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Stable carbon and nitrogen isotope analysis of archaeological human hair: Reconstructing diet and health of ancient individuals

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ABSTRACT

Stable isotope analysis is a powerful tool for reconstructing the diet and health of ancient individuals. The carbon and nitrogen stable isotope compositions of human tissues reflect those of the foodstuffs consumed and can be altered by physio-pathological stressors. The $\delta^{13}C$ and $\delta^{15}N$ values can be measured in the protein as a whole or in the amino acids constituting the protein by using a bulk or compound-specific isotope technique, respectively.

Human scalp hair is considered an ideal tissue in stable isotope studies because it is resistant to degradation, is predominantly composed of proteins (keratins), grows fast and at a 'known' rate (circa 1 cm/month when in anagen phase), and it does not remodel after deposition. The isotope signal is recorded sequentially as the tissue grows and remains unaltered through time, with the most recent information found at the hair root. The sampling procedure is minimally invasive and therefore comparative studies on living individuals can be performed.

Stable isotope analysis of sequential segments of scalp hair is a means of achieving a highly detailed and temporally resolved reconstruction of an individual's life. Dietary intake and health status of individuals can be reconstructed on a fortnightly basis when 0.5-cm-long hair segments are incrementally analysed.

1. Introduction

Humans have always been intrigued by the beauty of scalp hair. Hairstyles and their associated symbolism have evolved through time, while the significance of hair has kept thriving in life and after death (Bartlett, 1994). The treasuring of hair locks into jewellery (Lutz, 2011) and the treatments devoted to the hair of mummified individuals (Arriaza, 1995) are proofs of the emotional and ritual importance of scalp hair in the process of grieving, remembering, and celebrating the ancestors.

The ability of hair to survive 'intact' during the passing of time, including death and body decomposition, is fascinating. Hair, and other non-skeletal tissues, are preferentially preserved in windy and dry environments, such as the hyper-arid deserts (Lynnerup, 2007). Among those, the Atacama Desert (Peru, Chile) has always been an ideal environment for soft-tissue preservation. The oldest form of artificial mummification (5,050 BCE) was in fact discovered near Camarones, a funerary coastal site located in the northern Chilean segment of the Atacama Desert (Arriaza, 2005). Here, the Chinchorros, (semi-)sedentary hunting-gathering-fishing groups, initiated artificial treatments of the dead by mimicking the natural processes conducive to body preservation.

The unearthing of human hair in the archaeological record is a unique opportunity for accessing the detailed life history of the deceased individual, with the most recent information locked in the segment closest to the scalp and in the hair root. The biography 'written' along the hair fibres can be accessed by using a successful biomolecular technique that measures the stable isotope composition of the proteins in the hair – the keratins. Since the isotope compositions of the hair keratins reflect those of the ingested foods and drinks, it is possible to reconstruct the dietary intake of ancient individuals by measuring the keratin $\delta^{13}C$ and $\delta^{15}N$ values. The keratin isotope composition may also be indicative of a certain metabolic and physio-pathological status (Petzke et al., 2010).

Human scalp hair is considered an ideal bioresource in palaeodietary studies because (1) it is a robust tissue, (2) it is predominantly composed of proteins and therefore requires minimal sample preparation, (3) it grows fast and at a 'known' rate, (4) it does not remodel after deposition, (5) the isotope signal is recorded sequentially as the tissue grows and retained unaltered over time, (6) the sampling procedure is minimally invasive, and (7) comparative studies on living individuals can be easily performed.

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By undertaking stable isotope analysis of sequential segments of scalp hair, which are cut along the length of the hair fibre(s), it is possible to detect changes in the diet and health of individuals. The temporal resolution that can be achieved depends on the sampling strategy and the isotope technique applied. Traditionally, isotope studies of archaeological hair were (and often are) performed on a bundle of multiple hair fibres using an elemental analyser-isotope ratio mass spectrometer (EA-IRMS) that measures the isotope composition of the whole (*bulk*) protein.

In the last twenty years, novel compound-specific techniques have been developed, which permit the measurement of the isotope composition of the various amino acids constituting the protein (Dunn et al., 2011). An instrument such as a liquid chromatographer-isotope ratio mass spectrometer (LC-IRMS) requires a very limited amount of keratin sample, making it possible to analyse a 0.5-cm-long segment of a single hair fibre and therefore reconstruct a dietary intake on a fortnightly basis (Mora et al., 2017).

Several stable isotopes can be investigated in hair depending on the specific research questions, but herein we focus on carbon and nitrogen, which are the isotope ratios traditionally measured for reconstructing dietary intakes (and incidentally mobility), and for identifying physiological and pathological conditions. Moreover, carbon and nitrogen isotope ratios can be measured in either the protein as a whole or in the amino acids constituting the protein, depending on which technique (bulk or compound-specific) is applied. Compound-specific isotope analysis gives an interesting insight into the metabolism of the various amino acids and their associate isotopic fractionation, which significantly improves the information pertaining to the sourcing of the three macronutrients constituting the dietary intake (proteins, carbohydrates, lipids).

2. Stable isotopes

2.1. What are stable isotopes?

Isotopes are atoms of the same element having a different number of neutrons and thus different atomic mass. The difference in masses affects the properties of the isotopes and the way they behave in reactions. In nature, a variety of processes, with associated isotopic fractionation, take place, which result in a variation in the ratio of the two isotopes ($^{13}C/^{12}C$ for carbon; $^{15}N/^{14}N$ for nitrogen).

In the laboratory, the stable isotope ratios are measured by a mass spectrometer relative to their international standards (V-PDB: Vienna Pee Dee Belemnite for carbon; AIR: Ambient Inhalable Reservoir for nitrogen) and reported in parts per thousand (‰ per mil, $\delta^{13}C$ for carbon and $\delta^{15}N$ for nitrogen) (Hoefs, 2015).

2.2. Carbon stable isotopes

The carbon isotope ratio measured in the archaeological human proteins depends on those of the plant and animal products consumed, which in turn are derived by the specific plant ecosystem at the base of the food chain. The $\delta^{13}C$ value of the bone collagen of a plant consumer is roughly 5‰ less negative than that of the plants consumed (van der Merwe and Vogel, 1978), although it may vary depending on the diet. Moving up the food chain, for each trophic level, there is a very small fractionation associated (~1‰) (Schoeninger, 1985).

Plants are traditionally subdivided into two groups (C_3 and C_4), which follow distinctive photosynthetic pathways and therefore present non-overlapping ranges of $\delta^{13}C$ values. This is true if we exclude a series of environmental factors (e.g. temperature, altitude, latitude) that may affect the plant $\delta^{13}C$ value (Codron et al., 2005, Kohn, 2010). C_3 plants are typically found in temperate areas and include vegetables, legumes, cereals, and fruits. Their $\delta^{13}C$ values span from about –20‰ to –37‰ (Kohn, 2010, O'Leary, 1988). C_4 plant values generally range from –6‰ to –19‰ (O'Leary, 1988, Smith and Epstein, 1971). These plants are adapted to heat and aridity and include maize, sorghum, millet and sugar cane. There exists a third group of plants (CAM) that have photosynthetic flexibility (night- vs. day-time) to survive arid environments (Cushman, 2001) and present $\delta^{13}C$ values that are overlapping with those of C_3 and C_4 plants (Llano and Ugan, 2014, O'Leary, 1988). The Crassulacean Acid Metabolism plants include cacti, agave and pineapple (Cushman, 2001). In the aquatic environment, resources from marine and freshwater ecosystems present (mostly) non-overlapping ranges of $\delta^{13}C$ values, since the particulate organic carbon (POC) at the base of the food chain ranges from –18‰ to –24‰ in seawater and from –24‰ to –30‰ in fresh water (Fry and Sherr, 1989).

2.3. Nitrogen stable isotopes

The nitrogen isotope composition of human tissues depends on the type (plant or animal) and quantity of proteins ingested. Within the terrestrial ecosystems, leguminous plants present lower $\delta^{15}N$ values than those of non-nitrogen fixing plants because they can fix atmospheric nitrogen, which has a value close to 0‰. The non-leguminous plants use nitrogen available from the decomposed organic matter in the soil, which has more positive $\delta^{15}N$ values. When fertilisers are applied to the cultigens, the $\delta^{15}N$ values of plant tissues are increased to a certain amount depending on the type of manure (Fraser et al., 2011, Szpak et al., 2012, Szpak et al., 2014). In addition, the nitrogen isotope composition of plants may be affected by several environmental and physiological factors, mostly associated with heat, salinity, and water paucity (Britton et al., 2008, Evans, 2001, Heaton, 1987, Schwarcz et al., 1999). In hot and arid environments, the $\delta^{15}N$ value of animal tissues is also increased by consumption of those ^{15}N-enriched plants or by the animal physiology itself (Balter et al., 2006, Hartman, 2011). In the aquatic ecosystems, consumers present more positive $\delta^{15}N$ values than those of terrestrial carnivores because in marine food webs there are many more trophic steps than in terrestrial ones, and each step up in the food chain is associated with an increase of about 3‰ in the $\delta^{15}N$ values (Schoeninger and DeNiro, 1984).

The nitrogen isotope composition of human body tissues reflects the intake of dietary proteins, but it may be affected by physiological and pathological factors. When the nitrogen metabolism of an individual is altered by stressors, the body enters anabolism or catabolism, depending on whether the biochemical processes favour, respectively, the synthesis of proteins or their breakdown (Reitsema, 2013). A body in catabolic state (negative nitrogen balance) is characterised by an extensive breakdown of pre-existing proteins to recycle amino acids for the synthesis of new proteins in an affected area of the body. These processes of transamination and deamination, which are associated with isotope fractionation, produce nitrogenous waste (urea) depleted in the heavier isotope (^{15}N), leaving the body tissues ^{15}N-enriched. Examples of catabolism can be found in individuals experiencing infection (D'Ortenzio et al., 2015, Katzenberg and Lovell, 1999) or malnutrition (Mekota et al., 2006, Neuberger et al., 2013), and their body tissues are characterised by more positive $\delta^{15}N$ values. Conversely, a body in anabolic state (positive nitrogen balance) maximises protein synthesis and increases nitrogen retention, which may result in a decrease in the $\delta^{15}N$ value of body tissues. Pregnant women, who are not experiencing morning sickness, are a typical example of a body in anabolic state (D'Ortenzio et al., 2015, Fuller et al., 2004, Fuller et al., 2005).

3. Analytical techniques

3.1. Bulk stable isotope analysis

The conventional way to measure $\delta^{13}C$ and $\delta^{15}N$ values of proteinaceous tissues is by using an elemental analyser coupled to an isotope-ratio mass spectrometer (EA-IRMS). Both isotopes can be measured in the same sample run of the whole protein, which makes this technique quite rapid and affordable. The downside is that sample size in the order

of the milligram is required, and the bulk $\delta^{13}C$ and $\delta^{15}N$ values are a weighted average of all the amino acid fractions constituting the protein.

3.2. Compound-specific stable isotope analysis

Compound-specific isotope analysis measures the carbon, and/or nitrogen, isotope composition(s) of the various amino acid fractions constituting the whole protein. Firstly, the amino acids are freed from their protein structure, and then separated using a chromatographic method, either gas- or liquid-based (Dunn et al., 2011). Selecting a chromatographic column with a smaller diameter reduces the amount of sample needed and consequently improves the temporal resolution of the palaeodietary reconstruction. A hair segment as short as 0.5 cm can be measured on a liquid chromatographer-isotope ratio mass spectrometer (LC-IRMS) equipped with a Primesep A column (2.1 × 250 mm, 100 Å, 5 μm) (Mora et al., 2017).

Compared to bulk techniques, the compound-specific process is more complex, expensive and time-consuming, but the results are worth it. For instance, it is possible to trace the source (aquatic, terrestrial) of the various components of the diet at the macronutrient level. The differential isotope ratios of the several amino acids constituting the protein are indicative of the dietary, metabolic, and physio-pathological aspects characterizing the consumer's life since they are the result of the different fluxes from diet to consumer and of metabolic pathways within the consumer's body.

4. Human scalp hair

4.1. The hair fibre morphogenesis

The hair originates from a follicle, located a few millimetres below the surface of the skin. The first hair cells are synthesised at the base of the follicle (bulb), which then multiply and differentiate to form the various structures of the hair fibre. Pigmentation occurs at this stage. Extending upward towards the skin surface, the hair undergoes keratinization with dehydration and compression of the hair cells. At this point, the hair is a permanent fibre, ready to emerge from the skin, while moving through the inner root sheath and the hair canal (Harkey, 1993, Robbins, 2012a, Tobin, 2005a).

4.2. The hair fibre microstructure and pigmentation

The hair shaft is composed of three concentric layers, which are the cuticle, the cortex and (if present) the medulla. This multi-layered structure is bound together by a cell membrane complex, a mixture of cell membranes and adhesive material. The *cuticle* is the outermost layer of the hair shaft and protects the bulk of the hair. Cuticle scales have an appearance similar to that of roof tiles, being positioned slightly overlapping and pointing towards the tip end of the hair fibre (Harkey, 1993, Robbins, 2012a, Tobin, 2005b).

The *cortex* constitutes the bulk of the hair, accounting for up to 90% of its mass. Air spaces (cortical fusi) and pigments granules (melanins) are found within this layer (Harkey, 1993, Robbins, 2012a). Only two types of melanin exist (eumelanin and pheomelanin), but their quantity and ratio generate the wide spectrum of hair colours present in nature (Borges et al., 2001, Ito and Wakamatsu, 2011). Hair pigmentation changes with ageing. The greying of hair is likely caused by a reduction in the number of melanin-producing cells (melanocytes) in the hair bulb and a consequent decrease in melanins along the hair shaft (Commo et al., 2004, Tobin, 2008). Despite the evident differences between pigmented and grey hair, it has been shown that the reduction in melanin content in grey hair has no significant influence on carbon and nitrogen content, nor on the $\delta^{13}C$ and $\delta^{15}N$ values of scalp hair (Minagawa, 1992, O'Connell and Hedges, 1999). The fact that melanin granules contribute only minimally to the overall mass of the hair fibre (McKenzie et al., 2007) might be the reason. The *medulla* represents the porous core of the hair fibre. It may be continuous along the hair axis, discontinuous, or even absent (Harkey, 1993, Robbins, 2012a, Tobin, 2005b).

4.3. The macromolecular and chemical composition of hair

Human hair is largely constituted by proteins (65–95%, keratins), and in fewer amounts by water (15–35%), lipids (1–9%), pigment, and trace elements (Harkey, 1993, Robbins, 2012b). Approximately twenty amino acids have been identified as being the constituents of the human hair keratins. They slightly vary in abundance across the different structural components of the hair shaft (see Robbins, 2012b). The numerous disulfide bonds cross-linking the cysteine residues of the proteins (Tobin, 2005b) make human hair a stable, durable and resistant fibre. Since the predominant component of hair is protein, the cleaning and processing of the hair sample before isotope analysis is limited, and the sampling is restricted to the area of interest because there is minimal loss of mass as a result of protein 'extraction'.

At the elemental level, human hair consists of approximately 50% carbon, 22% oxygen, 16% nitrogen, 7% hydrogen, and 5% sulphur (by weight) (Popescu and Höcker, 2007). The fact that hair contains three times less nitrogen than carbon defines how small the hair fibre can be sampled in bulk stable isotope analysis. Since carbon and nitrogen isotope ratios are generally measured in a single run by elemental analyser-isotopic ratio mass spectrometry (EA-IRMS), it is the concentration of nitrogen that defines the minimum amount of sample required for generating reliable $\delta^{15}N$ (and $\delta^{13}C$) values.

5. Scalp hair growth

5.1. Hair growth cycle

Hair growth is thought to be a cyclical process that consists of subsequent phases of growth (anagen), regression (catagen), and rest (telogen). Eventually, the old hair is shed and the follicle re-enters in the actively growing phase producing a new hair fibre. This is, however, an oversimplified depiction of the hair follicle cycle, which likely includes many more steps (Bernard, 2012, Higgins et al., 2009, Paus and Foitzik, 2004).

At any given time, the majority of the hair follicles on an adult head are in the growing phase (~85%) (Harkey, 1993), since the anagen phase lasts significantly longer than the catagen and telogen phases (i.e. anagen, 2 to 6 years; catagen, a few weeks; telogen, 2 to 4 months) (Krause and Foitzik, 2006). If a hair fibre from a follicle in the telogen or catagen phase is sampled for isotope analysis, the isotope signal measured in the segment closest to the scalp may be delayed for several weeks or even a few months.

Humans present an asynchronous pattern of hair growth and shedding, meaning that each hair follicle follows its own internal clock, and it can be at a different growth stage than those surrounding it. This implies that contiguous hair fibres, sampled for bulk stable isotope analysis, could be out-of-phase. In addition, the length of the hair cycle phases may be affected by factors such as age (Robbins, 2012c), hormonal changes during pregnancy and after delivery (Gizlenti and Ekmekci, 2014), seasonality (Randall and Ebling, 1991), nutritional deficiencies (Goldberg and Lenzy, 2010), and disease (Wilson et al., 1992).

Conversely to adults, hair growth and loss are synchronous in infancy. During late fetal and early neonatal life, adjacent follicles (within the same region of the scalp) are in synch and therefore the hair fibres are grown and shed simultaneously (Barth, 1987, Furdon and Clark, 2003). At birth, newborns may present very fine and non-medullated hair (lanugo), which are soon replaced by fine and medullated hair (vellus). These intermediate hair persist up to two years of age, when they are eventually replaced by terminal hair (Duggins and Trotter, 1950).

5.2. Rate of growth

In humans, the average elongation rate for scalp hair is around 0.35 mm/day, spanning between 0.3 and 0.5 mm/day (Krause and Foitzik, 2006, Lehn et al., 2019, Tobin, 2005a). Many factors may affect the rate of hair growth, including age and sex (Pecoraro and Astore, 1990). White (unpigmented) hair grow faster than pigmented hair (Choi et al., 2011); hair located in the vertex region of the scalp (top of the head) grow slightly faster than those located in the temporal and occipital regions (Loussouarn et al., 2005).

6. Food intake and the body's isotope equilibration to the dietary signal

The isotope compositions of hair keratins have proven to be representative of human dietary choices. Distinctive keratin $\delta^{13}C$ and $\delta^{15}N$ values have been detected in the hair of individuals consuming an omnivorous, ovo-lacto-vegetarian, or vegan diet (Petzke et al., 2005).

The consumption of foodstuffs that differ from the routine dietary intake brings into the body an isotopically different pool of macronutrients, which are directly assimilated or subjected to metabolic processes. The resulting amino acids, either routed from food or biosynthesised within the body, are ultimately incorporated into the newly synthesised body proteins. The variation in the dietary isotope signal induces a rapid change in the keratin $\delta^{13}C$ and $\delta^{15}N$ values (Lehn et al., 2015, O'Connell and Hedges, 1999), which makes possible the identification of short-term dietary changes along the hair fibres. Although the initial response of the body to a dietary change is quite rapid, it takes at least several days for the growing hair to emerge from the skin and being accessible for sampling (Valkovic, 1988). Moreover, the process conducive to full isotope equilibration between the new dietary signal and the hair keratins is gradual, being slowed down by the recycling of endogenous amino acids, which are supplied via the breakdown of pre-existing proteins carrying the 'old' isotope signal (Lehn et al., 2019, O'Connell and Hedges, 1999). Recycling of amino acids for protein synthesis may be more extensive under an excessively high protein intake since the dietary amino acids would be preferentially catabolised. This would delay further the variation in the keratin $\delta^{13}C$ and $\delta^{15}N$ values (Petzke and Lemke, 2009).

Stable isotope studies on scalp hair of modern individuals have shown that it can take several months for carbon and nitrogen isotopes to achieve a steady state after a drastic change in diet, such as moving from a C_3- to a C_4-based diet (or vice versa) (Huelsemann et al., 2009, McCullagh et al., 2005, O'Connell and Hedges, 1999) or from an omnivorous to a vegan diet (O'Connell and Hedges, 1999). Generally, the complete isotope equilibration to the new dietary signal is reached faster by nitrogen isotopes than carbon isotopes (Huelsemann et al., 2009, O'Connell and Hedges, 1999). This is likely because nitrogen isotopes are incorporated into the body solely from dietary proteins, while carbon isotopes are derived from all three macronutrients (proteins, carbohydrates, lipids), which additionally generate interconversion products (Remien, 2015). As a result, the 'old' carbon isotope signal derived from the previous diet remains in circulation within the body for a longer period, acting as an isotopic buffer against the process of equilibration towards the new dietary isotope signal.

7. The fate of scalp hair: Degradation or survival?

The preservation of the original composition and structure of the hair fibres is a prerequisite for keratin stable isotope analysis. The chemical, physical, and mechanical properties of human hair are overall remarkable, fostering the survival of this tissue in very inhospitable environments. Nevertheless, there exist ante- and post-mortem degradative processes that may damage hair integrity.

7.1. Processes destructive to the hair

Invasive cosmetic procedures may fracture the cuticle and chemically alter the amino acid composition of hair fibres (Cruz et al., 2016). Artificial mummification treatments using embalming resins may introduce contaminants, which alter the isotope composition of hair (White et al., 1999).

If bodies were deposited above the ground, visible and ultraviolet light exposure might have induced photo-oxidative degradation of hair proteins. The rupture of disulphide bridges induces brittleness and loss of structural definition exposing the hair to potential contaminants. Melanin granules are eventually oxidized generating changes in hair colour (Fernández et al., 2012, Hoting et al., 1995, Ito et al., 2018, Nogueira and Joekes, 2007). The preferential preservation of yellow–red melanins (phaeomelanin) over the brown-black ones (eumelanin) would explain the peculiar blonde-reddish hair colour observed in certain mummies (Wilson et al., 2001).

Scalp hair retrieved from an aqueous environment might have adsorbed water molecules over time if the hydrophobic surface of cuticles had been damaged exposing the hygroscopic core of the fibres (Kempson et al., 2010, Popescu and Höcker, 2007). Although wet burials may induce significant macroscopic degradation, the keratin $\delta^{13}C$ and $\delta^{15}N$ values may be minimally affected (von Holstein et al., 2014).

Hair fibres buried in soil may become a source of nutrients for bacteria and fungi (Tridico et al., 2014). The attack of these microorganisms appears quite selective, favouring the hair components with lower degrees of keratinization, leaving the more keratinized structures apparently intact (Wilson et al., 2007a). Keratinolytic fungi can digest keratin molecules using specific enzymes (keratinases) and the by-products of keratin breakdown can then be targeted by keratinophilic fungi (Błyskal, 2009, English, 1963, 1965, Filipello Marchisio et al., 2000). The hair samples might be subjected to biodegradation even while held in museum collections (Hawks and Rowe, 1988), or undergo contamination while stored in laboratories, where the use of inappropriate storage materials containing additives might alter their isotope composition (Fraser et al., 2008).

7.2. Processes conducive to hair preservation

The post-mortem survival of human scalp hair is enabled by the concurrent occurrence of favourable environmental and biological conditions, and, sometimes, by anthropic activity. Extremely hot or cold environments, combined with dry-windy conditions, may foster the rapid dehydration/desiccation of body tissues (Lynnerup, 2007). By quickly removing the water content from soft tissues, the onset of the natural decay process, driven by destructive enzymes, is inhibited (Aufderheide, 2011). Desiccation is one of the several mechanisms conducive to natural mummification, which is the successful preservation of non-bony tissues of deceased individuals (Lynnerup, 2007). The extremely dry conditions of the Atacama Desert (Peru, Chile), the Sahara Desert (Egypt, Sudan), and the Gobi Desert (China, Mongolia) have favoured the natural preservation of human hair fibres over the millennia (Mora et al., 2017, Turner et al., 2012, White et al., 1999).

Dehydration can also be achieved at low temperatures. Freeze-drying is conducive to preservation through a combination of cold, dry, and windy conditions (Lynnerup, 2007). Ice-mummies with intact scalp hair were found on the peaks of the South American Andes (Wilson et al., 2007b), in the arctic regions (Siberia, Greenland, Alaska) (Britton et al., 2018), and on the peaks of the European Alps (Macko et al., 1999). This is not exhaustive of all the potential mechanisms conducive to natural preservation of human hair fibres.

Preservation of human remains can also be achieved by consciously treating the dead bodies (Allison et al., 1984, Aufderheide et al., 1999). A clear distinction between natural and artificial mummification practices may be challenging since bodies could have been purposely disposed of in environments where preservation would have naturally

taken place (Lynnerup, 2007). Ancient morticians likely observed the natural processes conducive to body preservation and subsequently mimicked those in their artificial treatments of the dead (Arriaza, 1995). This is why artificial mummification practices thrived in regions where tissue preservation was naturally favoured, such as Chile, Peru, and Egypt (Guillén, 2004, Jones et al., 2018). In these landscapes, ancient populations developed their own complex system of mummification treatments and the cult of ancestors, following particular beliefs and purposes (Aufderheide et al., 1999, Marquet et al., 2012).

8. Hair sample collection and laboratory processing

8.1. Hair collection

When feasible, the hair samples should be collected by plucking the fibres from the vertex of the head, which is the region of the scalp where hair is generally growing faster. Plucking the hair fibres ensures that the hair roots are sampled, making it possible to identify the hair cycle phase. When the bundle of hair is cut using a scalpel or scissors, the most recent isotope information recorded at the roots and in the first millimetres of the hair fibres is lost, and it is no longer possible to reconstruct the 'latest' dietary intake of the individual. This is true only if these fibres were in the growing-anagen phase.

8.2. Sampling strategies and implications: A comparison between bulk and compound-specific approaches

Whether conducting isotope analysis on multiple fibres or on a single fibre of scalp hair depends on research questions, amount of sample and its state of preservation, curators' regulations, analytical techniques, level of expertise, and ultimately on budget. Routine bulk carbon and nitrogen isotope analyses are conducted using an EA-IRMS, which measures the isotope ratios of both elements in a single run. Considering instrument sensitivity as well as hair mass and chemical composition, bulk carbon and nitrogen isotope analysis requires measuring multiple fibres of hair when working with 1-cm-long segments or shorter.

The main limitation of isotopically measuring multiple hair fibres is that even adjacent fibres can be out-of-phase. Collecting a bundle containing at least 25 fibres is a way to increase the likelihood that the sampled hair fibres are in growing-anagen phase (Mekota et al., 2006). Otherwise, after collection, actively growing fibres can be selected via microscope analysis, avoiding the out-of-phase error (Williams et al., 2011). Nevertheless, the potential variability in growth rates between the fast- and slow-growing hair fibres would persist (Lehn et al., 2019). Both approaches require the sampling of large quantities of scalp hair, which could be discouraged by curators of ancient human remains.

Statistical (Remien et al., 2014) and isotope studies (Lehn et al., 2019, Mora et al., 2017, Williams et al., 2011) have shown that measuring out-of-phase hair fibres in bulk isotope analysis poses some limitations to the palaeodietary reconstructions, hindering the detection of subtle variations in diet over time. The shift in isotope values (measured along a bundle of hair fibres) appears reduced in amplitude when compared to the effective variations in the original dietary isotope signal. Therefore, the extent of the dietary variations experienced by an individual could be underestimated in bulk isotope analysis of multiple hair fibres, although the timing of these changes could be still correctly estimated (Mora et al., 2017). The attenuation of the original isotope signal appears greater in the oldest hair segments (tip) (Lehn et al., 2019).

Investigating a single hair fibre, in place of a bundle of hair, avoids the measurement of misaligned fibres, either induced by growth cycle error or differential growth rates, while respecting the integrity of the archaeological collection. Thanks to the recent advancements in chromatography, it is possible to conduct compound-specific stable isotope analysis on a very small amount of keratin sample. Amino acid $\delta^{13}C$ values were successfully measured in keratin hydrolysates generated from a 0.5-cm-long segment cut from a single fibre of scalp hair (Mora et al., 2017). The longitudinal sampling of very short segments (cut along a single hair fibre) allows the high-resolution detection of seasonal diets, short-term nutritional changes, or occasional intakes to the full extent of their isotope variation. The hair fibre should be sampled in the growing phase to assure that the isotope composition measured in the hair segment closest to the scalp (root included) represents the most recent dietary information.

In brief, when the aim of the research project is to determine the food consumed by an individual during the period leading to their death, the analysis of the latest hair segment, cut from a bundle of 25+ fibres, may increase the likelihood of measuring metabolically active hair, which were recording the most recent dietary habits. In contrast, when investigating dietary changes over time, especially subtle/occasional changes or short-term/seasonal variations, compound-specific isotope analysis of longitudinal segments cut along a single fibre of hair should be preferred.

8.3. Sample orientation, identification of the hair cycle phase, and other morphological examinations

Palaeodietary reconstructions can be improved by undertaking a microscopic examination of the collected hair fibres. Optical and electron microscope analyses are useful tools for orienting the hair fibres, identifying the phase of the hair cycle, establishing the human origin, and assessing the structural integrity of the hair samples (Ogle and Fox, 1998, Wilson et al., 2010).

When the hair samples are provided without roots, it is possible to assess the orientation of the fibres by examining the morphological characteristics of the cuticle scales, which, resembling roof tiles, point towards the tip end of the hair fibres (Deedrick and Koch, 2004a). Moreover, the cortical fusi (air pockets) are generally located within the cuticle in the proximity of the root end (Oien, 2009). If unsure about the human origin of the sample, the morphological characteristics of cuticle, cortex and medulla, and the shape of the hair root should help to discriminate between human and animal fibres (Cortellini et al., 2019, Deedrick and Koch, 2004a, 2004b).

Assessment of the growth phase is achieved by studying the morphology of the hair roots (see Ogle and Fox, 1998, Petraco and Kubic, 2004). Hair fibres in the growing phase (anagen) usually display a fully pigmented elongated root, which appears stretched as a result of sample plucking. Some epithelial tissue may be found adhering at the surface (Ogle and Fox, 1998). By selecting and isotopically analysing only hair fibres in the anagen phase, the temporal resolution of the dietary reconstruction is significantly improved and the longitudinal variations in keratin isotope composition are clearly distinguishable (Williams et al., 2011).

Microscope analysis helps identify coatings applied to hair for aesthetic purposes, either in life (McCreesh et al., 2011) or post-mortem (Yatsishina et al., 2020). The scalp hair of mummies can be found infested by head lice, *Pediculus humanus capitis* (Arriaza et al., 2013). Their eggs are laid on the hair fibres, close to the scalp, as the nymphs rely on human blood for survival (Pray, 1999). The empty egg cases should be removed before undertaking stable isotope analyses.

8.4. Sample cleaning

Before proceeding with the cleaning process, the hair fibres should be oriented, aligned, and kept in phase using an appropriate inorganic wrapping/thread that does not affect the isotope composition of the sample nor alter during chemical washings (e.g. Knudson et al., 2007). Since the hair fibres are predominantly composed of keratin proteins, they do not require protein extraction as other tissues do (e.g. bone collagen, tooth dentine).

Each laboratory generally develops and standardises its own cleaning protocols. Usually, an initial cleaning wash (in an ultrasonic bath)

with ultrapure water is performed to remove loose particulate adhering to the surface (e.g. Britton et al., 2018), otherwise the hair fibres are wiped with wipes soaked in ethanol (e.g. Webb et al., 2013). Subsequently, samples are directly immersed in a solvent (ethanol or methanol) (e.g. Turner et al., 2013, White et al., 2009) or a mixture of solvents in various ratios (e.g. methanol, acetone, chloroform) to remove lipids and body fluids (e.g. Jackson et al., 2015, Knudson et al., 2015, Touzeau et al., 2014, Webb et al., 2013, Williams et al., 2011). Several cycles of washing in ultrapure water under sonication are eventually needed to remove the solvents.

Ad-hoc cleaning protocols are required to remove embalming resins from artificial mummies (Cockitt et al., 2020), which vary depending on the nature of the preservative material. Embalming substances could be isolated and isotopically analysed to exclude potential contamination to the hair sample (White et al., 1999). Once the cleaning process is completed, the hair fibres should be left to dry naturally in air or under a fume-hood (e.g. Webb et al., 2013), and/or dried in a freeze-drier for approximately 12 hrs (e.g. Britton et al., 2018).

8.5. Sample preparation

8.5.1. Sample preparation in bulk stable isotope analysis

Once the cleaning process is completed, the aligned hair fibres are sectioned into sequential segments, starting from the root, using a sterile scalpel. The length of the hair segment defines what temporal resolution can be achieved in the palaeodietary reconstruction. Highly time-resolved studies (conducted on very short hair segments) require sampling numerous hair fibres to achieve the minimum sample weight necessary for bulk analysis. For consistency, the chosen length should be kept constant along the hair bundle and across all individuals under investigation. The hair segments are then inserted into tin capsules for EA-IRMS analysis, which measures the carbon and nitrogen isotope ratios in the whole keratin protein.

8.5.2. Sample preparation in compound-specific stable isotope analysis

The hair fibre is sectioned into longitudinal segments, starting from the root, using a sterile scalpel. Before isotope analysis, the amino acids constituting the keratins need to be freed from the protein structure via acid hydrolysis. Each hair segment is inserted into a hydrolysis tube and heated under vacuum in 6 M hydrochloric acid to ~ 110 °C for at least 24 hrs. A longer reaction time is usually required to cleave the more resistant bonds. As a result of acid hydrolysis, asparagine and glutamine are deaminated to aspartic acid and glutamic acid (Fountoulakis and Lahm, 1998). The resulting mixture of amino acids is simply dissolved in ultrapure water prior to liquid chromatography-isotope ratio mass spectrometry (LC-IRMS). Instead, keratin acid hydrolysates require derivatization prior to gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS).

9. Assessment of hair keratin preservation

In bulk stable isotope analysis, the elemental integrity of the hair sample is conventionally assessed by verifying the carbon (%C) and nitrogen (%N) content by weight and calculating the C/N atomic ratio [C/N = (%C/C atomic weight)/(%N/N atomic weight)]. Archaeological hair samples should present a C/N comparable to that of modern hair, which ranges from 2.9 to 3.8 (O'Connell and Hedges, 1999). If the sample amount is too small, the nitrogen concentration would be insufficient and the calculated C/N ratio would fall outside the proposed range, even if the hair sample is well preserved.

Ideally, the content by weight should be around 44–46% for carbon and 13–14% for nitrogen (Roy et al., 2005, Valkovic, 1988). A higher content in carbon could be caused by contamination with carbon-rich substances such as lipids. If that is the case, a more stringent cleaning protocol should be followed.

In compound-specific isotope analysis, the integrity at the molecular level can be assessed by comparing the amino acid profiles of the archaeological keratins to those of modern hair samples (Mora et al., 2018). The accuracy of the amino acid isotope values can be evaluated by comparing the calculated mass balance value to the corresponding bulk value, if bulk measurements have been performed (Jim et al., 2006).

10. Reconstructing the palaeodiet

Traditionally, the palaeodiet is reconstructed by comparing the human keratin $\delta^{13}C$ and $\delta^{15}N$ values to the isotope values of the foodstuffs available to the individual in antiquity. Plant and animal $\delta^{13}C$ and $\delta^{15}N$ values should be corrected with respect to their edible portions (e.g. grains, meat) (e.g. Codron et al., 2005, Mateo et al., 2008, Warinner and Tuross, 2010). When the isotope values of ancient foods are not available, it is possible to build an ecological baseline by isotopically measuring modern foodstuffs, providing they existed in antiquity. However, their $\delta^{13}C$ values should be appropriately corrected to account for the Suess Effect, which is the decrease in the ratio of $^{13}C/^{12}C$ in atmospheric CO_2 due to fossil-fuel emissions (Dombrosky, 2020, Eide et al., 2017, Marino and McElroy, 1991).

As a result of macronutrient assimilation, there exists an isotope fractionation between the food consumed and the human body tissues. In past dietary studies, it was often considered a difference between diet and scalp hair of 3.5‰ for $\delta^{13}C$ values and of 3.0‰ for $\delta^{15}N$ values (e.g. Knudson et al., 2015, White et al., 2009). However, controlled dietary surveys across several contemporary populations (Hedges et al., 2009, Naito et al., 2015, O'Connell et al., 2012, Yoshinaga et al., 1996) have reported much greater fluctuations with offset values ranging from 1.8‰ to 4.8‰ (Yoshinaga et al., 1996) for $\delta^{13}C$ and from 3.9‰ (Naito et al., 2015) to 6.9‰ (Yoshinaga et al., 1996) for $\delta^{15}N$. This leads to average diet-to-keratin offsets of ~ 3.8‰ for $\delta^{13}C$ values and ~ 4.9‰ for $\delta^{15}N$ values (Mora et al., 2017). The most recent study by Yoshinaga and colleagues (2021), who followed a different approach in estimating the whole-diet isotope values ('market basket' method), has reported smaller isotope offsets (1.9‰ for $\delta^{13}C$; 4.3‰ for $\delta^{15}N$) between the Japanese diet and scalp hair.

The correct reconstruction of the dietary intake may be challenging when a variety of food combinations can result in equivalent isotope compositions of a consumer's tissue. For this reason, palaeodietary studies have been increasingly relying on Bayesian Stable Isotope Mixing Models (BSIMM) to interpret stable isotope data, circumventing the issue of isotopic equifinality. Stable isotope mixing models are able to estimate the percentages of the various foodstuffs constituting the diet because they take into account how the various macronutrients are differentially contributing to the carbon and nitrogen isotope compositions of the consumer's body tissues. Nitrogen is solely routed from dietary proteins, while carbon is retrieved in different proportions from the protein, carbohydrate and lipid fractions (Fernandes et al., 2012). Mora and colleagues (2017) have proposed that carbon in keratin is sourced by ~ 86% from dietary proteins and ~ 14% from carbohydrates and lipids. The inclusion of prior information and parameters' uncertainties helps in customising the mixing model to the specific dietary context and body tissues, which improves the quality of the dietary estimates. There exists a variety of SIMMs (Phillips et al., 2014) and some have been applied to reconstruct the diets of ancient individuals using keratin isotope data (Britton et al., 2018, Mora et al., 2017).

The significant advantage of measuring amino acid isotope values, over bulk values, is that the amino acids constituting the protein are involved in different metabolic pathways and, for this reason, they carry differential information pertaining to certain dietary macronutrients. Being able to track the source of the protein, carbohydrate and lipid portions of the diet is exceptionally useful, especially when reconstructing mixed marine-terrestrial dietary intakes, or mixed plant-animal dietary intakes.

With respect to nitrogen metabolism, amino acids can be broadly

divided into two groups: those undergoing minimal isotopic discrimination ('source' amino acids) and those undergoing, to a lesser or greater extent, isotopic discrimination as a result of deaminating reactions during transfer from dietary proteins to consumer ('trophic' amino acids) (Takizawa et al., 2020). This means that in a consumer, the 'source' amino acids (e.g. phenylalanine) have similar $\delta^{15}N$ values to those of their nitrogen sources at the base of the food web, while 'trophic' amino acids (e.g. glutamic acid) increase in their $\delta^{15}N$ values from prey to consumer. By applying an appropriate equation that contains the collagen $\delta^{15}N$ values of the 'source' phenylalanine and the 'trophic' glutamic acid from the same individual, it is possible to quantify the contribution of marine proteins to the diet (Naito et al., 2010, Styring et al., 2010), or the differential proportion of plant and animal proteins (Naito et al., 2013).

With respect to carbon metabolism, the 'essential' amino acids undergo minimal isotopic discrimination, being assimilated directly from dietary proteins to body tissues, while the 'non-essential' amino acids undergo (to a lesser or greater extent) isotopic fractionation, being (mainly) biosynthesised within the body. This implies that the $\delta^{13}C$ values of the 'essential' amino acids (e.g. phenylalanine, valine, leucine) in body tissues are similar to those of the ingested dietary proteins. In contrast, the $\delta^{13}C$ values of 'non-essential' amino acids (e.g. glycine, alanine, glutamic acid) are indicative of all three dietary macronutrients (especially of carbohydrates and lipids) since these amino acids are (mainly) *de novo* synthesised, following specific metabolic pathways.

Although the majority of dietary proxies have been developed from bone collagen isotope data, some appear to be applicable also to hair keratin datasets. For instance, the difference between valine and phenylalanine $\delta^{13}C$ values within the same individual ($\Delta^{13}C_{\text{Val-Phe}}$) helps to discriminate between high marine protein-, high freshwater protein-, terrestrial C_3-, and terrestrial C_4-consumers (Honch et al., 2012). In recent years, researchers have focused on developing dietary markers for keratin amino acid $\delta^{13}C$ values, proposing $\Delta^{13}C_{\text{Leu-Phe}}$ as a tool for discriminating between consumers of marine and terrestrial diets (Mora et al., 2018). This index is able to identify the predominant protein source even when the individual is consuming a mixed marine-terrestrial diet. When the keratin $\Delta^{13}C_{\text{Leu-Phe}}$ values are plotted against the $\delta^{13}C$ valine values, it is also possible to determine whether the terrestrial resources are of C_3 or C_4 origin (Mora et al., 2021).

11. Contributions of hair stable isotope analysis to archaeological studies

The combined carbon and nitrogen isotope analysis of hair fibres is a successful tool for reconstructing the recent life history of individuals. Its investigative power mainly depends on sampling strategy and isotope technique. The longitudinal study of intraindividual variations in keratin isotope compositions allows the reconstruction of an individual's diet, health status, travels and relocation happening during the last months/year of life. The concurrent investigation of other body tissues may extend the temporal reconstruction to the last decades of life or even up to early childhood (e.g. bone, teeth), depending on tissues' turnover rates and formation times (e.g. Frei et al., 2015, Hyland et al., 2021, Mora et al., 2021, Richards et al., 2007, Scaffidi et al., 2021, Turner et al., 2012). Interindividual comparison in keratin isotope compositions may shed light onto cultural, economic, and socio-political aspects of ancient populations.

11.1. Stability or seasonality in diet?

Stability in diet implies continuous intake of a defined range of foods, whose proportion may slightly change over time. Low dietary variability generates a keratin isotope signal consistent year-round. Stable diets were detected in the hair of coastal individuals relying predominantly on marine resources (e.g. Aufderheide et al., 1994, Britton et al., 2013, Mora et al., 2017), or agriculturalists living in an environment with limited seasonal fluctuations and no access to storage facilities or imported goods (e.g. White et al., 1999).

Seasonality in diet implies alternating intake of the seasonal C_3 and C_4 plant foods, which induces cyclical shifts in the keratin $\delta^{13}C$ values. C_3 plants are grown during the cool-wet months and their consumption generates more negative $\delta^{13}C$ values than the C_4 plants, which are planted and harvested in the warm-arid months. The characterization of the frequency, length and amplitude of the shifts in $\delta^{13}C$ values along the hair fibres may permit the reconstruction of the local agricultural cycle. The concurrent measurement of keratin $\delta^{15}N$ values is crucial for verifying that the changes in $\delta^{13}C$ values are driven by direct consumption of plant products.

Seasonality in diet can only be assessed in fast-growing, non-remodelling tissues. A cyclical consumption of seasonal C_3 and C_4 foods would be mistaken for intake of a combination of C_3 and C_4 resources if the diet was reconstructed by analysing bone collagen. Seasonal variations in diet were successfully detected along the hair of individuals relying on crops grown following the natural cycle of seasons without practicing extensive crop storage or food exchange (e.g. Basha et al., 2018, Mora et al., 2017, Schwarcz and White, 2004, Wang et al., 2022). Incidentally, the characterization of the carbon isotope signal (C_3 or C_4) pertaining to the hair segment closest to the scalp was a means of assessing the 'season of death' of the individual (e.g. White, 1993, Williams and Katzenberg, 2012), providing the hair follicles were in anagen-growing phase.

11.2. Temporary mobility and consumption of imported foodstuff

Occasional and short-term intraindividual variations in keratin isotope values, which are irregular in frequency and amplitude, may be evidence of consumption of non-local foodstuffs, grown in an ecologically different zone. This pattern was detected in individuals moving across an isotopically different landscape, or consuming non-local resources sourced from multiple production zones or through long-distance trade (e.g. Knudson et al., 2012, Mora et al., 2018, Webb et al., 2013, White et al., 2009).

11.3. Relocation preceding the death event

A gradual and discrete shift in isotope values along the hair fibre may be indicative of a change in diet as a result of recent relocation, with the segment closest to the scalp possibly displaying local isotopic values and the farthest segments reflecting the place of origin. The hair of the victims of pre-Columbian rituals, who were moved to the high peaks of the Andes to be sacrificed, often display this isotopic pattern (e.g. Fernández et al., 1999, Killian Galván et al., 2020, Panzer et al., 2014, Turner et al., 2013, Wilson et al., 2007b). Relocation can also be driven by socio-economic factors. Shifts in keratin isotope composition, moving from a non-local to a local dietary signal, were detected along the hair of individuals who relocated from the Pacific coast and the highlands to a mid-altitude Andean centre to tie alliances and control the long-distance trade of coastal and inland resources (Mora et al. 2018).

11.4. Beyond palaeodiet: Reconstructing health and disease

Physiological and pathological stressors may alter the carbon and nitrogen metabolism of the body, inducing a variation in the $\delta^{13}C$ and $\delta^{15}N$ values of hair keratins. Separating the effects of physio-pathological stressors from that of diet is not an easy task since all these factors concur in shaping the final isotope composition of body tissues.

Clinical, nutritional, and forensic studies on modern human hair have proven that keratin $\delta^{15}N$ values become more positive when the body preferentially breaks down pre-existing muscle proteins, freeing amino acids, with the aim of satisfying the increased need of protein synthesis. Evidence of catabolism (negative nitrogen balance) has been

detected in the hair of individuals suffering from infections and skeletal fractures (D'Ortenzio et al., 2015), or severe malnutrition (Mekota et al., 2006, Neuberger et al., 2013, Verostick et al. 2019). Variations in $\delta^{13}C$ values are more subtle and thought to be driven by the preferential sourcing of energy from adipose tissue, which presents more negative $\delta^{13}C$ values than those of muscles. More negative keratin $\delta^{13}C$ values were reported in the hair of nutritionally deprived individuals (Mekota et al., 2006, Neuberger et al., 2013). The concurrent depletion in ^{13}C and enrichment in ^{15}N was detected in the hair keratins of a young subadult (2–3 yrs) living in the Dakhleh Oasis (Egypt, Romano-Christian Period), which was interpreted as a potential sign of malnutrition and physical abuse in antiquity (Wheeler et al., 2013).

Extensive nutritional, metabolic and physiological changes permeate the early life of children, which result in variations in their tissues' isotope values (Reynard and Tuross, 2015). Clinical studies (De Luca et al., 2012; Fuller et al., 2006) on infant-mother pairs have shown that the keratinous tissues of breastfed infants present more positive $\delta^{15}N$ values (~1-3‰) and less negative $\delta^{13}C$ values (~0.5-1‰) than those of their respective mothers. When complementary foods are included in the infant diet, their keratin $\delta^{13}C$ and $\delta^{15}N$ values initiate a gradual, but asynchronous, shift towards maternal isotope values. Conducting studies on ancient infant feeding strategies through longitudinal analysis of hair fibres is challenging because it requires the retrieval of multiple remains of juveniles who died at different ages during infancy and early childhood. Despite the rarity of such collections, potential evidence of breastfeeding in antiquity has been detected along the hair of a pre-Columbian infant living on the coast of Peru (White et al., 2009).

12. Conclusion and future perspectives

The unique composition and properties of human scalp hair make this tissue an ideal bioresource in archaeological studies. Carbon and nitrogen isotope analysis of hair keratins unlocks the recent dietary history of ancient individuals. The elected sampling strategy and methodological approach define which temporal resolution can be achieved in the palaeodietary reconstruction.

Physio-pathological stressors, which may be frequent in the period leading to the death of the individual, can alter the carbon and nitrogen metabolism of the body, possibly affecting the hair keratin $\delta^{13}C$ and $\delta^{15}N$ values. For this reason, research should focus on understanding if, and to what extent, a specific physiological/pathological stressor affects the keratin isotope composition of scalp hair. Keratin isotope analyses should be performed along the hair of two groups of contemporary individuals, clinical and non-clinical, who would be ideally subjected to a controlled diet. The knowledge produced by these clinical isotope studies would improve the interpretation of the carbon and nitrogen isotope compositions of archaeological human hair by contributing to isolate the effects of nutritional factors from those of physiological and pathological stressors. Examples of clinical studies with direct applications to archaeological populations can be found in Canterbury et al. (2020) and Hatch et al. (2006), but more research is surely needed.

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