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Finding Britain's last hunter-gatherers: A new biomolecular approach to 'unidentifiable' bone fragments utilising bone collagen



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ABSTRACT

In the last decade, our knowledge of the transition from foraging, fishing, and hunting to agricultural food production has been transformed through the molecular analysis of human remains. In Britain, however, the lack of Late Mesolithic human remains has limited our understanding of this dietary transition. Here, we report the use of a novel strategy to analyse otherwise overlooked material to identify additional human remains from this period. ZooMS, a method which uses bone collagen sequences to determine species, was applied to unidentifiable bone fragments from 5th millennium deposits from the Late Mesolithic site of Cnoc Coig (Oronsay, Inner Hebrides) using an innovative new methodology. All samples bar one produced ZooMS results, with 14/20 bone fragments identified as human, and the remainder a mixture of pig and seal. 70% of bone fragments had sufficient collagen for stable isotope analyses, however none of three human bone fragments analysed had sufficient endogenous DNA. By conducting AMS dating and stable isotope analysis on this identified collagen, we provide new data that supports the view that the exploitation of marine resources partially overlapped with the earliest agricultural communities in Britain, and thus argues against the idea that forager lifeways in Britain were immediately replaced by agriculture c.4000 cal. BC. Unfortunately, we were unable to explore the genetic relationship between contemporaneous farmers and foragers. However, the more persistent bone protein could be used to identify species, determine date, and assess diet. This novel approach is widely applicable to other early prehistoric sites with fragmentary skeletal material.

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1. Introduction

Archaeology, according to Kristiansen (2014), is experiencing its third scientific revolution, driven by the application of new biomolecular methods. One of the most demonstrable signs of this revolution has been to transform our understanding of the transition from foraging, fishing and hunting to agricultural food production. Previously, stable isotope analysis of human bone collagen has been used to assess the degree of dietary change associated with the shift to farming, AMS dating of bone collagen has been used to determine the speed and trajectory of this shift, whilst ancient DNA analysis has provided new insights regarding the

extent of demographic change. In many parts of Europe, these methods have been applied with spectacular success (e.g. Bramanti et al., 2009; Haak et al., 2010; Lidén et al., 2004; Lightfoot et al., 2015; Richards et al., 2003a; Rasteiro and Chikhi, 2013; Shennan and Edinborough, 2007) triggering new and better informed debates regarding this key phase in human history (e.g. Richards, 2003; Rowley-Conwy, 2011, 2004; Tresset and Vigne, 2011). However, in Britain their application has been hampered by the near absence of human remains dating to the period immediately preceding the arrival of farming c.4000 cal. BC, i.e. the Late Mesolithic. Remarkably, the only directly dated sites from the whole of the 5th millennium BC with human remains are from the small Inner Hebridean island of Oronsay (Meiklejohn et al., 2011), severely restricting meaningful comparisons with more abundant Neolithic remains found across Britain.

The paucity of human remains from the Late Mesolithic of Britain is puzzling. Although the British Isles may have been less

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densely populated during this period compared to the Neolithic (Collard et al., 2010), many bone bearing sites have been identified. One possibility is that human remains became disarticulated and highly fragmented through cultural practices (Gray Jones, 2011) that rendered them unidentifiable using conventional osteological methods. Here, we revisit the site of Cnoc Coig, Oronsay, one of the few Late Mesolithic sites with human remains known in Britain. Despite the identification of only six individuals at the site (Meiklejohn et al., 2011, 2005), it has previously been pivotal to the argument for a rapid dietary change with the arrival of agriculture in Britain (Richards et al., 2003b; Schulting and Richards, 2002). We apply an innovative method (ZooMS), which uses bone collagen sequences to determine species, to investigate whether additional human remains can be identified amongst 5th millennium deposits of small, fragmentary 'loose' bone. The study also aimed to utilise this collagen to conduct AMS dating and stable isotope analysis on any identified bone samples, to enhance our understanding of the diet of Britain's last forager groups and their chronological relationship to the earliest evidence for agriculture, thereby contributing to larger debates regarding the transition in Britain.

1.1. Cnoc Coig

The site of Cnoc Coig is one of five Mesolithic shell middens on the island of Oronsay, Inner Hebrides. Cnoc Coig was first excavated in 1911–1912 (Wickham-Jones et al., 1982), and then more extensively in 1973–1979 (Mellars, 1987). During the latter excavations, 49 pieces of human bone were recovered, predominantly from the hands and feet, thought to represent at up to six individuals (Meiklejohn et al., 2011, 2005; Meiklejohn and Denston, 1987). Spatial analysis has suggested these human remains fall largely into seven circumscribed bone groups, although none are indicative of primary inhumation (Meiklejohn et al., 2005). Critically, AMS dating of the samples revealed that they date to the late 5th millennium BC, immediately prior to the emergence of agriculture in Britain; although slightly earlier dates (4300 cal. BC) have been proposed for both Neolithic monuments and pottery on the West Coast of Scotland (Sheridan, 2010). Although small and fragmented, the Oronsay remains represent one of the only Late Mesolithic human skeletal assemblages in Britain and, as such, have been subjected to a range of analyses aimed at establishing their date, circumstances of deposition, and diet (e.g. Meiklejohn et al., 2011, 2005; Mellars et al., 1980; Richards and Mellars, 1998; Richards and Sheridan, 2000; Wicks et al., 2014).

In particular, stable isotope analysis of the human bones from Cnoc Coig has shown a strongly marine isotopic signature, in contrast to the terrestrial signatures observed for humans from early 4th millennium sites along the west coast of Scotland and elsewhere in Britain (e.g. Schulting and Richards, 2002; Hedges et al., 2008; Milner and Craig, 2009). In the absence of other Late Mesolithic human remains, the Oronsay material has been pivotal to the argument for a rapid dietary change with the arrival of agriculture in Britain (Richards et al., 2003b; Schulting and Richards, 2002), despite being based on a very small number of individuals. However, from recent recalibration of the dates, it has been suggested the human remains may instead date to the early 4th millennium BC (Milner and Craig, 2009) and are therefore coeval with the earliest evidence for domestic crops and animals in Scotland and other parts of Britain (Brown, 2007; Rowley-Conwy, 2004). Therefore, further identification of human remains for dating and dietary analysis from Cnoc Coig has the potential to greatly clarify our understanding of the transition in Western Scotland and more generally across Britain.

2. Materials and methods

2.1. Samples

New biomolecular techniques have opened up the possibility of the identification of bone fragments to genera using collagen peptide mass fingerprinting (ZooMS; Welker et al., 2015). Twenty fragments of disarticulated and heavily fragmented bone from the 1973–9 excavations, originally classified as 'unidentifiable' or '? human', and which had therefore remained unstudied, were utilised within this research (Fig. 1). Although the trench number of the remains is known, little other contextual information is available. The majority of fragments ($n = 15$) derive not from the main midden structure, but instead lie just outside in a single outlying trench (Fig. 2). The remaining five 'unidentifiable' bones were selected from other areas within the main midden structure. This research was undertaken with permission from National Museums Scotland, to whom the Oronsay assemblage has been allocated.

2.2. A combined biomolecular approach

A multi-methodological approach was adopted in the study of these bone fragments, combining ZooMS, stable isotope analysis and AMS dating (Fig. S1). Collagen was extracted and isotopically analysed using published protocols (Richards and Hedges, 1999; Colonese et al., 2015). ZooMS, a qualitative analytical technique for taxonomic identification of archaeological materials (Buckley et al., 2009, 2010), was undertaken on a sub-sample of the extracted collagen (<1 mg), using a novel methodology, as outlined below. Four samples with adequate collagen preservation were submitted for AMS dating at the NERC radiocarbon facility (Oxford) and calibrated using the procedure detailed below. Three samples identified as human using ZooMS were also submitted for aDNA analysis. Protocols for each of the methodologies employed in this study are provided in the [Supplementary Information](#).

3. Results and discussion

Initially, collagen was prepared and extracted from all 20 bone fragments using previously published protocols (Richards and Hedges, 1999; Colonese et al., 2015), but yields varied, with only fourteen having sufficient collagen for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic analysis (Fig. 3). The range of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values obtained however indicated samples with both fully marine and fully terrestrial diets. van Doorn et al. (2011) have previously shown that it is possible to undertake ZooMS on samples soaked in ammonium bicarbonate buffer (AmBic), utilising macroscopic amounts of bone collagen. Due to this, we speculated that the emptied 15ml Falcon tubes previously utilised for lyophilisation following collagen extraction would have absorbed sufficient collagen to their surface to allow for ZooMS identification to be undertaken. Lyophilised collagen samples were therefore removed from Falcon tubes, and 75 μl 50mM AmBic was added to each 'empty' tube used during ultrafiltration and digested with 1 μl trypsin. Identification was based upon peptide matching as outlined in Welker et al. (2015). Nineteen of the twenty samples yielded identification information, including six that had insufficient collagen to undertake stable isotope analyses. Two fragments identified as *Pinnipedia* (seal) using ZooMS had $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic values indicative of a typical marine based diet expected of these animals. Indeed, all the seal bones analysed from Oronsay (Richards and Mellars, 1998; this study) are within analytical error (Pestle et al., 2014) and could therefore even be from the same skeleton.

Of the three bone fragments identified as *Sus scrofa* using ZooMS, two samples (at least one individual) showed $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$



Fig. 1. Selection of bone fragments from the Cnoc Coig assemblage used within this research; highlighting the range of sizes, elements and preservation. From top, L-R, ZooMS IDs: seal, pig, remainder human.

values indicative of a terrestrial herbivorous diet, whilst the third had isotope values consistent with a more marine/omnivorous diet, possibly deriving from the consumption of refuse from the shell midden, or being purposively fed marine foods by humans. Biometrically identified as wild boar (Grigson and Mellars, 1987), these animals are likely to have been purposively brought to Oronsay from the mainland or larger surrounding islands. As they had differential diets prior to death, we may hypothesise that they inhabited different areas, derived from two (geographically) distinct populations, or were managed differently prior to their death, mirroring interpretations of deer at the site (Grigson and Mellars, 1987).

Remarkably, fourteen of the fragments were identified as human, increasing the number of known human bone fragments from all five Oronsay middens from 55 (Meiklejohn et al., 2005) to 74 (including five fragments recently recovered at NMS) (Sheridan, *pers. comm.*). Of the fourteen bone fragments identified as human here, nine yielded sufficient amounts of collagen for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic analysis (Fig. 3). Generally, the isotope values of the human samples are similar to those from previous study, confirming

marine protein rich diets (Fig. 3; Table S1). However, variation in the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values indicates that the human bone samples are unlikely to be from the same individuals as previously analysed. At least two of the new human samples are outside the error expected by replicate analysis of a single individual (Pestle et al., 2014). Conservatively, if we use these errors, combining this new human isotopic data with previous analysis (Richards and Mellars, 1998) suggests a potential minimum of seven human individuals are represented isotopically, from thirteen pieces of bone (Fig. 3).

Given the scarcity of British Late Mesolithic human remains and the unique nature of these samples, aDNA analysis was attempted at two independent laboratories on three fragments of bone identified as human here using ZooMS. Unfortunately, this was unsuccessful due to low endogenous DNA content and inhibition of the samples (Barnes & Brace, *pers. comm.*; Reich & Harney, *pers. comm.*; see Supplementary Information). However, this does highlight that in sites which do not yield DNA, the bone protein collagen can still provide useful biomolecular information, through identifying species, determining date, and assessing diet.

Finally, importantly, all the human remains identified here

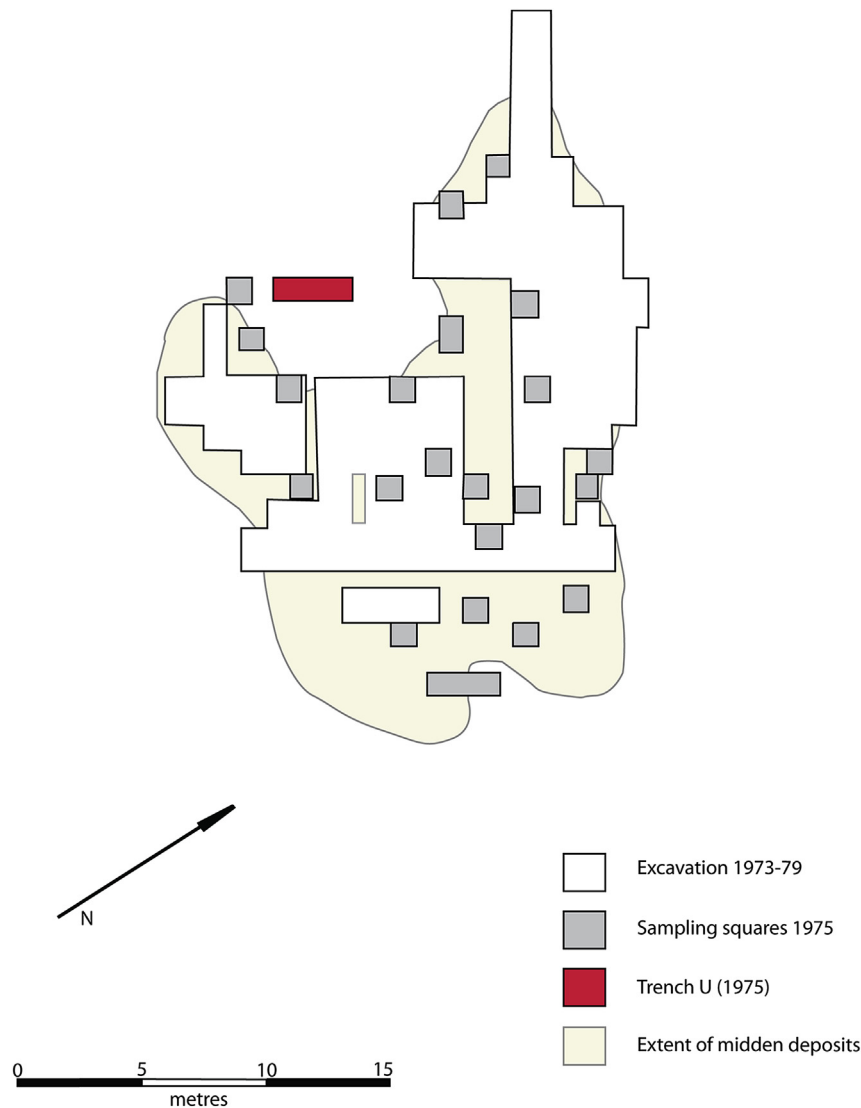


Fig. 2. Plan of excavated areas of Cnoc Coig (adapted from Mellars, 1987, 215), indicating the extent of the midden as defined by Mellars. Trench U, where the human remains identified in this study were found, is highlighted in red.

originate from outside the main midden structure (Fig. 2) and may represent a different depositional event. This raises interesting questions as to whether deposition in this location was intentional, or is a product of taphonomic processes – and can perhaps contribute to discussions surrounding the deposition of human remains in the late 5th-early 4th millennium BC in Britain. Given the ubiquitous nature of disarticulated human remains with the Mesolithic burial record, potential degrees of intentionality with regards to these kinds of deposits have previously been discussed. Gray Jones (2011), for example, has suggested that ‘loose bone’ or disarticulated remains may in fact be the result of deliberate acts, and thus a part of, rather than separate from, other types of mortuary practice. Additionally, only one of the bone fragments identified here as human appears to originate from the hands or feet, which have previously been noted to be the dominant element types within the midden deposits, and have led to suggestions of excarnation at the site (Meiklejohn et al., 2005).

3.1. Re-dating human remains at Cnoc Coig

Previous AMS dates on human remains dated Cnoc Coig to

4300–3800 cal. BC (Milner and Craig, 2009; Richards and Sheridan, 2000). However, the marine carbon isotope signatures of the human remains mean they are subject to uncertainties associated with the marine reservoir effect (MRE). Additional ¹⁴C dates previously obtained from bulk charcoal (Switsur and Mellars, 1987) could have derived from ‘old wood’ (Schiffer, 1986), adding to the uncertainty regarding the dating of the site. The first dates on short-lived terrestrial mammals, as identified by ZooMS, were therefore undertaken here, along with dates on the newly identified human remains (Table 1).

Calibration of all dates was undertaken using OxCal v.4.2 (Bronk Ramsey, 2009). As the human samples had marine isotopic signatures however, they were calibrated using a mixed marine-terrestrial curve (Marine13/IntCal13; Reimer et al., 2013) in a proportion determined by marine/terrestrial carbon contribution to collagen (as in Barrett and Richards, 2004; following best practice outlined in Cook et al., 2015). The latter was estimated for each individual from their $\delta^{13}\text{C}$ values following linear interpolation from the observed marine and terrestrial endpoints after Schulting and Richards (2002) (–12‰ and –21‰ respectively; Table S1). We placed a 10% error on this value following Hedges (2004).

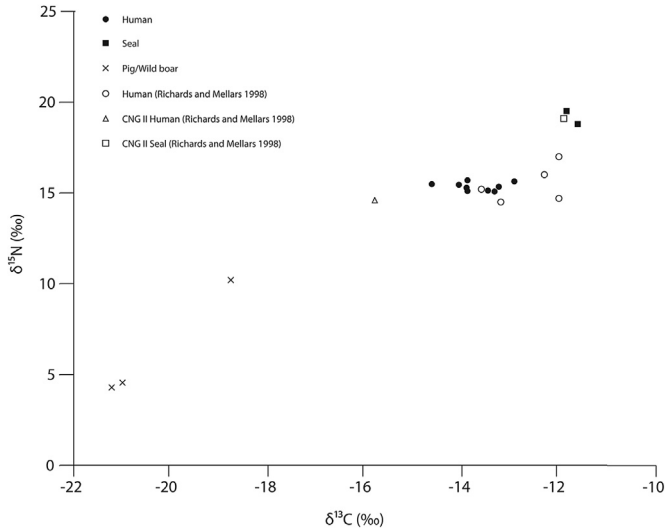


Fig. 3. Stable carbon and nitrogen isotope values from Cnoc Coig and Caisteal nan Gillean human and fauna. The analytical error on data obtained in this study was $<0.2\text{‰}$ (1σ) for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Data from this study and Richards and Mellars (1998).

Calibration of AMS dates from Cnoc Coig using this approach has previously been successfully undertaken by Gordon Cook (Milner and Craig, 2009). MRE and ΔR values are known to vary both temporally and geographically, caused by palaeoclimatic, environmental and oceanographic changes (Ascough et al., 2007, 2004). As the MRE has not been assessed at Oronsay itself, a calculated mean ΔR value for Scotland was utilised ($\Delta R = 47 \pm 52$ 14 C yr) (Russell et al., 2015) following best practices (Cook et al., 2015). The two pig samples dated were calibrated using only the terrestrial (IntCal13) calibration curve.

Intriguingly, the dates on two pig bone samples with purely terrestrial diets fall within the 4th millennium BC at 95% confidence (Table 1). After calibration of both new dates presented here and those previously obtained from Oronsay using the approach outlined above, it is clear that all the humans overlap with the terrestrial fauna and fall within the early part of the 4th millennium BC (Table 1 and Table S1; Fig. 4). This is a significant result as the Oronsay human dates, with marine isotope signatures, overlap with humans from other parts of Western Scotland with fully terrestrial isotope signatures (Fig. 4) and with the earliest evidence for domesticated animals and plants in Britain. We suggest that there was considerable heterogeneity in human diets in the early part of the Neolithic reflecting specialisation in subsistence

Table 1

AMS dates obtained within this study from newly identified humans and fauna. A ΔR value of 47 ± 52 was used to calibrate samples 8257 and 8267.

Sample number	Trench	ZooMS ID	Lab Ref. No.	¹⁴ C date BP	Date cal. BC (95.4%)
8257	U III	Human	OxA-29939	5391 ± 30	3991–3702
8267	U III	Human	OxA-29938	5379 ± 29	3944–3649
10494	P (E)	Pig	OxA-29937	5122 ± 30	3982–3803
17050	H/13	Pig	OxA-29936	5117 ± 29	3977–3803

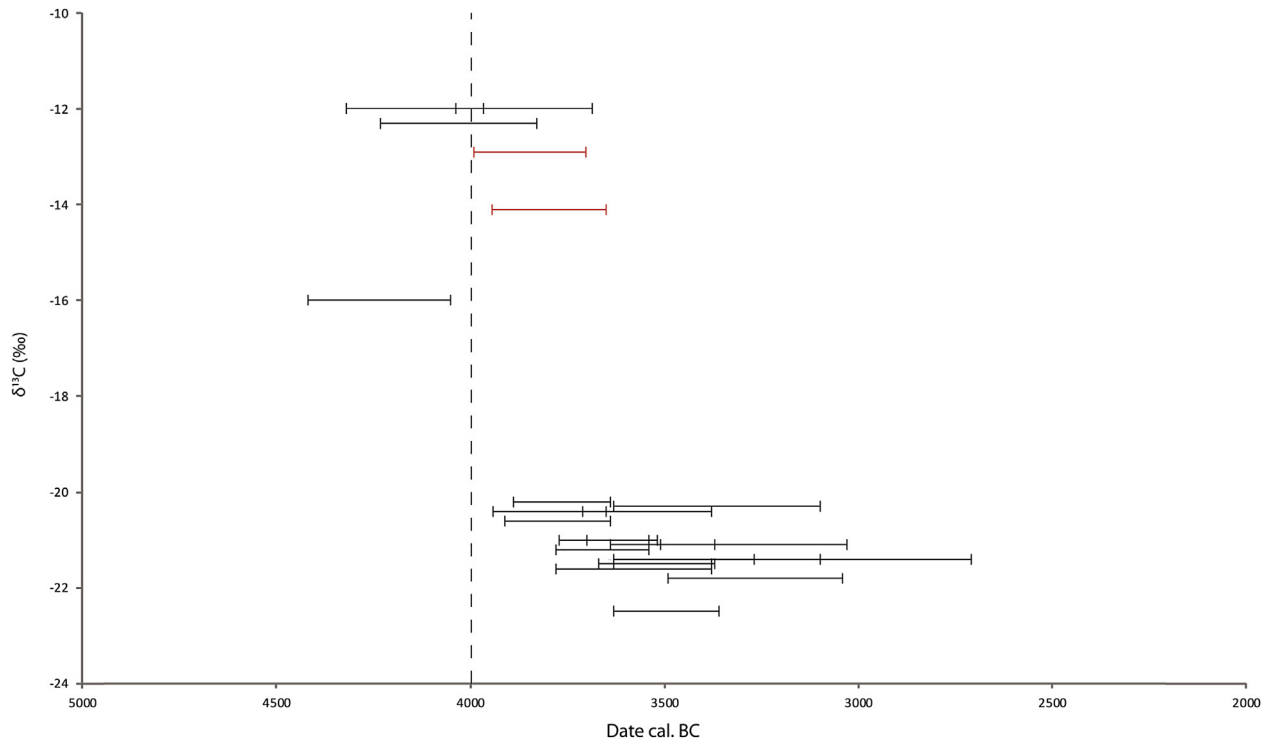


Fig. 4. Plot of $\delta^{13}\text{C}$ values against radiocarbon dates for humans from Scottish West Coast sites, c.4500–3000 cal. BC (data from this study (Table 1 and Table S1); Richards and Sheridan, 2000; Schulting and Richards, 2002). New human data obtained for Cnoc Coig within this study is highlighted in red.

practices across the landscape, and the continuity of foraging, hunting and fishing into the period traditionally associated with agriculture and pastoralism. Sheridan (2010) argues for the arrival of a 'Breton Neolithic' in this region from around 4300–4200 cal. BC, and Collard et al. (2010) suggest that farming emerged in western Scotland c.6100 cal. BP. These dates, combined with the data obtained here, would imply that both hunter-gatherer-fisher and farming lifestyles potentially co-existed on the West Coast of Scotland for several hundred years. However, it should be noted that we have very little isotopic evidence for human subsistence practices in 5th millennium Britain.

4. Conclusions

This study adopted an innovative biomolecular approach to bone fragments from the site of Cnoc Coig, and highlights the archaeological information which can be obtained from bone protein alone. By combining a variety of scientific techniques (all of which target bone collagen) and applying them in tandem to the same samples, this research has aimed to illustrate the information which can be obtained from previously overlooked fragmented material. As such, this study importantly highlights the research potential currently dormant within osteologically unidentifiable bone fragments from prehistoric contexts. There is consequently significant potential for future application of the method to other prehistoric sites with fragmentary or loose bone, such as caves and middens. We therefore call for widespread application of ZooMS to similar Mesolithic assemblages across Britain.

As demonstrated here for the first time, the ability to be able to obtain taxonomic information from 'empty' tubes previously utilised within the collagen extraction process also appears to hold great future potential - particularly as it does not require the use of collagen reserved for isotopic analysis or AMS dating. It also presents a potential opportunity to retrospectively analyse empty tubes previously utilised within collagen extractions to gain taxonomic information. This may be of particular use with samples which produce AMS dates or isotopic values that are distinctly different from what is anticipated.

Overall, this research has detected extremely rare human bones from the Mesolithic-Neolithic transition which can be used to further elucidate issues surrounding the period. In total, fourteen new fragments of human bone have been identified, increasing the number of known human bone fragments from the five Oronsay middens from 55 (Meiklejohn et al., 2005) to 74 (including five fragments recently recovered at NMS) (Sheridan, pers. comm.). The human remains identified here provide additional data comparable to isotopic analyses undertaken previously at Cnoc Coig. The isotopic results also provide additional evidence of a high marine protein diet along the west coast of Scotland – but AMS dates obtained from these samples suggest that this marine diet may have extended into the 4th millennium BC and the 'Neolithic' period. However, the presence of a marine isotopic signature within one *Sus scrofa* sample suggests the need for better characterisation of faunal baselines within the British Mesolithic, in particular when considering interpretations of marine resource consumption by humans.

Finally, unfortunately, insufficient endogenous DNA content within the samples analysed here meant that it was not possible to explore the genetic relationship between the Cnoc Coig humans and the earliest known agricultural communities in Britain. In future, analysis of the single human petrous bone known from Cnoc Coig may be worth exploration, as this element is known to provide significantly higher endogenous DNA yields (Pinhasi et al., 2015).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jas.2016.07.014>.

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