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Proteomic profiling of archaeological human bone

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Ancient protein analysis provides clues to human life and diseases from ancient times. Here, we performed shotgun proteomics of human archeological bones for the first time, using rib bones from the Hitotsubashi site (AD 1657–1683) in Tokyo, called Edo in ancient times. The output data obtained were analysed using Gene Ontology and label-free quantification. We detected leucocyte-derived proteins, possibly originating from the bone marrow of the rib. Particularly prevalent and relatively high expression of eosinophil peroxidase suggests the influence of infectious diseases. This scenario is plausible, considering the overcrowding and unhygienic living conditions of the Edo city described in the historical literature. We also observed age-dependent differences in proteome profiles, particularly for proteins involved in developmental processes. Among them, alpha-2-HS-glycoprotein demonstrated a strong negative correlation with age. These results suggest that analysis of ancient proteins could provide a useful indicator of stress, disease, starvation, obesity and other kinds of physiological and pathological information.

1. Introduction

Ancient proteins have been studied with the aim of revealing the life and diseases of past humans, species identification and evolution [1–3]. Immunological methods were applied to archaeological human bones as early as the 1980s [4]. Haemoglobin, serum albumin (ALB) and immunoglobulin G (IgG) were studied using immunological methods for species identification and for detecting immune responses [5–7].

In recent years, proteomics analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) has attracted more attention owing to its accuracy and robustness and to the remarkable improvement in instruments and protein databases; this method has also been applied to ancient remains [8–11]. The first LC-MS applied to archaeological remains was conducted by Schweitzer *et al.* [12]. Then shotgun proteomics analysis was performed on a 43 000-year-old woolly mammoth bone [8]. Since then, the method has been applied to other species [9–11,13–16]. With regard to archaeological human bones, ancient protein analyses have been performed to detect disease and immune response [17,18]; however, to date, shotgun proteomics analysis has not been used to profile the bone proteome of archaeological human remains. In this study, we present the results of shotgun proteomic profiling of archaeological human bones. We performed shotgun proteomics of human remains using eight rib bones excavated from the Hitotsubashi site of the Edo period, expecting to acquire physiological information.

We used rib samples from eight skeletons excavated from the Hitotsubashi site (AD 1657–1683) in Tokyo, Japan. The Hitotsubashi site is located in the city of Edo, the former name for Tokyo, and is known for its rapid urbanization and stressful living environment [19–21]. Our findings suggest that the bone proteome represents a source of information about immune system activity and age-related changes. Our work demonstrates that the proteomes of archaeological bone represent a source of biomolecular information for the reconstruction of physiological and pathological processes that occurred in antiquity but cannot be detected using other bioarchaeological methods.

2. Material and methods

2.1. Human bone samples

Two infants and six adults, including four elderly adults (more than 60 years old), were obtained from the skeletal collection of the Hitotsubashi site (AD 1657–1683) in Tokyo, Japan. The chronological age was determined on the grounds of the stratigraphic layer, which is between the Great fire of Meireki (AD 1657) and the fire of Tenna (AD 1684). The individuals buried in this graveyard are townspeople, known from the fact that the largest proportion of graves consisted of the wooden coffins typically used for the lower social classes. Nagaoka & Hirata [21] had reported the sex and age at death of the adult skeletons used in this study. Sex was determined by the sexual dimorphism of the pelvis using the dimorphic criteria of the pelvis including the greater sciatic notch [22–24], preauricular sulcus [23–25], ventral arc [26], subpubic concavity [26] and medial aspect of the ischiopubic ramus [26]. Age at death for each individual was estimated by the observation of the auricular surface of the ilium using the method of Buckberry & Chamberlain [27]. The auricular surface of the ilium is resistent to decay in forensic and archaeological contexts, and it seems to cover a wide range of ages up to elderly individuals [28]. The age estimation at death of adults aged more than 15 years was based on the chronological metamorphosis of the auricular surface of the ilium. Buckberry & Chamberlain [27] developed a quantitative system of 5–19 composite scores according to five morphological traits of the auricular surface: transverse organization, surface texture, microporosity, macroporosity and apical change. The 5–19 composite scores of the five traits are then classified into seven stages. Each obtained stage of the auricular surface of the ilium is then transformed into an average age at death with standard deviation shown in the data of Buckberry & Chamberlain [27]. Although the age at death estimation is more difficult in adults than in sub-adults, Murlhern & Jones [29] tested the reliability of the revised method using 309 individuals from the Terry Collection and detected that the Buckberry and Chamberlain's method [27] improved the accuracy of age estimation for elderly adults. The age at death of sub-adults was estimated by the degree of formation and eruption of teeth [30,31].

2.2. Protein sample preparation

In order to prevent contamination, surfaces were removed from bone samples with sandpaper. Samples (30–50 mg) were wrapped in clean aluminium foil and fragmented into powder using a conventional

hammer. The hammer was cleaned with bleach and ethanol, repeatedly. Powdered bone samples were washed three times with 300 µl 0.5 M EDTA (pH 8.0), followed by incubation at 4 °C for 24–48 h with agitation. Samples were washed again three times with 100 µl 0.1 M Tris (pH 8.0). Samples were suspended in 300 µl 6 M guanidinium hydrochloride (GuHCl), 10 mM Tris (2-carboxyethyl) phosphine, 20 mM chloroacetamide and 200 mM Tris (pH 8.0). Soft organic pellets eventually released after demineralization were disrupted using single-use individually sealed disposable micropestles (Eppendorf), cleaned before use with a 5% bleach solution and 95% ethanol (v/v). Without separating supernatants and the residual bone powder, samples were heated at 80 °C for 2 h, and cooled to room temperature. LysC-Trypsin mix, 1/100 by amount of protein, was added to the samples for protein digestion. Mixed samples were incubated at 25 °C for 30 min then diluted to 2 M GuHCl with 25 mM Tris (pH 8.0), followed by incubation at 37 °C overnight with agitation. Digestion was terminated with 10% trifluoroacetic acid to a final concentration of 1%. After centrifugation at $14\,000\,g$ for 10 min, the tryptic peptides in the supernatant were immobilized on C18 stage tips as previously described [8]. Peptide mixtures were analysed by online nanoflow reversed-phase C18 LC-MS/MS, as described previously [32]. Briefly, peptides were separated on a 50 cm PicoFrit column (75 mm inner diameter) in-house packed with 1.9 mm C18 beads (Reprosil-AQ Pur, Dr. Maisch) on an EASY-nLC 1000 system connected to a Q-Exactive HF (Thermo Scientific) on a 165 min gradient. The Q-Exactive HF was operated in data-dependent top 10 mode. Full scan mass spectra were recorded at a resolution of 120 000 at m/z 200 over the m/z range 300–1750 with a target value of 3×10^6 and a maximum injection time of 20 ms. HCD-generated product ions were recorded with a maximum ion injection time set to 108 ms through a target value set to 2×10^5 and recorded at a resolution of 60 000 with a fixed first mass set to m/z 100.

2.3. Data analysis

MS/MS spectra in '.raw' file format were processed with MAXQUANT v. 1.5.3.28 [33] and ANDROMEDA [34] against the human reference proteome (UniProtKB, downloaded on 13 December 2015). In every search, spectra matched against the common contaminants database were removed except ALB, a common protein in bones [35].

Although bovine serum albumin (BSA) can be a common contaminant in proteomics laboratories, it has been previously demonstrated that endogenous ALB from extinct species can be confidently retrieved from ancient bones [8,16,35]. We validated the authenticity of the human serum albumin retrieved in this study by checking for the absence of BSA peptides in all the samples. All but one peptide of ALB had the same sequence as human, not bovine (the one peptide sequence was the same as both human and bovine). This indicates that the contamination of BSA had little impact, if any. We also checked the evidence of deamidation in ALB peptide, so we included ALB in data analysis.

Cysteine carbamidomethylation was used as a fixed modification. Oxidation (M and P), Gln → pyro-Glu (N-term Q), Glu → pyro-Glu (N-term E) and deamidation (N and Q) were used as variable modifications. The peptide tolerance, in the main search, was set to 5 ppm, while the tolerance for MS/MS match was set to 20 ppm. Up to seven modifications per peptide and up to two missed cleavages were allowed. The false-discovery rate was set at 1%. Other parameters were used as those pre-set in the software. The first protein of major protein IDs was used as a protein name. The deamidation rate of collagen (COL1A1 and COL1A2) was calculated as the total number of deamidated glutamine residues divided by the total number of glutamine residues. The mass difference between the deamidated monoisotopic peak and the 13C peak of the amidated form of the same peptide is 19.34 mDa. Previous studies demonstrated that the use of an orbitrap mass spectrometer with high mass measurement accuracy and high resolving power, like the one used in this study (Q-Exactive HF), and a peptide tolerance value like the one we imposed, 5 ppm, enable correct assignment of deamidated peptides [36].

In order to compare the amount of proteins among samples, detected proteins were quantified using the exponentially modified protein abundance index (emPAI) score [37]. The emPAI score is calculated using the number of detected peptides normalized by the number of theoretically observable ones. The emPAI score of basic salivary proline-rich protein 3 (PRB3) was quite high because the number of theoretically observable peptides is two, which is too small for calculating emPAI. We therefore excluded this protein. The emPAI scores of the remaining proteins were normalized, respectively, by the score of collagen (COL1A1). In order to detect proteins that correlated with age, Pearson's correlation analysis between age and normalized emPAI score was performed on the proteins that included more than 10 unique peptides in total samples. As multiple comparisons were performed, Bonferroni's correction was used for correlation analysis. We used the corr.test and p.adjust functions in R for statistical analysis.

Table 1. Summary of the proteins extracted from samples. Proteins: number of identified unique proteins from each sample. Peptides: the number of identified unique peptides from each sample. Deamidation (%): the deamidation rate of glutamine calculated using COL1A1 and COL1A2.

sample ID	sex	age (years)	proteins	peptides	deamidation (%)
H-9	—	0.75	173	794	10.1
H-142	—	4	162	497	13.3
H-91	male	29 ± 6.7	158	341	4.1
H-206	male	38 ± 13.1	188	782	9.2
H-24	female	60 ± 13.0	127	387	10.0
H-88	male	60 ± 13.0	175	483	10.0
H-160	male	60 ± 13.0	160	520	10.9
H-162	female	72 ± 12.7	145	554	10.9

To reveal protein function, we used the Gene Ontology (GO) term. The PANTHER statistical over-representation test [38] was conducted to identify functional GO annotations that were enriched in each individual. We used the 'GO experimental only' database for reliable annotation. Focusing on the fold change of proteins associated with developmental function, we calculated the correlation test between age and fold change of proteins that have the 'developmental process' term (GO: 0032502). We also used the STRING database [39] to create a network of functional relationships between the identified top 30 proteins.

3. Results

3.1. Proteins detected from archaeological human bones

We applied shotgun proteomics using LC-MS/MS to the skeletal samples excavated from the Hitotsubashi site in Tokyo (AD 1657–1683, Edo period, Japan). The number of detected proteins varied among individuals from 127 to 188 (table 1). There were no significant correlations between the estimated age at death and the number of detected proteins/unique peptides (Pearson's $r = -0.4082$, $p = 0.3154$ for proteins; $r = -0.3480$, $p = 0.3983$ for unique peptides). Venn diagrams indicated that infants and adults shared 33 proteins, while adults and elderly adults shared 46 proteins (figure 1a). Infants and elderly adults shared 21 proteins. The common proteins between females and males were less than those among males (figure 1b). Elderly females shared 25 proteins with adult males and 23 proteins with elderly males. Adult males and elderly males shared 48 proteins. The composition of proteins was shown in figure 2. This indicates that approximately half of total proteins were collagens and extracellular matrix. Plasma proteins accounted for 10–25% of total proteins. Proteins expressed in granules, the intracellular region and membrane were also detected from each bone sample.

With regard to the presence of unique peptides, COL1A1 had the largest number of unique peptides in all individuals, followed by COL1A2. Other collagens, such as COL2A1, COL5A1, COL5A2, COL12A1 and COL22A1, were also detected in almost all samples. Plasma proteins, such as ALB, prothrombin (F2) and coagulation factor IX (F9) were also detected. The top 30 proteins, with the exception of the olfactomedin-like protein (OLFML3), were functionally connected to at least one other protein in the STRING network (electronic supplementary material, figure S1). Osteocalcin (BGLAP), one of the major components of bone, was detected in all samples. Two proteins related to osteoclast regulation were detected in some of the samples: macrophage colony-stimulating factor 1 (CSF1), which plays an essential role in the proliferation and differentiation of osteoclasts [40], and tumour necrosis factor receptor superfamily member 11A (TNFRSF11A), which is essential for RANKL-mediated osteoclastogenesis [41].

3.2. Leucocyte-derived proteins in archaeological human bones

We identified proteins derived from leucocytes, such as neutrophils and eosinophils, in archaeological human bones. Warinner *et al.* [42] found leucocyte-derived proteins in human dental calculus; however, to date, no research detecting these proteins in archaeological human bones, including immunological
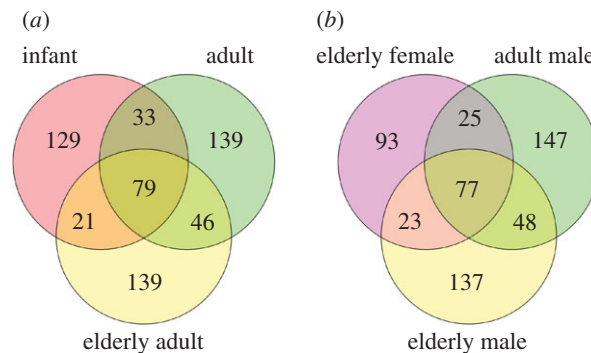
Figure 1. Venn diagrams of proteins between (*a*) life stages and (*b*) sex. (*a*) Infant: H-9 and H-142; adult: H-91 and H-206; elderly adult: H-88 and H-160. (*b*) Adult male: H-91 and H-206; elderly female: H-24 and H-162; elderly male: H-88 and H-160.
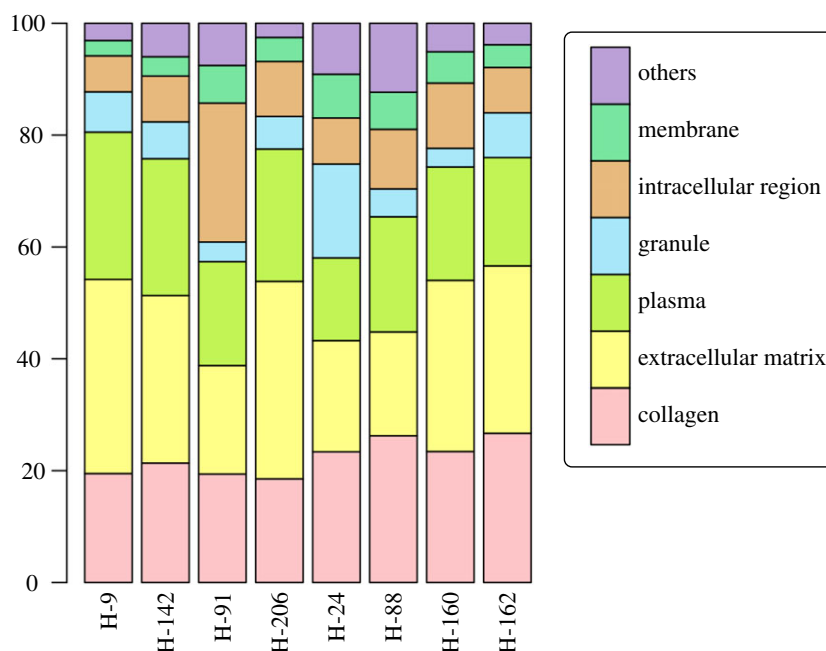

Figure 2. The composition of detected proteins. *X*-axis corresponds to sample ID of each individual.

analysis, has been performed. To our knowledge, this is the first study detecting leucocyte-derived proteins in archaeological human bones.

Neutrophils are the most abundant white blood cells and are the first-responders among inflammatory cells to migrate towards the site of inflammation during its beginning phase, particularly as a result of bacterial infection [43]. A variety of neutrophil-derived proteins were detected from the Hitotsubashi samples, including neutrophil defensin 1/3 (DEFA1/3), neutrophil elastase (ELANE), myeloperoxidase (MPO), azurocidin (AZU1) and cathepsin G (CTSG). DEFA1/3, ELANE and MPO are characteristic proteins in azurophilic granules, which are a type of granule found in neutrophils [43,44]. The unique peptides in these proteins were significantly correlated to each other (electronic supplementary material, table S1).

Eosinophils have a key role in allergic and inflammatory processes, including asthma and host resistance to parasites, such as helminths, but they also exhibit antimicrobial activities towards bacterial, viral and protozoan pathogens, and mediate hypersensitivity diseases [45]. In this study, eosinophil peroxidase (EPX) was detected in all samples; EPX is uniquely expressed in eosinophils and not in other cells [45]. Another protein expressed in eosinophil granules, proteoglycan 2 (PRG2), was also detected, and the number of unique peptides was significantly correlated with that of EPX (Pearson's $r = 0.7782$, $p < 0.05$). These results indicate the presence of eosinophils in archaeological human bones. Eosinophil-derived neurotoxin (EDN/RNASE2) and eosinophil cationic protein (ECP/RNASE3) were not detected,
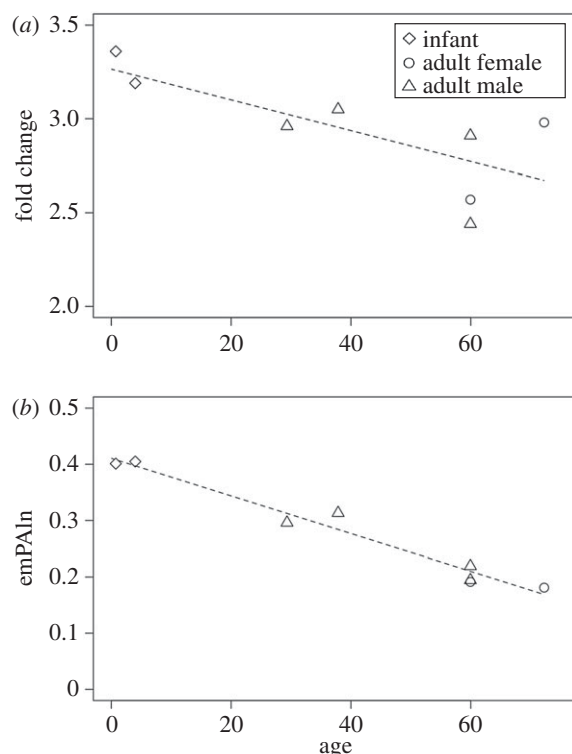
Figure 3. Age-related changes of proteins. (*a*) Relationship between age and fold change of the number of proteins annotated with the GO term 'developmental process' (GO: 0032502) (Pearson's $r = -0.7386, p = 0.03638$). (*b*) Relationship between age and the normalized emPAI value of AHSG (Pearson's $r = -0.9826, p = 1.295 \times 10^{-5}$).

likely because of the small amount of the proteins or the difficulty of detecting proteins with relatively short sequences [46].

3.3. Gene Ontology enrichment analysis

We performed GO enrichment analysis in order to estimate what kinds of processes were over-represented. Bone formation processes, such as 'collagen fibril organization' and 'ossification', were enriched (electronic supplementary material, figure S2). There were also processes associated with non-collagenous proteins (e.g. 'extracellular structure organization', 'extracellular matrix organization' and 'cell adhesion'). The processes most enriched in infants were associated with developmental processes, including 'tissue development', 'multicellular organismal development', 'single-organism developmental process', 'developmental process' and 'anatomical structure development', Age at death was significantly negatively correlated with the fold change of the 'developmental process' (Pearson's $r = -0.7386$, $p < 0.05$; figure 3*a*). The numbers of proteins involved in the 'ossification' process in infant and adult samples were twice as large as that in the elderly adult (electronic supplementary material, figure S2). In GO Cellular Component terms, the 'extracellular matrix' was enriched most remarkably in the infant (electronic supplementary material, figure S3). In GO Molecular Function terms, 'growth factor binding' was clearly enriched in the infant (electronic supplementary material, figure S4).

3.4. Protein quantitation using exponentially modified protein abundance index

In order to compare the amount of proteins in each sample, we used the emPAI score, one of the most common label-free approaches used for absolute quantification of shotgun proteomics [37]. EmPAI scores of all proteins were normalized by that of COL1A1. The sum of the normalized emPAI scores for non-collagenous proteins showed significantly negative correlation with age (Pearson's $r = -0.7088, p < 0.05$). There were proteins with normalized emPAI scores showing a significantly negative correlation with age (table 2). In particular, the normalized emPAI score of alpha-2-HS-glycoprotein (AHSG) showed a strong, significantly negative correlation with age (Pearson's $r = -0.9826$, $p = 1.295 \times 10^{-5}$; figure 3*b*). The normalized emPAI scores of ALB, insulin-like growth factor-binding protein 5 (IGFBP5), kininogen-1 (KNG1) and pigment epithelium-derived factor (SERPINF1) also showed a significantly negative

Table 2. Correlation between normalized emPAI scores and age. *p*-Value after Bonferroni's correction is marked with an asterisk.

gene name	*p*-value	*p*-value*	Pearson's *r*
SERPINF1	0.01933536	0.8894264	−0.7912284
ALB	0.0168598	0.775551	−0.8010759
AHSG	1.29×10^{-5}	5.95×10^{-4}	−0.9826241
KNG1	0.007853061	0.3612408	−0.8477033
IGFBP5	0.001842309	0.08474621	−0.9075273

correlation with age (electronic supplementary material, figure S5). Among these proteins, AHSG was the only protein whose emPAI score was still statistically significant after multiple test correction (table 2). There were no emPAI scores that significantly and positively correlated with age. AHSG, also known as fetuin-A, is produced not only in the liver but also in osteocytes [47], and is characterized by particularly high mineral affinity. IGFBP5 is an IGF-binding protein involved in the regulation of cell growth and is the most abundant IGFBP stored in bone, where it controls osteoblast differentiation and osteoblast–osteoclast crosstalk [48]. Kininogens are inhibitors of thiol proteases with primarily two isoforms, high-molecular-weight kininogen (HMWK) and low-molecular-weight kininogen (LMWK). HMWK is essential for blood coagulation and the assembly of the kallikrein–kinin system, while LMWK is not involved in blood coagulation. SERPINF1 is a multifunctional secreted protein with anti-angiogenic, anti-tumorigenic and neurotrophic functions. A form of osteogenesis imperfecta, a heritable bone dysplasia characterized by bone fragility and deformity and growth deficiency with a mineralization defect, is caused by mutations in SERPINF1 [49].

There are proteins highly expressed only in infants. Periostin (POSTN), formerly named osteoblast-specific factor-2, was originally cloned from a mouse osteoblast cell line [50]. Its function is as a cell-adhesion molecule for preosteoblasts and it is thought to be involved in osteoblast recruitment [50, 51]. POSTN was remarkably high in the youngest infant (unique peptides = 11, normalized emPAI = 0.0685), although no peptide was found in adult and elderly adult samples. Apolipoprotein A-1 (APOA1) is the major protein component of high density lipoprotein (HDL) in plasma. The expression level of APOA1 in blood has been reported to decrease with age [52,53]. In this study, APOA1 was detected only in the youngest infant (0.75 years old), which is consistent with the previous research [52,53].

4. Discussion

4.1. The authenticity of the proteomic data

In general, the problems of ancient biomolecule analysis are contamination from the modern environment and the preservation of endogenous molecules. Discerning contaminant molecules from endogenous molecules is especially problematic, particularly for ancient DNA analysis. However, in the case of ancient protein analysis, there is no amplification process (e.g. PCR for DNA); thus, contaminant proteins have less of an effect on the endogenous proteins. In addition, the expression patterns of proteins are tissue-specific (e.g. keratin from human epidermis), making the identification of contaminant proteins easier. The proteomic analysis software MAXQUANT also provides a list of common protein contaminants [33]; thus, we removed proteins included in the list, with the exception of albumin, which is a common protein detected from archaeological bones [8,35]. To address the issue of endogenous molecule quality, proteomic data from the samples were assessed using two indices: the rate of deamidation and the proportion of type I collagen. Deamidation is an indicator of the preservational quality, and the rate of deamidation of glutamine range is 0–20% in bones of 0–2000 years ago [54]. In this study, the rate of deamidation was 10% on average (range 4–13%; table 1), which is compatible with bones dating *ca* 500 years before present. Type I collagen accounts for 90% of the organic matter of bone, and the collagen triple helix is composed of two alpha 1 chains and one alpha 2 chain. Therefore, it is expected that the proportion of type I collagen is dominant in archaeological bones. In our samples, collagen type I alpha 1 (COL1A1) had the largest number of unique peptides followed by collagen type I alpha 2 (COL1A2). The number of unique COL1A1 peptides showed significant correlation with that of COL1A2 (Pearson's $r = 0.9715$, $p = 5.636 \times 10^{-5}$). These findings indicate that the proteomics data accurately reflect the composition of the modern bone proteome. In addition, the atomic C/N ratios and gelatin yields, the proxies for the preservation of collagen used in isotope analysis, indicated that
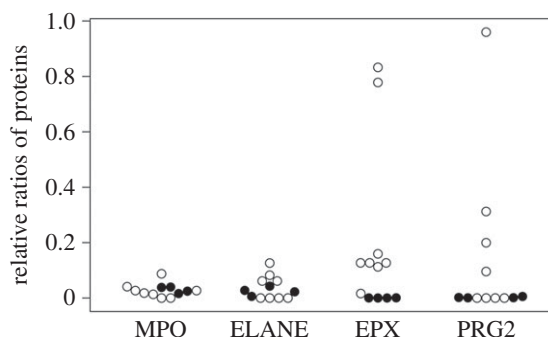
Figure 4. Relative ratios of proteins expressed in neutrophils (MPO and ELANE) and eosinophils (EPX and PRG2). Expression levels of DEFA1/3 were used as a standard. Open circles are from the Hitotsubashi samples. Closed circles are from reference data of The Human Protein Atlas [61].

the samples from the Hitotsubashi site were well preserved [55]. From these results, we confirmed the preservation of the proteins in our samples.

4.2. The presence of leucocyte-derived proteins

An interesting finding in this study was the presence of proteins derived from leucocytes, such as neutrophils and eosinophils. These proteins have not been found in previous studies of archaeological bone [3,8,9,16]. Leucocytes play a critical role in the immune system, so this finding shows the potential of ancient protein analysis for reconstructing the disease, health status and stress profiles of past humans.

These proteins were possibly derived from red bone marrow in the rib samples. Red marrow is haematopoietically active and contains erythrocytes and leucocytes, whereas yellow marrow is made almost entirely of fat, with few haematopoietic elements [56]. All bone marrow is haematopoietic red marrow during infancy. With age, red marrow is gradually converted to the fatty yellow type. By early adulthood, the yellow type makes up two-thirds of the total bone marrow, while red bone marrow remains specifically in the ribs, vertebrae and os coxae [57,58]. In this study we used rib samples, which may explain why we could detect ample leucocyte-derived proteins. For instance, these proteins are scarcely detected in the femur [59].

Although eosinophils constitute only approximately 1–4% of leucocytes of human bone marrow [60], we detected adequate numbers of unique peptides of EPX in all samples. In addition, some individuals showed higher ratios of eosinophil-derived proteins (EPX and PRG2) compared with bone marrow reference data (figure 4). We also found that the number of unique peptides was significantly correlated with that of EPX (Pearson's $r = 0.7782$, $p < 0.05$). This may be due to the increase of eosinophils in rib samples. The number of eosinophils increases in association with pathogenesis during asthma, allergic and, particularly, parasitic disease. In the city of Edo, high population pressure led to overcrowding and unhygienic living conditions. It has been reported that there were repeated epidemics among the citizens of Edo at that time [62]. Regarding the Hitotsubashi site, Hirata [20] found that severe cribra orbitalia resulting from iron deficiency anemia was prevalent in the population. In addition, Nagaoka & Hirata [21] reported a relatively short lifespan tendency for the people of Hitotsubashi. Yamamoto [19] reported that a high incidence of dental enamel hypoplasia was found in Hitotsubashi samples. These authors concluded that people residing at the Hitotsubashi site lived under a highly stressful environment [19–21]. According to the historical literature, helminth infection was prevalent during the Edo period [63–65]. These reports support our observation that eosinophil-derived proteins were highly expressed in individuals excavated from the Hitotsubashi site and this may reflect the presence of infectious disease, such as parasite infections.

4.3. Age-related changes in the proteins of human bones

The protein profile, structure and composition of bone change with age [66]. Indeed, levels of certain proteins in bone are related to age. For example, the content of non-collagenous proteins, such as AHSG and ALB, decreases with age in bone [67,68]. A recent study sought to determine protein groups whose levels change with development in zebrafish bone [69]. However, whether archaeological bones also show these age-related changes is unknown.

Our analysis indicates, for the first time, that proteomics data from archaeological bones reflect the life stages of individuals. This is supported by three levels of analysis: whole profiling by Venn diagram, the functional protein groups by GO enrichment analysis, and individual protein levels by quantification analysis.

First, the Venn diagram analysis among life stages (infant, adult and elderly adult) showed that the number of shared proteins between infant and elderly adult was the smallest among the three comparisons (figure 1a). The number of proteins shared by infant and adult was more than that shared by infant and elderly adult, and proteins were shared most between adult and elderly adult. This indicates that the component of proteomics differs most between infants and elderly adults (figure 1a).

Second, GO analysis suggests that the proteomic profile reflects the life stage of individuals. GO Biological Process terms associated with development were most abundant in infant bones (electronic supplementary material, figure S2). Specifically, the age of the individuals was significantly negatively correlated with the fold change of the 'developmental process' (figure 3a). For the 'ossification' GO term, the number of proteins involved in that process in infants and adults was twice as large as that of the elderly adult. In GO Cellular Component terms, the 'extracellular matrix' was enriched most remarkably in the infant (electronic supplementary material, figure S3). In GO Molecular Function terms, 'growth factor binding' was clearly enriched in the infant (electronic supplementary material, figure S4). These results suggest that infants have more proteins involved in development and growth.

Third, quantitative analysis using emPAI suggests that there are some proteins whose expression undergoes age-related changes. Sum emPAI scores for non-collagenous proteins in total showed significantly negative correlation with age (Pearson's $r = -0.7088$, $p < 0.05$). In regard to each protein, the emPAI score of AHSG correlated most strongly with age (figure 3b). The normalized emPAI scores for ALB, IGFBP5, KNG1 and SERPINF1 also negatively correlated with age (electronic supplementary material, figure S5).

It is noteworthy that AHSG, ALB and IGFBP5 reportedly show age-related changes in modern bone. Wilson et al. [70] reported that the levels of AHSG and ALB in fetal bone were at least one order of magnitude greater than those of adult cortical bone. Quelch et al. [67] reported that AHSG, ALB and sialic acid were present in higher concentrations in neonatal bone compared with bones from children and adults. The concentration of AHSG in neonatal bone was approximately three times higher than that in bone from children, and seven times higher than that in adult bone. The ALB concentration in neonatal bone was one-and-a-half times higher than that in child bone and twice as higher than that in adult bone. With regard to IGFBP5, Mohan et al. [71] reported that a decrease in IGFBP5 has been linked to age-related bone loss. Our results from archaeological human bones indicated that the levels of AHSG were correlated most strongly with age; ALB and IGFBP5 also showed a tendency to decrease with age. These results are consistent with the previous research in modern human bone [67,70,71].

Owing to high concentrations of AHSG in fetal serum, multiple functions related to development have been suggested [72,73]. Following collagen, AHSG is one of the most abundant proteins in bone and its ability to bind calcium and other minerals supports the notion that it is essential for osteogenesis [74,75]. Current studies suggest that circulating AHSG is highest during infancy, declines in childhood and varies in adults owing to genetic factors, obesity and diet [73]. AHSG might also be impacted by the natural ageing process [76]. It has been demonstrated that the level of AHSG in bone is also highest in infants and declines with age [67,68,70]. From these findings, we believe our results, indicating high levels of AHSG in infants that decline with age, are consistent and generalizable to other archaeological bones.

4.4. Insights of proteomics analysis in archaeological human bones and future perspective

Shotgun proteomics enabled us to analyse more than 100 proteins with accuracy and robustness. Our findings confirmed by multiple proteins that leucocytes such as neutrophils and eosinophils remained in archaeological rib bones. We also found certain proteins to be significantly correlated with age. In addition, we used samples of rib bone for which morphological information related to disease and age did not previously exist. Collectively, our study has demonstrated significant achievement in ancient proteomics, revealing biological processes not visible on the exterior.

Leucocyte-derived proteins provide new insights of paleopathological interest. IgG has been studied by enzyme-linked immunosorbent assay (ELISA) [77], Western blot [78] and LC-MS/MS [18] for detecting the immune responses of the past; however, some studies found it difficult to detect IgG in archaeological bone [6,18]. By contrast, leucocyte-derived proteins such as DEFA1/3 and EPX were prevalently detected in archaeological rib bones in this study. This suggests that these proteins may be better target molecules for studying the immune system.

Furthermore, our results suggest that rib bone is suitable for detecting proteins abundant in bone marrow, particularly for leucocyte proteins. Vertebrae and os coxae are also promising bone elements owing to the remaining red bone marrow [57]. Currently, no method exists to detect an infection in an individual from their bones, except severe stages of some diseases morphologically apparent, such as syphilis and tuberculosis. The particularly prevalent helminths are difficult to detect, because they do not seriously damage the human body. In this study, eosinophils, involved in allergic reactions or parasitic infections, were prevalently detected, which could be related to infectious disease during the Edo period. However, while highly suggestive, our results are insufficient to verify the existence of an infection, thus more rigorous quantitative methods, such as selected reaction monitoring (SRM), would be favourable as a next step.

Our results indicate that AHSG emPAI scores correlate strongly with age. Previous studies have suggested that AHSG exists prevalently in archaeological bone [35], which might suggest that we can estimate life stages of other species or chronologically older samples. There were other proteins that correlated with age or that were expressed only in infants, such as ALB, IGFBP5, POSTN and APOA1. Even though the sample size was small, the data were consistent with previous findings of modern samples. Based on the relative amount of these proteins, we can confirm and further narrow down the estimated age range for each individual, especially when dealing with remains from sub-adults. The full potential of the innovative approach we present here should, however, be fully validated with future research.

The analysis of ancient proteins could provide a useful indicator of stress, disease, starvation, obesity and other kinds of physiological and pathological information. For example, ancient proteins have revealed past diseases such as osteogenic sarcoma and prostate carcinoma [17,79]. Scott *et al.* [80] reported that osteocalcin analysis is promising for detecting stress. The analysis of multiple proteins is preferable to confirm these results.

The field of ancient DNA has entered a new era of genomics and has provided valuable genetic information about past human history [81]; nevertheless, ancient DNA is unable to answer many questions, which makes it difficult to obtain a comprehensive picture of ancient human life. The method of ancient proteomics analysis undoubtedly complements the techniques of ancient DNA analysis. This novel study sheds light on the potential of ancient proteomics for obtaining biological information such as age-related changes or immune system processes, which cannot be extracted from conventional bioarchaeological methods.

References

1. Corthals A, Koller A, Martin DW, Rieger R, Chen EI, Bernaski M, Recagno G, Dávalos LM. 2012 Detecting the immune system response of a 500 year-old Inca mummy. *PLoS ONE* **7**, e41244. (doi:10.1371/journal.pone.0041244)

2. Schmidt LØ, Brandt AL, Mannering U, Sarret M, Kelstrup CD, Olsen JV, Cappellini E. 2014 Species identification of archaeological skin objects from danish bogs: comparison between mass spectrometry-based peptide sequencing and microscopy-based methods. *PLoS ONE* **9**, e106875. (doi:10.1371/journal.pone.0106875)

3. Welker F *et al.* 2015 Ancient proteins resolve the evolutionary history of Darwin's South American

ungulates. *Nature* **522**, 81–84. (doi:10.1038/nature14249)

4. Ascenzi A, Brunori M, Citro G, Zito R. 1985 Immunological detection of hemoglobin in bones of ancient Roman times and of Iron and Eneolithic Ages. *Proc. Natl Acad. Sci. USA* **82**, 7170–7172. (doi:10.1073/pnas.82.21.7170)

5. Smith PR, Wilson MT. 1990 Detection of haemoglobin in human skeletal remains by ELISA. *J. Archaeol. Sci.* **17**, 255–268. (doi:10.1016/0305-4403(90)90023-X)

6. Cattaneo C, Gelsthorpe K, Phillips P, Sokol RJ. 1992 Detection of blood proteins in ancient human bone using ELISA: a comparative study of the survival of IgG and albumin. *Int. J. Osteoarchaeol.* **2**, 103–107. (doi:10.1002/oa.1390020202)

7. Cattaneo C, Gelsthorpe K, Sokol RJ, Phillips P. 1994 Immunological detection of albumin in ancient human cremations using ELISA and monoclonal antibodies. *J. Archaeol. Sci.* **21**, 565–571. (doi:10.1006/jasc.1994.1055)

8. Cappellini E *et al.* 2012 Proteomic analysis of a Pleistocene mammoth femur reveals more than one hundred ancient bone proteins. *J. Proteome Res.* **11**, 917–926. (doi:10.1021/pr200721u)

9. Orlando L *et al.* 2013 Recalibrating *Equus* evolution using the genome sequence of an early Middle Pleistocene horse. *Nature* **499**, 74–78. (doi:10.1038/nature12323)

10. Cappellini E *et al.* 2014 Resolution of the type material of the Asian elephant, *Elephas maximus* Linnaeus, 1758 (Proboscidea, Elephantidae). *Zool. J. Linn. Soc.* **170**, 222–232. (doi:10.1111/zoj.12084)

11. Hill RC, Wither MJ, Nemkov T, Barrett A, D'Alessandro A, Dzieciatkowska M, Hansen KC. 2015 Preserved proteins from extinct *Bison latifrons* identified by tandem mass spectrometry; hydroxylysine glycosides are a common feature of ancient collagen. *Mol. Cell. Proteomics* **14**, 1946–1958. (doi:10.1074/mcp.M114.047787)

12. Schweitzer MH, Hill CL, Asara JM, Lane WS, Pincus SH. 2002 Identification of immunoreactive material in mammoth fossils. *J. Mol. Evol.* **55**, 696–705. (doi:10.1007/s00239-002-2365-6)

13. Wadsworth C, Buckley M. 2014 Proteome degradation in fossils: investigating the longevity of protein survival in ancient bone. *Rapid Commun. Mass Spectrom.* **28**, 605–615. (doi:10.1002/rcm.6821)

14. Cleland TP, Schroeter ER, Schweitzer MH. 2015 Biologically and diagenetically derived peptide modifications in moa collagens. *Proc. R. Soc. B* **282**, 20150015. (doi:10.1098/rspb.2015.0015)

15. Cleland TP *et al.* 2016 Peptide sequences from the first *Castoroides ohioensis* skull and the utility of old museum collections for palaeoproteomics. *Proc. R. Soc. B* **283**, 5252–5262. (doi:10.1098/rspb.2016.0593)

16. Welker F *et al.* 2016 Palaeoproteomic evidence identifies archaic hominins associated with the Châtelperronian at the Grotte du Renne. *Proc. Natl Acad. Sci. USA* **113**, 1162–1167. (doi:10.1073/pnas.1605834113)

17. Bona A, Papai Z, Maasz G, Toth GA, Jambor E, Schmidt J, Toth C, Farkas C, Mark L. 2014 Mass spectrometric identification of ancient proteins as potential molecular biomarkers for a 2000-year-old osteogenic sarcoma. *PLoS ONE* **9**, e87215. (doi:10.1371/journal.pone.0087215)

18. Kendall R, Hendy J, Collins MJ, Millard AR, Gowland RL. 2016 Poor preservation of antibodies in

archaeological human bone and dentine. *Sci. Technol. Archaeol. Res.* **2**, 15–24. (doi:10.1080/20548923.2015.1133117)

19. Yamamoto M. 1989 Enamel hypoplasia in the deciduous teeth of Edo Japanese. *J. Anthropol. Soc. Nippon* **97**, 475–482. (doi:10.1537/ase1911.97.475)

20. Hirata K. 1990 Secular trend and age distribution of cribra orbitalia in Japanese. *Hum. Evol.* **5**, 375–385. (doi:10.1007/BF02437251)

21. Nagaoka T, Hirata K. 2007 Reconstruction of paleodemographic characteristics from skeletal age at death distributions: perspectives from Hitotsubashi, Japan. *Am. J. Phys. Anthropol.* **134**, 301–311. (doi:10.1002/ajpa.20670)

22. Genovés S. 1959 L'estimation des différences sexuelles dans l'os coxal: différences métriques et différences morphologiques. *Bull. Mem. Soc. Anthropol. Paris* **10**, 3–95. (doi:10.3406/bmsap.1959.2750)

23. Ferembach D, Schwidetzky I, Stloukal M. 1980 Recommendations for age and sex diagnoses of skeletons. *J. Hum. Evol.* **9**, 517–549. (doi:10.1016/0047-2484(80)90061-5)

24. Bruzek J. 2002 A method for visual determination of sex, using the human hip bone. *Am. J. Phys. Anthropol.* **117**, 157–168. (doi:10.1002/ajpa.10012)

25. Houghton P. 1974 The relationship of the pre-auricular groove of the ilium to pregnancy. *Am. J. Phys. Anthropol.* **41**, 381–389. (doi:10.1002/ajpa.1330410305)

26. Phenice TW. 1969 A newly developed visual method of sexing the os pubis. *Am. J. Phys. Anthropol.* **30**, 297–301. (doi:10.1002/ajpa.1330300214)

27. Buckberry J, Chamberlain A. 2002 Age estimation from the auricular surface of the ilium: a revised method. *Am. J. Phys. Anthropol.* **119**, 231–239. (doi:10.1002/ajpa.10130)

28. Storey R. 2007 An elusive paleodemography? A comparison of two methods for estimating the adult age distribution of deaths at late Classic Copan, Honduras. *Am. J. Phys. Anthropol.* **132**, 40–47. (doi:10.1002/ajpa.20502)

29. Mulhern DM, Jones EB. 2005 Test of revised method of age estimation from the auricular surface of the ilium. *Am. J. Phys. Anthropol.* **126**, 61–65. (doi:10.1002/ajpa.10410)

30. The Japanese Society of Pedodontics. 1988 The chronology of deciduous and permanent dentition in Japanese children. *Jpn. J. Pediatr. Dent.* **26**, 1–18. (In Japanese.)

31. Ubelaker D. 1978 *Human skeletal remains. Excavation, analysis, interpretation*. Washington, DC: Taraxacum.

32. Demarchi B *et al.* 2016 Protein sequences bound to mineral surfaces persist into deep time. *Elife* **5**, e17092. (doi:10.7554/eLife.17092)

33. Cox J, Mann M. 2008 MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **26**, 1367–1372. (doi:10.1038/nbt.1511)

34. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. 2011 Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.* **10**, 1794–1805. (doi:10.1021/pr101065j)

35. Buckley M, Wadsworth C. 2014 Proteome degradation in ancient bone: diagenesis and phylogenetic potential. *Palaeogeogr.*

Palaeoclimatol. Palaeoecol. **416**, 69–79. (doi:10.1016/j.palaeo.2014.06.026)

36. Nepomuceno AI, Gibson RJ, Randall SM, Muddiman DC. 2014 Accurate identification of deamidated peptides in global proteomics using a quadrupole orbitrap mass spectrometer. *J. Proteome Res.* **13**, 777–785. (doi:10.1021/pr400848n)

37. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, Mann M. 2005 Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Mol. Cell. Proteomics* **4**, 1265–1272. (doi:10.1074/mcp.M500061-MCP200)

38. Mi H, Poudel S, Muruganujan A, Casagrande JT, Thomas PD. 2016 PANTHER version 10: expanded protein families and functions, and analysis tools. *Nucleic Acids Res.* **44**, D336–D342. (doi:10.1093/nar/gkv1194)

39. Szklarczyk D *et al.* 2015 STRING v10: Protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* **43**, D447–D452. (doi:10.1093/nar/gku1003)

40. Cecchini MG, Hofstetter W, Halasy J, Wetterwald A, Felix R. 1997 Role of CSF-1 in bone and bone marrow development. *Mol. Reprod. Dev.* **46**, 75–84. (doi:10.1002/(sici)1098-2795(199701)46:1<75::aid-mrd12>3.3.co;2-f)

41. Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Yano K, Morinaga T, Higashio K. 1998 RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. *Biochem. Biophys. Res. Commun.* **253**, 395–400. (doi:10.1006/bbrc.1998.9788)

42. Warinner C *et al.* 2014 Pathogens and host immunity in the ancient human oral cavity. *Nat. Genet.* **46**, 336–344. (doi:10.1038/ng.2906)

43. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. 2012 Neutrophil function: from mechanisms to disease. *Annu. Rev. Immunol.* **30**, 459–489. (doi:10.1146/annurev-immunol-020711-074942)

44. Faurschou M, Borregaard N. 2003 Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect.* **5**, 1317–1327. (doi:10.1016/j.micinf.2003.09.008)

45. Acharya KR, Ackerman SJ. 2014 Eosinophil granule proteins: form and function. *J. Biol. Chem.* **289**, 17406–17415. (doi:10.1074/jbc.R113.546218)

46. Ezkurdia I, Juan D, Rodriguez JM, Frankish A, Diekhans M, Harrow J, Vazquez J, Valencia A, Tress ML. 2014 Multiple evidence strands suggest that there may be as few as 19 000 human protein-coding genes. *Hum. Mol. Genet.* **23**, 5866–5878. (doi:10.1093/hmg/ddu309)

47. Mattinzoli D *et al.* 2016 FGF23-regulated production of Fetuin-A (AHSG) in osteocytes. *Bone* **83**, 35–47. (doi:10.1016/j.bone.2015.10.008)

48. Peruzzi B, Cappariello A. 2012 c-Src and IL-6 inhibit osteoblast differentiation and integrate IGFBP5 signalling. *Nat. Commun.* **3**, 630. (doi:10.1038/ncomms1651)

49. Marini JC, Blissett AR. 2013 New genes in bone development: what's new in osteogenesis imperfecta. *J. Clin. Endocrinol. Metab.* **98**, 3095–3103. (doi:10.1210/jc.2013-1505)

50. Horiuchi K, Amizuka N, Takeshita S, Takamatsu H, Katsuura M, Ozawa H, Toyama Y, Bonewald LF, Kudo A. 1999 Identification and characterization of

a novel protein, periostin, with restricted expression to periosteum and periodontal ligament and increased expression by transforming growth factor beta. *J. Bone Miner. Res.* **14**, 1239–1249. (doi:10.1359/jbmr.1999.14.7.1239)

51. Kruzynska-Frejtag A, Machnicki M, Rogers R, Markwald RR, Conway SJ. 2001 Periostin (an osteoblast-specific factor) is expressed within the embryonic mouse heart during valve formation. *Mech. Dev.* **103**, 183–188. (doi:10.1016/S0925-4773(01)00356-2)

52. Ohta T, Hattori S, Murakami M, Nishiyama S, Matsuda I. 1989 Age- and sex-related differences in lipoproteins containing apoprotein A-I. *Arterioscler. Thromb. Vasc. Biol.* **9**, 90–95. (doi:10.1161/01.ATV.9.1.90)

53. Nakamura T, Fox-Robichaud A, Kikkawa R, Kashiwagi A, Kojima H, Fujimiya M, Wong NC. 1999 Transcription factors and age-related decline in apolipoprotein A-I expression. *J. Lipid Res.* **40**, 1709–1718.

54. Schroeter ER, Cleland TP. 2016 Glutamine deamidation: An indicator of antiquity, or preservational quality? *Rapid Commun. Mass Spectrom.* **30**, 251–255. (doi:10.1002/rcm.7445)

55. Tsutaya T, Nagaoka T, Sawada J, Hirata K, Yoneda M. 2014 Stable isotopic reconstructions of adult diets and infant feeding practices during urbanization of the city of Edo in 17th century Japan. *Am. J. Phys. Anthropol.* **153**, 559–569. (doi:10.1002/ajpa.22454)

56. Moulopoulos LA, Koutoulidis V. 2015 *Bone marrow MRI: a pattern-based approach*. Milan, Italy: Springer.

57. Cristy M. 1981 Active bone marrow distribution as a function of age in humans. *Phys. Med. Biol.* **26**, 389–400. (doi:10.1088/0031-9155/26/3/003)

58. Valentin J, Streffer C. 2002 Basic anatomical and physiological data for use in radiological protection: reference values—ICRP Publication 89. *Ann. ICRP* **32**, 1–265. (doi:10.1016/S0146-6453(03)00002-2)

59. Alves RDAM, Demmers JAA, Bezstarosti K, Van Der Eerden BCJ, Verhaar JAN, Eijken M, Van Leeuwen JPTM. 2011 Unraveling the human bone microenvironment beyond the classical extracellular matrix proteins: a human bone protein library. *J. Proteome Res.* **10**, 4725–4733. (doi:10.1021/pr200522n)

60. Terstappen LW, Levin J. 1992 Bone marrow cell differential counts obtained by multidimensional flow cytometry. *Blood Cells* **18**, 311–330.

61. Uhlén M *et al.* 2015 Tissue-based map of the human proteome. *Science* **347**, 1260419. (doi:10.1126/science.1260419)

62. Fujikawa Y. 1969 *Nihon-shippei-shi*. Tokyo, Japan: Heibonsha. (In Japanese.)

63. Motoi S. 1794 *Hiden eiseiron* (In Japanese.)

64. Tsuge R. 1802 *Man'nanroku* (In Japanese.)

65. Maki J, Sakagami H, Kuwada M, Caceres A, Sekiya H, Tamai E. 2009 Infections with gastrointestinal parasitic helminths indigenous to Japan and their treatment historically studied in an attempt to control the diseases in countries where they are still rampant: (1) the Jomon to Edo periods. *Yakushigaku. Zasshi.* **44**, 18–23.

66. Zimmermann EA *et al.* 2011 Age-related changes in the plasticity and toughness of human cortical bone at multiple length scales. *Proc. Natl Acad. Sci. USA* **108**, 14416–14421. (doi:10.1073/pnas.1107966108)

67. Quelch KJ, Cole WG, Melick RA. 1984 Noncollagenous proteins in normal and pathological human bone. *Calcif. Tissue Int.* **36**, 545–549. (doi:10.1007/bf02405363)

68. Dickson IR, Bagga MK. 1985 Changes with age in the non-collagenous proteins of human bone. *Connect. Tissue Res.* **14**, 77–85. (doi:10.3109/03008208509089845)

69. Kessels MY, Huitema LAF, Boeren S, Kranenbarg S, Schulte-Merker S, Van Leeuwen JL, De Vries SC. 2014 Proteomics analysis of the zebrafish skeletal extracellular matrix. *PLoS ONE* **9**, e90568. (doi:10.1371/journal.pone.0090568)

70. Wilson JM, Ashton B, Triffitt JT. 1976 The interaction of a component of bone organic matrix with the mineral phase. *Calcif. Tissue Res.* **22**, 458–460. (doi:10.1007/BF02064129)

71. Mohan S, Farley JR, Baylink DJ. 1995 Age-related changes in IGFBP-4 and IGFBP-5 levels in human serum and bone: implications for bone loss with aging. *Prog. Growth Factor Res.* **6**, 465–473. (doi:10.1016/0955-2235(95)00027-5)

72. Pedersen KO. 1944 Fetuin, a new globulin isolated from serum. *Nature* **154**, 575. (doi:10.1038/154575a0)

73. Robinson KN, Teran-Garcia M. 2016 From infancy to aging: biological and behavioral modifiers of fetuin-A. *Biochimie* **124**, 141–149. (doi:10.1016/j.biochi.2015.12.016)

74. Binkertt C, Demetriou M, Sukhu B, Szweras M, Tenenbaum HC, Dennis JW. 1999 Regulation of osteogenesis by fetuin. *J. Biol. Chem.* **274**, 28514–28520. (doi:10.1074/jbc.274.40.28514)

75. Brylka L, Jahnen-Dechent W. 2013 The role of fetuin-A in physiological and pathological mineralization. *Calcif. Tissue Int.* **93**, 355–364. (doi:10.1007/s00223-012-9690-6)

76. Laughlin GA, Barrett-Connor E, Cummins KM, Daniels LB, Wassel CL, Ix JH. 2013 Sex-specific association of fetuin-A with type 2 diabetes in older community-dwelling adults: the Rancho Bernardo study. *Diabetes Care* **36**, 1994–2000. (doi:10.2337/dc12-1870)

77. Kolman CJ, Centurion-Lara A, Lukehart SA, Owsley DW, Tuross N. 1999 Identification of *Treponema pallidum* subspecies pallidum in a 200-year-old skeletal specimen. *J. Infect. Dis.* **180**, 2060–2063. (doi:10.1086/315151)

78. Schmidt-Schultz TH, Schultz M. 2004 Bone protects proteins over thousands of years: extraction, analysis, and interpretation of extracellular matrix proteins in archeological skeletal remains. *Am. J. Phys. Anthropol.* **123**, 30–39. (doi:10.1002/ajpa.10308)

79. Schultz M, Parzinger H, Posdnjakov DV, Chikisheva TA, Schmidt-Schultz TH. 2007 Oldest known case of metastasizing prostate carcinoma diagnosed in the skeleton of a 2,700-year-old Scythian King from Arzhan (Siberia, Russia). *Int. J. Cancer* **121**, 2591–2595. (doi:10.1002/ijc.23073)

80. Scott AB, Choi KY, Mookherjee N, Hoppa RD, Larcombe LA. 2016 The biochemical signatures of stress: a preliminary analysis of osteocalcin concentrations and macroscopic skeletal changes associated with stress in the 13th–17th centuries black friars population. *Am. J. Phys. Anthropol.* **159**, 596–606. (doi:10.1002/ajpa.22915)

81. Der Sarkissian C *et al.* 2015 Ancient genomics. *Phil. Trans. R. Soc. B* **370**, 20130387. (doi:10.1098/rstb.2013.0387)

82. Vizcaíno JA *et al.* 2016 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res.* **44**, D447–D456. (doi:10.1093/nar/gkv1145)