleogenomics, paleoproteomics, ancient lipids and stable isotope analyses with the aim of providing a technical and historical background on ancient biomolecules, and examples of their application in the Arabian Peninsula and Middle East in general.

Results: The progress seen in the past decade with regard to the study of ancient biomolecules has led to a dramatic expansion of the studies that apply those analyses. Increasing attention has also been paid to the development and optimization of protocols aimed at reducing and/or preventing the risk of contamination. While extensively applied to Western areas, the study of ancient biomolecules in the Middle East and the Arabian Peninsula has been limited.

Conclusions: Research on ancient biomolecules represents the most valuable source of information to understand our evolutionary past at an inconceivable level of detail, especially when applied to areas so far underrepresented in this field, such as the Middle East and the Arabian Peninsula in particular.

Keywords (3-5): Paleobiology; ancient DNA; Gulf; stable isotopes; strontium

Introduction

The Nobel Prize in Medicine and Physiology last year (2022) has been awarded to Svante Pääbo, one of the pioneers in paleo genomics research, whose studies underlined the importance of our evolutionary history not only for our present but also for our future [1]. The scientific community has recently seen a rapid expansion of ancient biomolecular studies, which have allowed previously unsolved questions on our human history to be addressed [2,3]. Cappellini et al. have provided a review of the most recent applications of the ancient biomolecules [2]. By applying ancient biomolecular analyses, bioarchaeologists were able to shed light on the pathways and evolutionary processes that contributed to the current biodiversity [2]. The field of ancient biomolecules covers a broad spectrum of techniques, including the examination of ancient nucleotide and amino acid sequences, as well

as the analysis of lipids and stable isotopes [2,3]. The development and application of ancient biomolecular analyses has the potential to help bioarchaeology studies to be carried out in geographical areas potentially challenging, due to suboptimal environmental conditions, including heat and humidity [4]. The advent of next-generation sequencing (NGS) technologies has revolutionized the field of ancient DNA (aDNA), allowing an increased number of ancient genomes to be analyzed and the possibility to study extremely ancient remains [2,5-8]. As reviewed in early studies, the last decades also recorded a growing interest in environmental DNA (eDNA) [2,8-10]. Environmental DNA (eDNA) pertains to the genetic material gathered from environmental samples, which may include soil, seawater, or even samples taken directly from organisms [11]. The field of eDNA has also benefited from the introduction of NGS technologies as well as from the subsequent application of paleometagenomics [2,12,13]. Along with the advancement of sequencing technologies, increasing efforts have been focused on the development of protocols aimed at maximizing the retrieval of aDNA as well as reducing and/or preventing the risk of contamination [5,14-17].

Long bones and teeth dentine have historically been recognized as the most employed substrates for aDNA studies; however, in the last years, researchers have pointed out other skeletal elements such as petrous bones and tooth cementum as samples richer in aDNA [5,14,15,18]. Moreover, advancements in the paleogenomics field have facilitated the retrieval of a vast amount of genetic data from various substrates that were previously unexplored, such as archaeological materials, dental calculus, archaeobotanical remains, and more [18]. In addition to the advances in aDNA studies, investigations on ancient proteins recently underwent a significant expansion [2,19]. Paleoproteomics is the discipline that aims at identifying and characterizing the proteins of ancient remains [20]. Unfortunately, the field of paleoproteomics has advanced at a slower pace as compared to the field of aDNA [20]. Although the survival of ancient proteins has been investigated since the 1950s [21], it is only since the 2000s that technical advancements in mass spectrometry enhanced the possibility of recovering and characterizing ancient proteins [20]. The paleoproteomics field has not been spared from technical challenges. In fact, efforts aimed at directly sequencing ancient proteins have failed because of the need to start from highly purified, unmodified, and concentrated proteins [22,23]. Despite the challenges, there is a growing number of paleoproteomics studies being conducted, which significantly enhances the field's potential to deepen our understanding of species evolution and human culture [24].

Another class of biomolecules is represented by ancient lipids. As recently reviewed by Cappellini and colleagues [2], ancient lipids were firstly analyzed in the 1960s, but their application in archaeology was registered only in the following decade [2,25,26]. These molecules have the potential to elucidate past human activities [27,28]. Ancient lipids can be recovered from a broad range of materials as demonstrated by wide research [27-31]. Moreover, unlike aDNA and proteins, lipid biomarkers can be retrieved in high concentrations as proved by the analyses of potteries and bog butters [28,32,33]. Similar to ancient lipids, the examination of stable isotopes has seen significant growth in recent decades and has become one of the most prevalent methods in bioarchaeological research. This approach enables the investigation of past human diet and residential mobility [34-37]. In the present day, stable isotope analysis finds application in a wide range of areas, including plant and livestock management, examination of past population mobility, understanding social dynamics, exploring development trends, and studying the variation of subsistence strategies [37-55].

The Middle East lies at the crossroads of the Old World, where Africa, Europe and Asia meet. Due to this geographical location, the Middle East has played a central role in human evolution. At the same time, the often-inhospitable nature of the climate in this region has contributed to geographic structuring both within the region and between the Middle East and other parts of the World [56]. Nevertheless, it is thought that despite the Middle East being characterized by an arid desertic environment, there have been humid periods in the past that resulted in what has been called "Green Arabia" and which might have facilitated human dispersals [57]. The Middle East contains some of the earliest evidence of modern humans outside of Africa, with fossils dating back approximately 180,000 years ago from the Levant and around 85,000 years ago from North West Arabia. [58, 59]. However, our understanding of the prehistoric societies and population movements of Middle Easterners remains limited. [56]. In this article we describe the different classes of ancient biomolecules, highlighting their potentialities and shortcomings and providing examples of their application in the Middle East and the Arabian Peninsula.

Stable Isotope Analysis: Investigating Human Diet and Mobility

Stable isotope signatures represent a widely employed method for investigating dietary habits and migrations of past human populations [37,60]. This well-known method has been frequently utilized in reconstructing the dietary habits of human populations from Prehistory to the present day (e.g. [41-43,49,50,55,61-69]) also represents a useful tool to explore broader issues such as the domestication of plant and animal species, the patterns of the Neolithization process etc. [37,70-73]. Moreover, stable isotopes provide evidence of population mobility allowing inferences on the structure, dynamics, and economy of human groups [37,74-76]. Situated in a strategically central location, the Arabian Peninsula has facilitated the movement of its inhabitants and traders, leading to the exchange and spread of animal and plant species across Africa, Europe, and Asia. However, due to its arid and often inhospitable climate, the Arabian Peninsula (and the Middle East in general) has received limited attention in stable isotope analysis studies aimed at understanding the Arabian paleoenvironment [77]. We report some examples below.

Carbon and nitrogen stable isotopes: dietary pattern reconstruction

Diet represents the convergence of social and cultural values that display a strong influence on human habits [78, 79]. The

analysis of carbon and nitrogen stable isotopes has received a growing interest from the scientific community as it provides a powerful tool to reconstruct dietary patterns in past populations by providing dietary data at the individual level [80]. The isotopic composition of bone proteins reflects that of the protein component of food consumed in the last 10-15 years prior to death [81,82]. Because stable isotope analysis refers to single individuals, the obtained data can be useful for evaluating variation and social dynamics within the analyzed samples [83]. As widely discussed in the literature, the isotopic values are reported as the ratio of heavy/light (C^{13}/C^{12}; N^{15}/N^{14}) isotope expressed in delta (δ) per mil (‰) relative to internationally defined standards [84], the Pee Dee Belemnite, PDB [85] limestone fossil, then substituted by the Vienna Pee Dee Belemnite, V-PDB [86] and atmospheric nitrogen, AIR [87]. Photosynthesis is the main natural process responsible for carbon isotopic fractionation during which the preferential exchange of the lighter carbon isotope (^{12}C) is responsible for the depletion of $\sim$ -7‰ in $\delta^{13}C$ from CO_2 to plants [88, 89].

Terrestrial plants follow three main photosynthetic pathways. The crassulacean acid metabolism (CAM) will not be discussed here since these plants are not relevant to human diets. Plants known as C3 plants (such as trees, shrubs, tubers, wheat, rice, flowering plants, and grasses from temperate regions) follow the Calvin-Benson photosynthetic pathway, which results in the synthesis of a three-carbon compound; they discriminate against the heavier isotope of carbon, and thus their tissues are enriched in ^{12}C showing a mean $\delta^{13}C$ value of -26.5‰ [90, 91]. Maize and other plants from hot and arid environments (e.g., savannah grasses, amaranths, sedges, millet, sugarcane, sorghum, etc.) synthesizing a four-carbon compound are generally classified as C4 plants; They have higher carbon ratios, resulting in more positive $\delta^{13}C$ values (mean $\delta^{13}C$ value of -12.5‰) [92-95]. Temperature, variation in atmospheric CO_2, nutrients, water availability and the amount of light can affect the isotopic ratios of C3 plants whereas C4 species generally exhibit less variability [96,97]. The fractionation process continues in consumers implying an enrichment factor of approx. +5‰ into their bone proteins [98] plus a further enrichment of ca. 1‰ for each trophic level [99].

A similar small increase in ^{13}C has also been attested in the marine environment by previous research [98,100-103]; in fact, marine organisms possess higher $\delta^{13}C$ values for the main source of carbon in the environment is represented by dissolved bicarbonates ($\delta^{13}C$ value of about 0‰) [104]. The consumption of marine food webs, on the other hand, can complicate the assessment of C4 plant consumption [98] leading to an overlap of the carbon isotopic signatures of marine and terrestrial environments [61,90]. Another aquatic resource which is worth considering is freshwater (lacustrine and riverine) fish which was consumed by past human populations as attested by different isotopic archaeological studies [105-112]. Though aquatic, freshwater fish has $\delta^{13}C$ values more similar to terrestrial animals, although extremely variable because carbon can come from organic and inorganic carbon sources [113].

Nitrogen is present in the Earth's atmosphere (approx. 78%) however, numerous biochemical processes (e.g., fixation, ammonification, nitrification, denitrification, assimilation) are necessary to convert it from gas (N_2) to the most usable forms and vice versa, clearly influencing its fractionation [114]. Plants from the terrestrial environment may alternatively use nitrogen as ammonium (NH4+) thanks to the symbiosis with Rhizobium bacteria (e.g. pulses) or in the form of ammonia (NH3) or nitrate (NO_3) [115]. As a consequence of the use of different forms of nitrogen these plants also differ in terms of $\delta^{15}N$ values which result very low in the former group (ca. 1‰) and higher in the latter one (ca. 9‰) [115]. Although nitrogen ratios can vary due to the use of different nitrogen sources, various environmental factors (such as humidity, pH, salt concentration, etc.) can influence the isotopic values [115]. Through successively higher trophic levels, an enrichment of 3-5‰ in $\delta^{15}N$ values is observed [116].

The source of nitrogen also determines a variation in $\delta^{15}N$ ratios between marine and terrestrial ecosystems as the use of ^{15}N enriched sources (e.g. nitrates from water) is responsible for the increase in nitrogen values in marine organisms with respect to the terrestrial ones [89,109]. A different situation may be observed for freshwater environments as in this case they show a higher variability due to the use of both terrestrial and aquatic materials [115]. Moreover, the $\delta^{15}N$ value also varies according to biological and cultural factors as breastfeeding practices [117-122], nutritional stress or metabolic disease [43,49,50,123-125]. The stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) of plants and animals consumed by humans could be useful for investigating dietary patterns, environments and the food groups consumed [126-129] although with some limitations [130,131]. Clearly, these data should refer to the specific environment in which past human population lived therefore it is extremely important to reconstruct the reference baseline by analyzing coeval faunal and plant remains if available [132-141].

Despite the potentialities of isotopic analysis from bone proteins, it may be possible that exposure to arid environments prevents proteins' extraction [54]. In this case, radiocarbon dating and stable isotope analysis on hydroxyapatite represent a valid substitute in the reconstruction of dietary patterns. The former relies on the absence of equilibrium between the marine environment and atmosphere where ^{14}C is produced, leading to the development of the marine effect reservoir (MRE) due to the differential age of the carbon in these two environments being the marine ca. 400 years older than that in the atmosphere [54]. Marine resources intake increases the individual's ^{14}C age with respect to the biological one. The difference in the estimates of the time of burial and MRE are thus useful for investigating the amount of marine sources' intake [54]. Alternatively, the isotopic $\delta^{13}C$ ratio of hydroxyapatite is useful to discriminate between subsistence strategies mainly based on C3 (average $\delta^{13}C$ values -12‰) or C4 (average $\delta^{13}C$ values -1‰) plants [142-144]. Moreover, unlike bone proteins, hydroxyapatite reflects the total diet and not only the protein components [143,145-148].

At present, there is a scarcity of studies on diet reconstruction in the Arabian Peninsula. However, Roberts and colleagues addressed this gap by utilizing 21 mammal tooth enamel samples and applying stable carbon and oxygen isotope analysis to investigate the middle Pleistocene paleoenvironment of Ti's al Ghadah in Saudi Arabia [77]. The $\delta^{13}C$ signatures from the fossil mammals associated with hominin presence showed a prevalence of C4 vegetation in the diets of herbivores. Additionally, the consistent C4 consumption by all animals was indicative of the high consumption of C4 grasslands. Even the $\delta^{18}O$ signature suggested that in the past the region probably experienced higher humidity [77]. The data produced in this study represents a clear example of how stable isotope analysis from fauna helps gain insights into paleoaridity and paleovegetation during periods of hominin migration [77]. Recently, another study employed stable carbon and nitrogen isotope analysis to examine the dietary patterns of adult human and animal bones excavated from Qalat al-Bahrayn [149]. Interestingly, they found that the diets consumed were dominantly terrestrial although samples were retrieved in proximity to the coast [149]. The diets consumed were heterogeneous, spanning from terrestrial diets to more mixed diets, which included C4 resources [149]. The same heterogeneity was found in the sheep, suggesting diversity also in feeding practices that might have included the use of dried fish [149]. The study is particularly relevant since it highlights diversity in the economic and ecological systems that one would not expect from a small island [149].

Stable isotopes of strontium and oxygen for the study of residential mobility

The mobility and migrations of past human groups represent an important topic for both the archaeological and forensic fields. Phenomena such as changes in economic patterns, social complexity, landscape, post-marital residence, population origins, mortuary practices, and economic activities are all considered motivating factors for movement. For this reason, population movement influences events such as subsistence, health, demography, socio-political organization, and economy. Even in this case, the human remains serve as a valuable source of information for investigating an individual's history by analyzing stable isotopes [150]. As widely reported in the literature, isotopes in bone and tooth remains reflect the chemical composition of the consumed foods and drinks which in turn are dependent on the local environment [151,152]. As a result, isotope ratios like $^{87}Sr/^{86}Sr$ ($\delta^{87}Sr$) and $^{18}O/^{16}O$ ($\delta_{18}O$) are extensively utilized to explore the origins and mobility patterns of ancient populations [150].

The fundamental principles behind employing combined strontium and oxygen isotope analysis of human tissues are rooted in their connection with the geological composition of the soils where food was cultivated and the origin of the drinking water. Strontium is naturally present in various isotopic variants (e.g. ^{88}Sr, ^{87}Sr, ^{86}Sr, ^{84}Sr etc.) [153, 154]; nonetheless, the only isotope suitable for comparing modern and archaeological data is ^{87}Sr, as it possesses both stable and radiogenic characteristics, being the decay product of the long-lived natural beta-emitting isotope ^{87}Rb, which has a

half-life of 48.4 billion years [154-156]. The unique geology of each region is reflected by the strontium abundance in the weathered rocks from which it can pass into the local ecosystems through its release into groundwater to the food chain maintaining the same $^{87}Sr/^{86}Sr$ ratio without appreciable fractionation [157-159]. As the amount of strontium varies among different areas, its uptake into the skeletal tissue reflects the geologic features and the bio-availability of the chemical element, providing evidence of the area of residence and mobility [160-162] as well as the presence of non-local individuals because it can be incorporated into enamel hydroxyapatite due to its similarity to calcium [161,163]. The Sr isotopic ratio of dental enamel provides the signal of the place of birth because it represents the individual's Sr intake throughout the crown formation and results in a static tissue extremely resistant to diagenetic alteration in the post-burial environment. Also, the teeth formed post-weaning accurately reflect the food and water consumed by individuals, therefore, it can be considered a birthplace signal. [161,163]. Oxygen's signatures depend on multiple pathways. However, although the overall oxygen signature is determined by different factors, the isotopic ratios in tooth enamel and skeletal remains are a proxy of the drinking water consumed [164-166] and its variations are ascribable to geographic and climatic factors [167-171]. The fractionation processes of oxygen isotopes are known and therefore the isotopic analysis allows the reconstruction of the consumed water sources ($\delta_{18}Ow$) and thus past human mobility [166-169,172-174].

As enamel formation takes place during childhood without any further remodeling, the strontium and oxygen signatures it contains mirror those of the environment in which individuals lived during their infancy [45]. Specifically, the development of the crowns of the permanent first molars initiates in the womb and reaches completion between the ages of 2.5 to 3.0 years. The formation of the second molars, otherwise, starts at 2.5-3.0 years to be completed in 7.0-8.0 years old children [175, 176]. Consequently, individuals drinking water from local sources will possess isotopic values in line with those of the environment, conversely the use of non-local water sources will cause an inconsistency between environmental and individual isotopic signatures. Despite the potentials of these isotopic analyses, the interpretation of data could be problematic. For instance, with regard to strontium, local environments generally display different inputs that do not necessarily reflect the $\delta^{87}Sr$ ratio of the geological substrate [161]. Therefore, it is important to distinguish strontium values from rocks and the bio-available one [62]. The local baseline could be assessed by several methods such as analyzing environmental samples including soil, freshwater, and coeval remains and/or modern ones; in this case however attention should be paid to the potential effect of human activities (e.g. fertilizers) on isotopic ratios [161,177-179].

A possible solution for data interpretation relies on the use of ancient faunal isotopic values as the baseline from which human values can be inferred [62,161,177,180,181]. Animals' (e.g. herbivores) subsistence is based on plants distributed in quite wide areas, therefore they possess more homogenized isotopic ratios

that more likely reflect local isotopic values [61,62]. Conversely, a broad array of factors, including flowing water, fluctuations in seasonal and annual rainfall, particular cooking and beverage preparation methods, diet, and others, can lead to inconsistencies and variations in the $\delta^{18}O$ of skeletal remains within a single archaeological site, consequently affecting the oxygen isotopic signatures [182-187]. Moreover, the reservoir effects that cause an enrichment in ^{18}O can also play a role in introducing heterogeneity within a local environment [185]. Furthermore, the isotopic incorporation may vary due to the number of years during which tooth enamel develops, leading to isotopic variability among teeth of the same individual [188-190]. Similar to the carbon and nitrogen stable isotope data, studies on strontium stable isotope analysis in the Arabian Peninsula are limited. We provide some examples below. Al-Shorman and El-Khouri applied strontium isotope analysis from rural sites in Barsinia, in Northern Jordan [191]. The earlier archeological excavations of the regions revealed that its occupation started during the Iron Age and continued until the Ottoman period. The site is particularly relevant as it was characterized by economic wealth, suggesting an enhancement of population dynamics. Yet, the study showed that all the individuals analyzed were raised in the area and their food was taken from spatially restricted localities in the region [191].

Recently, Ryan and colleagues applied strontium analysis to investigate the origin and the production of cotton in the ancient site of Mleiha, in the United Arab Emirates [192]. The authors found different isotopic signatures of cotton seeds and textiles as compared to modern plants retrieved from the same area. Hence, they should be considered "non-local". Along with archaeobotanical and archaeological evidence, the authors proved the existence of a trade network between Southeaster Arabia and India [192]. Evidence on human migration from strontium and oxygen stable isotope analysis in the Arabian Peninsula is also limited. Human skeletal remains from Neolithic sites within the United Arab Emirates have been subjected to investigation in a recent article [193]. The isotope ratios of strontium ($^{87}Sr/^{86}Sr$) and oxygen ($^{18}O/^{16}O$) of the remains assessed suggest the population was resident in the coastal area. Yet, the data suggested that mobility occurred between the coast and the inland regions [193]. A very recent study from Wang and colleagues has applied a combined approach encompassing isotopic and genetic analyses of samples recovered from Nevali Çori to study the Neolithization in the Fertile Crescent [194]. The authors analyzed 44 molar enamel from 28 human individuals thought to have lived between ca. 8700 to 7500 BCE for $^{87}Sr/^{86}Sr$, $\delta^{18}O$ and $\delta^{13}C$ analyses. The isotopic data indicated a reduction in mobility and growing resilience on domesticates by ca. 8300 BCE and contributed to the understanding of the Neolithization process in the Near East [194]. Regarding the Arabian Peninsula, a comprehensive investigation is necessary to establish a robust repository of Sr and O isotope variations within this region. This will serve as a foundational resource for archaeological studies focusing on human and animal mobility.

Ancient DNA (aDNA) analysis

Both the production and the analysis of human genetic data have undergone a complete transformation in the era of NGS, where genomes are being read at an unprecedented pace [195] and have allowed the completion of the human genome [196]. This phenomenon not only promises to transform the field of genetics but has far-reaching effects in understanding human prehistory when applied to the aDNA field. The study of aDNA is a powerful tool for discovering the origins, migration patterns, kinships, admixtures, and cultural shifts in past populations but it also allows retracing the origin of the modern mosaic of DNA sequences [197]. As stated above, archaeological skeletal series represent an important source of information about our past. The discovery of long-term DNA survival in skeletal remains [198, 199] and sediment [200, 201] has allowed human genetics to infer evolutionary processes [202]. Nevertheless, aDNA studies face specific challenges, one of which is represented by the retrieval of enough DNA endogenous content [202]. This is strictly dependent on post-mortem degradation processes. DNA starts decaying immediately after death as a result of the exposure to unmitigated insults by endonucleases, bacteria and fungi as well as by oxidation and hydrolysis in absence of the enzymatic repair mechanisms that help maintain the genomic integrity in living cells [203, 204]. DNA fragmentation is partly caused by depurination, a continuous process causing the increase of DNA molecule fragmentation with the age of the sample [205], although the deamination of cytosine residues towards the end of the molecules (resulting in apparent C to T or G to A substitution on 3' and 5' strand respectively) also represents one of the main damages observed on ancient DNA [203]. As a consequence, the endogenous DNA extracted from archaeological samples possesses features directly linkable to the abovementioned factors (e.g. reduction of fragments' length; lesions preventing the replication; lesions causing nucleotide misincorporation during the replication), moreover its content in archaeological specimens is often extremely low (<1%) [206]. Long-term DNA preservation is influenced by the environment; in particular it seems better in "niche" microenvironments forming during fossilization. Pruvost and colleagues [207] analyzed bones that were subjected to different post-excavation preservation conditions and discovered that freshly excavated fossil bones and teeth are the most suitable samples for amplifying aDNA.

Due to the difficulties related to aDNA retrieval and analysis, recent research focused on the identification of new substrates, the optimization of extraction methods as well as the control of contamination [208-210]. aDNA studies have been generally performed on teeth or dense bone fragments (e.g. cortical region of long bones) as those samples have been considered more enriched in endogenous DNA than the trabecular bone consisting of fewer osteocytes per gram [211]. Nevertheless, the petrous pyramid and the cementum layer in tooth root have been recently proved to be the most suitable substrates for aDNA analysis, as they display the highest degree of preservation of viable DNA [211, 212]. Certainly,

an important factor to evaluate is the invasiveness of the sampling procedures, especially for those skeletal elements not easily accessible (e.g., petrous bone). In fact, the extent of damages caused by a disruptive sampling of bone specimens should be limited especially for museum collections. Sirak and colleagues proposed an innovative minimally invasive method for accessing the petrous bone by drilling the cranial base [14]. A minimal-invasive protocol dedicated to sampling the tooth cementum of the roots has also been recently proposed and relies on the direct digestion of the target portion of the teeth [16]. Additionally, it has been proved that aDNA yield from petrous bone was in the order of hundreds-fold higher than other skeletal elements tested (including rib, tooth or long bones) and overall, it performed better than tooth cementum [213, 214]. Recently, another interesting study pointed to ear ossicles as an alternative source of aDNA, able to produce a DNA recovery similar to the cochlea; however, they offer the advantage of sampling (from intact skulls without significant damage to the surrounding material) [215].

aDNA is usually more preserved in permafrost or extremely cold climates [216-222] whereas high temperatures weaken DNA preservation directly and indirectly by enhancing the proliferation of fungi, bacteria and other microorganisms that contribute to endogenous DNA degradation and contamination [216, 217, 221, 223]. Despite that, researchers recently provided aDNA data from arid environments [214]. Contamination is of paramount concern in aDNA analysis due to the nature of aDNA itself (highly degraded and fragmented molecules) that can be easily mixed with exogenous molecules if strict precautions are not taken [224]. The problems of contamination may be partly solved by using ad-hoc facilities for aDNA extraction, however it is of paramount importance to pay extreme attention to avoid any carry-over between pre- and post-PCR laboratories [225]. The publication of the first ancient genome in 2010 was followed in the last years by an unprecedented and unexpected amount of genomic data [226-228]. The advent of NGS has significantly propelled the field of human aDNA forward. This progress has moved beyond the analysis of only a few hundred base pairs of mtDNA, which was susceptible to contamination and offered limited biological insights. Instead, NGS has enabled large-scale population studies, leading to a transformative shift in our understanding of human history [214,229]. Although inconceivable, partial and complete genomic sequences have been obtained from modern humans [230-232], Neanderthal [233, 234] and even Denisovans [235, 236]. Some of the genomes possess an extremely high depth as compared to that achieved in contemporary humans [228].

NGS has also played a vital role in enhancing our comprehension of the degradation processes that impact aDNA as time passes. For instance, the phenomenon of cytosine deamination, leading to C>U changes at regular cytosines and C>T changes at 5-methylated cytosines, has facilitated the improvement of distinguishing between endogenous and contaminant sequences. Additionally, it was also reported that the frequency of cytosine deamination increase with the age of a sample [237]. However, despite its numerous advantages, NGS is not exempt from encountering technical challenges when applied to the field of aDNA. During the initial application of NGS methods in aDNA studies, standard library construction protocols were used. However, it soon became evident that these protocols resulted in a significant loss of DNA. There are two main approaches to NGS library construction: direct sequencing (shotgun metagenomics) and enrichment for specific sequence regions using hybridization capture, followed by sequencing. Hybridization enrichment becomes necessary when the endogenous DNA content is low in a given sample.

Among these methods, whole exome capture of ancient samples has shown great promise for aDNA studies. Exomes, which encompass the protein-coding regions of the genome, tend to be more conserved than other parts of the genome. Consequently, exome capture has the potential to be particularly suitable for investigating extinct species, where no closely related genome sequence information is available [238].

In a very recent study Wang and colleagues analyzed six individuals retrieved from Nevali Çori to study the inner-community structure during the late Neolithic in the Southern Levant. The study indicated consanguinity and raised questions on how the Late Neolithic societies were structured internally and whether they were characterized by endogamy in terms of cultural behavior and social practices [194]. Despite the Arabian Peninsula played a critical role in the early structuring of modern humans, it is still underrepresented by large-scale genomic studies [4,56,239]. Unfortunately, the poor environmental conditions that characterize the Arabian Peninsula have not facilitated the recovery of aDNA, hence the aDNA evidence of the Arabian Peninsula is inferred from the application of modeling statistical and bioinformatic tools on modern genomic data [239,240]. DNA studies applied to the Arabian Peninsula have revealed that Arabians were subjected to a population bottleneck parallel to the peninsula's aridification around 6 kya. In fact, despite Arabia being characterized by arid weather, there have been several humid periods resulting in what has been called "green Arabia" [57]. In a recent study, Almarri and colleagues conducted an analysis of a high-coverage dataset comprising samples from the Arabian Peninsula, the Levant, and Iraq. Their aim was to gain insights into the population structure, demographic history, and admixture patterns of both modern and ancient humans in these regions. By employing a model based on published ancient genomes, the researchers successfully identified distinctions between the Levant and Arabia. Specifically, the Levant exhibited a higher European/Anatolian ancestry, while Arabia displayed higher African and Natufian ancestries [56]. However, it should be noted that this study made inferences on past populations based on modern DNA data. Additional studies applying aDNA are warranted to better understand the origin and connections of Arabian populations that are currently underrepresented in genome-wide studies [56].

Dental Calculus, a Valuable Tool to Investigate Microbiome

aDNA can be retrieved in a wide variety of substrates [241] some of which, like dental calculus, represent a valuable source

of ancient biomolecules that have the potential to provide useful information on oral ecology, metabolites, and ancient microbiomes. Dental calculus – tartar or mineralized dental plaque – is a mineral matrix covering the tooth surface is composed of bacteria, food, and environmental debris forming during time [242]. The development of dental calculus depends on the trapping of all the mentioned materials by the inorganic salts of saliva [243, 244]. Dental calculus is commonly detected in all human populations both past and present, and it is generally well preserved in archaeological contexts [242, 245] even if, due to dietary variations, its retrieval has been proven more challenging in ancient and modern foraging or hunter-gatherer groups [246] as despite the poor oral hygiene and the genetic pre-disposition the accumulation of larger deposits is related to the consumption of soft carbohydrates [245,247,248].

Dental calculus was partly overlooked until the 1960s-1970s whereas, afterwards, it received particular attention as it offers the chance to reconstruct the oral microbiome, diet, medicinal therapies, and paleoenvironment [43,242,244,249-257]. The analysis of archaeological calculus deposits can be performed following different methodologies. The most common approach is certainly the morphological examination, by light microscopy, of the entrapped material in samples decalcified by hydrochloric acid (HCl). In the 1990s and 2000s, the analysis of starch granules trapped in dental calculus deposits provided fundamental contributions to the reconstruction of the starchy components – roots, tubers, seeds – of the hominin and human diet [249-251,255,258,259], moreover, pyrolysis profiles were used to infer about cooking practices [249]. Furthermore, the presence of non-dietary debris (e.g. textile fiber) could also help in reconstructing craft activities and trade [260].

More recent approaches include DNA analysis and gas-chromatography that allow the identification of specific compounds attributable to the ingestion or consumption of plants and or volatile substances' inhalation [43,244,249,261-265]. Nowadays, the application of shotgun metagenomics determined a change in the field, because, as the bacterial community changes over different stimuli (e.g. diet, hygiene practices etc.), the analysis allows to investigate past human microbiome and also to make inferences on health status and diet during human evolutionary history [256,266,267]. The application of high-throughput sequencing has allowed researchers to move from the identification of calcified bacterial cells [268], dietary micro debris, host mtDNA [269] and biomolecules from a small range of selected species [269, 270] to the investigation of entire microbial communities [256,266]. The initial characterization of the microbiota of the ancient oral cavities was made possible by the target characterization of the 16S rRNA gene. This gene contains hypervariable regions that are informative of specific microbial communities [271]. Nevertheless, 16S rRNA sequencing has proved to generate skewed taxonomic data that can introduce biases when reconstructing the ancient microbiome [271]. For this reason, shotgun metagenomic sequencing is recommended for the analysis of the ancient microbiome over 16S rRNA sequencing [272, 273]. The reconstruction of a partial genome of an uncultured TM7 bacterium by Liu et al. [274] also pointed at shotgun metagenomics sequencing as a useful tool for the identification of uncultivable bacteria as well as for the recovery of extinct microbial genomes for which there are no reference sequences [242].

The recent advancement of molecular methods made dental calculus accessible to a wide range of analyses, including metagenomics and metaproteomics; the latter, although less explored, has recently been applied to dental calculus samples [253,256]. The metaproteomic approach allows the simultaneous characterization of the levels of individual proteins expressed by both microbial community and host, providing the direct assessment of protein functions but also allowing the elucidation of the interactions between potential disease-causing microorganisms and their host [253,275,276]. The work by Christensen et al. (2018) led to the identification of oral disease-susceptible individuals that were not apparent from the macroscopic examination of the skeletal remains demonstrating that the molecular investigation of dental calculus represents a valuable source of additional data useful to enhance our understanding of the health status of past human populations [253]. Despite the challenges of the emerging techniques, metaproteomics promises to yield a unique understanding of the role of the microbiome in the health status and diseases of past populations [242]. Dental calculus has the potential to become one of the most invaluable sources of information concerning ancient skeletal remains. Its analysis allows the investigation of various aspects, including diet, health status, diseases, microbial presence, and even the cultural affinity of past human populations. [267]. When integrated with data acquired from various methods, the examination of dental calculus holds the potential to offer a comprehensive reconstruction of our history.

To the best of our knowledge, the analysis of dental calculus has not been utilized in the context of the Arabian Peninsula.

Ancient proteins

Ancient proteins represent another class of biomolecules commonly referred to as "paleoproteomics" [24]. While the discipline has been introduced in the 1950s [277], it is only in the 2000s that techniques based on mass spectrometry shaped this field [24, 278]. Despite still at its infancy, the study of ancient proteins has many applications in evolutionary biology and archaeology [24]. Its application to biological tissues (e.g., ancient bones and teeth) allows phylogenetic studies when aDNA could not be used because degraded [279]. Proteins are indeed better preserved than DNA [24]. This enabled the proteomic analysis of samples substantially older than the experimented aDNA temporal limit, with the oldest sample so far analyzed being 1.77 million years old [279]. Beyond being a valuable tool in taxonomic and phylogenetic studies, ancient proteins can be leveraged for a number of other different applications. During the life of an organism, proteins are usually the expression of a specific tissue or physiological condition [24]. Hence, their study can give, for example, invaluable information concerning the origin of the material analyzed and the conditions surrounding the death of an individual [280]. In a study of 2013, Maixner and colleagues were able to identify many proteins in the brain tissue of the Tirolean mummy Ötzi, some typically expressed

by brain cells and others in situations of stress, opening up again the possibility of the Tirolean man being injured at the head before his death [280].

Furthermore, paleoproteomics has been used in archaeology to investigate past cultures. Despite some limitations, mainly due to the high impact of degradation processes on proteins removed from their original tissue and the lack of references required for accurate identification of the vegetal species [23,24], rests of proteins in ancient pottery and dental calculus have indeed the potential to reconstruct the main foodstuff used in the past. With respect to lipids, they are more concentrated in plant foods and can better distinguish between different food when mixed, thanks to their being taxonomic- and tissue-specific [23,24]. For instance, in a study by Hendy and colleagues from 2018, ancient proteins extracted from 8000-year-old ceramic vessels from West Mound in Anatolia revealed precisely the diet of the population. Interestingly, the authors were also able to discover that specific vessels were used only for certain types of food [23,24]. However, just a few studies were able to successfully study paleoproteins from ceramics thus far [23,24].

Both animal and vegetal proteins serve as raw materials for the production of clothes and they were used in building materials, paintings and glue. Hence, the peloeproteomics analysis of these materials can help the interpretation of some cultural aspects and habits of our past [23,24]. For instance, proteins such as fibroin and keratins help identify materials such as silk and wool [23,24] and the proteomics analysis of glues and paintings has allowed the detection of a wide range of proteins from different sources [281, 282]. Amino acid racemization (AAR) dating relies on the detection of amino acids. In living tissues, amino acids naturally occur in the L-configuration. However, following death, they undergo racemization and convert into the D-configuration [20]. The time since death may be estimated by analyzing the rate of D- and L-amino acids [20,283]. The AAR dating works well in highly mineralized substrates, such as tooth enamel [20,284]. The technique has also been applied to the study of early human activity in Europe [284].

Several methods are employed for the study of ancient proteins [20]. One of the methods for assessing ancient proteins is through immunoassays, which however can generate false positives and negatives, especially when analyzing degraded samples. These assays use the reaction of a specific antibody binding to an antigen. Their advantage is their effectiveness on challenging materials, such as ceramics [23, 285], as shown in a study of 2000 by Craig and colleagues where their application has allowed the detection of milk proteins on ceramic pots, unravelling the issue concerning dairy diffusion on the Scottish Atlantic coast during the Iron Age [285]. On the other hand, they require pre-knowledge of the target proteins and the conservation of specific epitopes [23,285]. Therefore, because of their limitations, they have been replaced by other techniques for the study of ancient proteins [20]. The introduction of mass spectrometry (MS) has revolutionized the paleoproteomics field. MS is more reliable as compared to immunoassays because

the mass and charges of the ionized molecules are detected precisely [20]. Matrix-assisted laser desorption/ionization MS (MALDI-MS) was applied initially in 2000 by Ostrom and colleagues [278]. In this study, peptides were identified from the osteocalcin of a 53,000-year-old bison [278]. Subsequently, the practicality of applying MALDI-time-of-flight (TOF) MS to collagenase-digested and trypsin-digested bone collagen surpassed that of using MALDI-TOF on purified osteocalcin [24]. This led to the development of the so-called ZooMS (Zooarchaeology by MS) [24], whose low cost and high sample throughput advanced its application in archaeology, ecology and cultural heritage [286]. ZooMS produces peptides mass fingerprints that are checked against reference fingerprints of known taxa; thus, it is used to detect the taxonomies of archaeological remains. Nevertheless, even though ZooMS has proved useful to discriminate between family and genera-level taxa, it is not as efficient in differentiating species due to the lack of sufficient protein variability [20].

Successively, the use of tandem mass spectrometry (MS/MS) led to an improvement in data accuracy and taxonomic resolution. This technique is indeed able to analyze a mix of proteins and, thanks to the parallel development of new software, to define de novo sequences, allowing the determination of proteins sequences of extinct species for which genomic data could not be obtained [24]. This is evidently valuable for phylogenetic studies. The MS/MS method has been applied to obtain proteome sequences from a Pleistocene specimen of Stephanorhinus of 1.77 million years [279]. Multiple methods are currently available for sample preparation for mass analysis. The main steps usually include the demineralization of the tissue, protein solubilization and buffer exchange, protein digestion and peptide purification. The protocols must be adjusted by considering multiple factors, from the status and kind of sample to the target amount and types of proteins [24]. Numerous programs for paleoproteomics analysis have been developed, improving the accuracy of the results and the applicability of the field [24].

Similar to the aDNA field, the paleoproteomics field needs to deal with contamination issues specific to the ancient biomolecules field. The contamination discrimination is based on the use of markers of degradation that can differentiate truly endogenous ancient proteins from potential modern contaminants [20]. Protein fragmentation can occur due to enzymatic damage (especially from the burial environment) or through hydrolysis via bond cleavage. Mass shifts are detected as an effect of protein degradation [20]. As an example, during degradation, glutamine and asparagine undergo deamidation and are transformed into glutamic acid and aspartic acid, respectively. Environmental factors like temperature and pH can influence these deamidation modifications. However, despite these influences, deamidation serves as a significant marker of protein degradation[20]. Ramsøe and colleagues have recently developed a method for the authentication of ancient proteins, called deamiDATE 1.0 [287]. The method was utilized on shotgun proteomic data of bone collagen derived from modern, archaeological, and extinct taxa. Through this approach, the researchers successfully distinguished authentic ancient proteins from contaminants in a case study involving dental calculus from

the Neolithic period [287]. Moreover, further developments in the method could enable the study of cooking practices in the past by assuming that the cooking process accelerates deamination. This strategy could be, in principle, applied to proteins extracted from animal bones but also pottery and dental calculus [287].

Paleoproteomics is a promising field of bioarcheology of recent and fast development. It has the potential to elucidate our history and evolution being applicable to a number of different and very old (at least if mineralized samples) substrates. paleoproteomics studies are still limited. However, we expect to see further technological improvements parallel to the increasing interest in the field. To the best of our knowledge, there have been no human paleoproteomics studies conducted in the Arabian Peninsula. This lack of research is likely attributed to the extreme environmental conditions in the region, which can lead to the deterioration of the organic components of archaeological remains.

Ancient Lipids

Lipids are an important class of organic substances that can be recovered in association with a wide range of archaeological artifacts and substrates that can be biomolecularly and isotopically characterized. The research in this field focused on a wide variety of substrates. Pottery artifacts are archaeological findings allowing inferring food production, storage, and processing, even if their original function is not always obvious [288,289]. These objects usually absorb organic residues entrapping and preserving organic biomolecules, including ancient lipids within the substances cooked and/or stored in them [290]. It has been demonstrated that the chemical analysis of these residues may help to understand the function of these archaeological artifacts as well as to allow the investigation of the culinary, cultural and social practices of past human populations parallelly to the transitions in human history [291-293]. The analysis of steroidal compounds in soil, coprolites and sediments has been proved helpful in investigating waste disposal, dairy, and agricultural activities as well as dietary patterns [294-304]. Furthermore, lipids from bog bodies, mummies and human skeletal remains [305-311], as well as from plants have also been analyzed [312,313].

The incorporation pattern of lipids taken from the diet as well as the distribution of lipid tissues are both well-known [314,315]. The analysis of isotopic signatures of bone lipids represents a valuable source of additional information for the investigation of past dietary patterns [307,310,311,316-319], and it allows not only to implement data from the analysis of bone proteins but also to shorten the investigated timeframe as they possess a faster turnover than proteins [320-322]. In addition, as previously described, the isotopic composition of bone proteins allows inferring information on diet's proteins intake, whereas that of lipids returns the individual's whole diet [146,323,324]. Ancient lipids are less susceptible to degradation as compared to aDNA and proteins, due to their chemical structures (e.g., limited presence of functional groups, high abundance of aliphatic chains, rings and branches). Moreover, the entrapment in organic and mineral matrices enhances their preservation even more, reducing the microbial degradation and the diffusion of the biomolecules [325].

Generally, the research in this field focuses on steroidal compounds and more hydrophobic molecules that possess a higher resistance to microbial and chemical degradation. This is due to fatty acids that are only rarely retrieved from archaeological samples, with the exception of specimens collected from arid environments [316]. Lipid analysis follows well-established protocols [326-328]. The most employed method to extract lipids from different substrates is the solvent extraction using intermediate polarity systems. According to the substrate's nature different preparation treatments may be needed; for instance, bone remains need pulverization whereas others (e.g. wax, fat, and other substances as resinous and bituminous residues) may be directly dissolved in solvents [28]. GC and GC-MS are employed to evaluate which compounds they originate from [27, 329], whereas stable carbon isotope analysis through GC-combustion-stable isotope ratio MS (GC-c-IRMS) identifies animal lipids in archaeological potsherds [290,330-333]. Currently, the use of acidified methanol extraction is commonly paired with gas chromatography-mass spectrometry (GC-MS) and gas chromatography combustion mass spectrometry (GC-C-MS) for analysis [334-336]. The two-step extraction procedure allowed the retrieval of a wider range of lipids with an appreciable amount of endogenous fatty acids from samples retrieved in hot environments [334].

The traditional interpretation approach based on the comparison with modern fat references [29,327,330,331,333,337,338], showed some limitations related to a higher variation in the diet of omnivores compared to herbivores and to variations in the faunal diet due to environmental, temporal, and cultural changes, etc. [339-342]. Moreover, it should be noticed that the ancient lipids recovered from archaeological artifacts do not necessarily derive from lipid tissue; for instance, the boiling of animal bones for preparing soups may cause the transfer of bone marrow lipids [336]. The application of GC-MS analysis has been proved useful to understand Nabataen funeral practices of monumental tombs of ancient Hegra, in Saudi Arabia [343]. A mixture of fatty acids and triterpenic compounds was retrieved from four textile samples and suggested the presence of vegetable oil and probably elemi resin. Although the exact botanic origin of the resin could not be identified, the results of these experiments suggested that the resinous material belonged to the genus Canarium, providing insights into how Nabataeans prepared dead bodies for their funerary practices [343]. GC and GC-MS have also been applied in the field of petroleum geochemistry to explore bituminous mixtures found in archaeological excavations [344]. Bitumen from natural sources in Iraq, Syria, Iran, Bahrain, and Kuwait has been utilized as a reference for calibrating biomarkers in hydrocarbon fractions [344]. The Middle East is rich in oil-stained rocks, deposits of solid bitumen and oil and gas shows, particularly in Iran in the Zagros mountain [344]. Evidence from Syria (Tell Atij, 6800 BCE) and Israel (Netiv Hagdud, 8900-7800 BCE) has shown that in the Neolithic time bitumen was used as an adhesive to glue flint elements to several tools [344]. Bitumen mixtures retrieved from Tell el'Oueili in Iraq have revealed the routes of Mesopotamian traders over time [344]. In Syria bitumen has been found to be used

as a hafting material during the Middle Paleolithic [344]. Bitumen was also found in balms of Egyptian mummies, dated between 1000 BCE to 400 BCE [344].

The use of GC-MS has allowed the characterization of mummies samples from Yemen and the elucidation of Yemeni methods for body preservation [345]. A more recent study has analyzed 31 samples retrieved from the ancient Harbour of Qâni' (Yemen) which led to the identification of frankincense and bitumen which was thought to have been imported from Iran [346]. Another study has also applied GC-MS along with other techniques to reconstruct the Yemeni commercial routes during the Middle Ages [347]. The majority of samples analyzed were made of a diterpenoid substance similar to East African copal [347]. Commercial routes and exchange networks represent an important subject to study as they can provide informative clues on past societies. In a very recent study, Suryanarayan and colleagues analyzed ceramic lipid residues from Hili, al Ain in the United Arab Emirates to shed light on the organic products that were part of the exchange networks that occurred in Arabia during the Bronze Age [348]. Their results indicated that the vessel studies were associated with the handling of products of animal and plant origin [21,348]. Although studies that apply GC and GC-MS in the Middle East do exist, they have been mainly focused on non-human remains. The Middle East and the Arabian Peninsula in particular could significantly benefit from additional studies aiming at analyzing lipid substrates in human archaeological samples.

Concluding Remarks

The technical advancements occurred during the last few decades determined a dramatic and previously inconceivable expansion of the availability of data from progressively older samples at a constantly increasing level of detail. The application of ancient biomolecular analysis is paving the way toward a refined characterization of population dynamics and socio-cultural changes. This review provides an up-to-date overview of the current methods for the study of ancient biomolecules. We hope that our review will facilitate the application of ancient biomolecules to studies to be carried out in underrepresented regions like the Arabian Peninsula.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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