Devlin Maureen (Orcid ID: 0000-0003-0845-3090)
Eick Geeta (Orcid ID: 0000-0001-7512-3265)

The utility of dried blood spot measurement of bone turnover markers in biological anthropology

Maureen J. Devlin[1], Geeta N. Eick[2], and J. Josh Snodgrass[2,3]

[1]Department of Anthropology, University of Michigan
[2]Global Health Biomarker Laboratory, Department of Anthropology, University of Oregon
[3]Center for Global Health, University of Oregon

Running title: Bone turnover markers

Word count
Text: 6501
Abstract: 250

Figures: 0
Tables: 1

Address correspondence to:
Maureen J. Devlin, PhD
University of Michigan, 101 West Hall
1085 S. University Ave
Ann Arbor, MI 48108
mjdevlin@umich.edu
ph: 734-615-3293; fax: 734-763-6077

ABSTRACT

Objectives: Bone is a dynamic organ under continual turnover influenced by life history stage, energy dynamics, diet, climate, and disease. Bone turnover data have enormous potential in biological anthropology for testing evolutionary and biocultural hypotheses, yet few studies have integrated these biomarkers. In the present paper we systematically review the current availability, future viability, and applicability of measuring bone turnover markers (BTMs) in dried blood spot (DBS) samples obtained from finger prick whole blood.

Methods: Our review considers clinical and public health relevance, biomarker stability in DBS, assay availability, and cost. We consider biomarkers of bone formation such as osteocalcin (bone matrix protein), PINP (N-terminal propeptide of type I collagen), and alkaline phosphatase (osteoblast enzyme), as well as biomarkers of bone resorption such as CTX (marker of collagen breakdown) and TRACP5b (tartrate-resistant acid phosphatase 5b; osteoclast enzyme).

Results: Two BTMs have been validated for DBS: osteocalcin (formation) and TRACP5b (resorption). Prime candidates for future development are CTX and PINP, the formation and resorption markers used for clinical monitoring of response to osteoporosis treatment.

Conclusion: BTMs are a field-friendly technique for longitudinal monitoring of skeletal biology during growth, reproduction and aging, combining minimized risk to study participants with maximized ease of sample storage and transport. This combination allows new insights into the effects of energy availability, disease, and physical activity level on bone, and questions about bone gain and loss across life history and in response to environmental factors; these issues are important in human biology, paleoanthropology, bioarchaeology, and forensic anthropology.

Keywords: biomarkers; skeletal biology; bone turnover; osteoporosis

INTRODUCTION

Skeletal biology is a central element in many longstanding issues in biological anthropology, from reconstructing the locomotion and life history of hominin species, to understanding ecogeographic variation in body shape and limb proportions in human populations, to considering how demographic shifts in reproduction affect skeletal health and risk for osteoporosis (Barak et al., 2013; Lockwood, Menter, Moggi-Cecchi, & Keyser, 2007; Macintosh, Pinhasi, & Stock, 2016, 2017; Madimenos, Liebert, Cepon-Robins, Snodgrass, & Sugiyama, 2015; Madimenos, Snodgrass, Liebert, Cepon, & Sugiyama, 2012; Pearson, 2000; Raichlen et al., 2015; Ruff, 1994, 2005; Shaw & Stock, 2013). Bone is a phenotypically plastic organ that models and remodels throughout life in response to mechanical loading, nutrition, disease, and reproduction (Allen & Burr, 2019; Pearson & Lieberman, 2004; Siddiqui & Partridge, 2016). As such, bone morphology preserves information about an individual's growth patterns, health, nutrition, and physical activity level. These data are essential for testing hypotheses about how environmental factors and physiological processes influence skeletal phenotype, as well as for reconstructing behavior and life history in past populations of humans and our fossil relatives. Furthermore, evolutionary and biocultural approaches to skeletal biology have increasing clinical relevance for the study of osteoporosis, a condition that is increasingly prevalent in societies around the world (Sambrook & Cooper, 2006), but understudied in traditionally living groups despite evidence of fracture (Madimenos, 2015; Stieglitz et al., 2015).

Hypothesis testing in human skeletal biology is challenging due to the constraints on measuring bone's response to stimuli in living individuals. Measuring bone phenotype from skeletal material is straightforward, but behavior, diet, and other external factors must be inferred or known through records. In living individuals, external stimuli are easier to quantify (though this can still be complicated) but obtaining data on bone size and shape is more challenging, requiring imaging. Furthermore, longitudinal tracking of bone formation and resorption is ideal

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for many research questions in skeletal biology, yet such studies are methodologically challenging because the gold standard modalities for measuring skeletal change over time, such as computed tomography (CT) scans or dual x-ray absorptiometry (DXA), involve repeated radiation exposure and/or are challenging to perform in remote locations (Choksi, Jepsen, & Clines, 2018). These data collection challenges may be one reason why skeletal phenotyping in human biology is often limited to stature and limb proportions, which can easily be measured with standard anthropometry but are less informative about dynamic changes in bone mass or morphology. These methodological limitations have prevented integrative studies that combine skeletal data with rich data on biology, behavior, sociodemographics, and environment.

In the present paper, we review a new approach for tracking bone gain and loss using bone turnover markers (BTMs) measured in dried blood spots (DBS), which overcomes several of the constraints on quantifying bone gain and loss in living individuals. BTMs are byproducts of bone formation and resorption that are produced in proportion to the actions of osteoblasts and osteoclasts. Serum BTMs have been used clinically to monitor bone turnover for decades (Delmas, Eastell, Garnero, Seibel, Stepan, et al., 2000), but there is a dearth of data from population-based research, especially in low- and middle-income countries. After briefly reviewing the basics of bone biology and methods used to quantify bone strength, we discuss key biomarkers of bone formation and resorption used in human clinical studies and in animal models. We then describe recent progress in measurement of BTMs in DBS, before turning our attention to questions in biological anthropology that could be addressed using these data, particularly when combined with field-friendly bone imaging techniques such as ultrasonography. The integration of BTMs, measures of bone strength, and behavioral and environmental variables has significant explanatory power both for testing hypotheses and for clinical and public health initiatives to improve bone health and decrease the risk of osteoporosis.

BONE BIOLOGY REVIEW

Bone strength—the ability to resist deformation and fracture—depends on bone properties at multiple levels of organization (Bouxsein & Seeman, 2009). The mass of the skeleton consists of about 80% cortical (compact) bone, e.g. in long bone diaphyses, and about 20% trabecular (cancellous or spongy) bone found near joints and in the axial skeleton (Clarke, 2008). In long bones, resistance to compressive loading depends on the amount of bone present, such as the cross-sectional area of cortical bone or the volume of trabecular bone, while resistance to deformation from bending and twisting depends on the cross-sectional geometry, or shape of the bone, with wider bones generally having greater strength than narrower bones (Burr, 2019). At the tissue level, bone mineral density (BMD; the amount of bone mineral per area or volume of tissue) contributes to bone stiffness, and material properties at the microscopic level, such as collagen cross-links, contribute to bone toughness and ability to resist propagation of microcracks, which can lead to fracture (van der Meulen, Jepsen, & Mikic, 2001).

Bone is a dynamic tissue that is constantly being formed and resorbed throughout life (Burr, 2019; Currey, 2002). Bone turnover refers to the coordinated processes of bone formation and resorption that, together, may lead to a net increase, stasis, or decrease in bone mass, depending on the relative rates of gain and loss (Allen & Burr, 2019; Zhou, Lu, & Dempster, 2010). Some of this turnover occurs within existing bone tissue to repair microdamage, through the actions of the Basic Multicellular Unit (BMU), in which bone resorption by osteoclasts and formation by osteoblasts are mechanistically coupled to maintain bone mass (Frost, Vilanueva, Jett, & Eyring, 1969; Rauch, Travers, & Glorieux, 2007). Bone mass and bone turnover also change in response to physiology, nutrition, physical activity level, reproduction, and aging (Cooper et al., 2007; Weaver & Peacock, 2019; Zebaze et al., 2010). For example, in growing children, bone resorption along the diaphysis (shaft) and deposition at the metaphyses (ends)

maintains the shape of bones as they elongate (Allen & Burr, 2019). During pregnancy and lactation, resorption allows liberation of calcium from bone tissue, which is typically replaced by deposition after weaning (Weaver & Peacock, 2019). Changes in bone shape, such as those that occur in response to exercise-induced mechanical loading, also require bone turnover through periosteal deposition and endosteal resorption (Pearson & Lieberman, 2004; Robling, Daly, Fuchs, & Burr, 2019). Bone resorption occurs with decreased mechanical loading, as in bedrest, microgravity, or significant weight loss (Konda et al., 2019; Krez & Stein, 2020; Schafer, 2016). Thus, the ability to track patterns of bone gain and loss over the life course is crucial for testing hypotheses about how health and disease, activity, nutrition, reproduction, and aging affect the skeleton. Furthermore, such research is essential for improving the accuracy of form-function inferences in paleontology, bioarchaeology, and forensics, such as more precisely reconstructing biological profiles and lifeways using data from the skeleton. When behaviors cannot be observed directly, controlled studies are essential in order to reliably link bone morphology to habitual activities, occupational stressors, and environmental influences.

MEASURES OF BONE STRENGTH

As noted above, bone strength is the product of multiple properties (e.g., mass, shape, density) at multiple organizational levels from macroscopic to microscopic. Given the interactions among these properties, it would be ideal to measure all of them. However, imaging of cortical bone cross-sectional area or trabecular bone microarchitecture requires computed tomography (CT) or peripheral quantitative computed tomography (pQCT), which are expensive and require radiation exposure, which may be a concern for vulnerable populations in research (Choksi et al., 2018). Many studies of bone strength use dual energy x-ray absorptiometry (DXA) to measure BMD as a proxy for bone strength, since in wealthy nations this technology is more commonly available in clinical settings and requires a much smaller radiation dose. However, to

date, dynamic changes in bone mass have been difficult to measure; bone grows slowly, so changes in bone mass or shape following a given stimulus may not be detectable via imaging for months (Clarke, 2008; Greenblatt, Tsai, & Wein, 2017). When *in vivo* measurements of bone mass, size, and shape are available, they tend to be cross-sectional rather than longitudinal, often focus on a limited number of skeletal sites, and are often biased towards populations in high-income nations—the so-called WEIRD populations (Gurven & Lieberman, 2020; Henrich, Heine, & Norenzayan, 2010). Some recent studies have started to address these challenges by using portable calcaneal ultrasonometry to measure bone mineral density (BMD), for example in the Shuar and *Colono* in Ecuador (Madimenos et al., 2015; Madimenos et al., 2011) and Tsimane of Bolivia (Stieglitz et al., 2015; Stieglitz, Madimenos, Kaplan, & Gurven, 2016). Because these studies couple skeletal data with high-resolution data on sociodemographic, lifestyle, dietary, and environmental variables, they allow the investigation of questions such as how reproductive factors (e.g., age at menarche, age at first parturition, and number of pregnancies) affect skeletal health. The research among the Shuar is particularly useful for understanding the effects of reproduction on skeletal homeostasis in a natural fertility population (Madimenos et al., 2012). Nevertheless, a limitation of all imaging methods is that they are static measurements and do not provide data on the underlying rates of bone gain and loss.

A complement to imaging-based methods is to track bone formation and resorption using BTMs in blood or urine; these BTMs are byproducts generated by bone formation by osteoblasts or bone resorption by osteoclasts. From a biological perspective, the main advantage of using these markers is they provide whole-body data on bone turnover at the cellular level, rather than being limited to the specific skeletal locations being imaged. BTMs can detect changes in osteoblast and osteoclast activity more quickly than could be detected by imaging (Greenblatt et al., 2017). The main disadvantage is the lack of data about changes in bone size, shape, and BMD. For example, elevated BTMs might reflect high levels of localized bone turnover such as

fracture healing or orthodontics (e.g. (Tang, Meikle, MacLaine, Wong, & Rabie, 2013), so recent skeletal and dental history should be included on participant questionnaires. However, combining traditional BMD measures with BTMs would be a particularly powerful approach because it would provide information about bone mass and about the current rate of bone turnover and balance between bone formation and resorption, connecting current skeletal phenotype to skeletal health trajectories and the mechanisms of skeletal growth, maintenance, or degeneration over the life course.

From a practical perspective, a major strength is that BTMs can be measured in longitudinal studies and in field settings at minimal risk to participants, making it possible to ask research questions that would be difficult to investigate using only imaging methods. However, BTMs have not been widely used in biological anthropology due to uncertainty about how to link these bone turnover markers to bone mass and to practical issues such as preserving and transporting frozen serum or urine from field locations. A solution to the first problem is to combine BTMs with BMD measurements by heel ultrasonometry, as both are field-friendly techniques. A solution to the second problem is to measure BTMs from DBS samples. This method is minimally invasive, requiring only a simple finger prick, with capillary whole blood collected onto standardized filter paper that does not require post-collection treatment and can often be stored at room temperature for days or weeks (McDade, Williams, & Snodgrass, 2007). However, a non-trivial challenge with current immunoassays designed to measure BTMs in whole blood is that they sometimes lack the sensitivity needed to measure BTM levels in DBS, which are effectively blood microsamples. In developing or modifying new assays for BTMs in DBS, validation studies should focus on assays with extremely high sensitivity. In the future, other technology platforms could be explored to create validated assays for BTMs that are able to detect in DBS markers that circulate in low concentrations. Following such validation, the promise of this method lies in its potential to reveal how social and environmental factors not

only 'get under the skin' but also 'into the bone', offering new opportunities to test hypotheses about the influence of chronic psychosocial stress, exercise, nutrition, temperature, disease burden, and more on the skeleton.

BONE TURNOVER MARKERS

Bone formation

At the cellular level, bone formation by osteoblasts begins with deposition of unmineralized osteoid, which consists of 90% type I collagen fibers and 10% extracellular matrix (also known as ground substance); mineralization follows several weeks later, as osteoblasts deposit hydroxyapatite onto the collagen fibers (Salhotra, Shah, Levi, & Longaker, 2020). Each of these steps generates byproducts that can be used as a proxy for bone formation rate.

Biomarkers of bone formation from blood and urine samples. Commonly measured bone formation markers include N-terminal propeptide of type I collagen (PINP), bone-specific alkaline phosphatase (BSAP), and osteocalcin (Table 1) (Szulc, 2018).

PINP is a peptide that is cleaved from type I procollagen during osteoid deposition by osteoblasts (Gillett, Vasikaran, & Inderjeeth, 2021). PINP has several benefits as a BTM: its levels are directly proportional to type I collagen production and thus bone formation; it has good stability in blood at room temperature for up to 5 days (Garnero, Vergnaud, & Hoyle, 2008); and it is relatively unaffected by time of day or food intake (Gillett et al., 2021). Clinically, PINP is most often used to track the response to anabolic (bone-forming) or antiresorptive (resorption-suppressing) therapies for osteoporosis. For example, in postmenopausal women, teriparatide (synthetic parathyroid hormone) treatment was associated with both increased BMD at the lumbar spine and higher PINP levels (P. Chen et al., 2005).

Bone-specific alkaline phosphatase (BSAP) is an important regulator of mineralization, and thus its levels reflect the extent of bone mineral deposition as well as osteoblast number. However, alkaline phosphatase is also produced by the liver, so the measurement assay used must be specific to bone. BSAP levels are elevated in diseases of high bone turnover such as Paget's disease of bone (Singer et al., 2014). It is sometimes used clinically to monitor response to therapy for metabolic bone disease, but assay cross-reactivity with liver-derived alkaline phosphatase limits its utility (Greenblatt et al., 2017).

Bone formation biomarkers from DBS. We recently developed and validated a DBS assay for osteocalcin by comparing OC values from matched plasma, venous DBS, and fingerstick DBS samples from 158 adults, and found linear relationships between plasma and DBS OC, with sample stability at room temperature or colder (Eick et al., 2020). Osteocalcin, which is synthesized by osteoblasts, is the major non-collagenous protein found in the bone matrix, and circulating osteocalcin levels reflect the rate of bone formation (A. J. Lee, Hodges, & Eastell, 2000; Manolagas, 2000). High osteocalcin levels are a marker of either rapid bone formation, as seen in adolescence, or increased bone turnover (resorption followed by formation), as seen in osteoporosis, while low osteocalcin levels are associated with decreased bone turnover (Brown et al., 1984; Szulc, Seeman, & Delmas, 2000). Clinically, osteocalcin levels are used to monitor bone turnover in metabolic diseases such as osteoporosis and growth hormone deficiency (Brown et al., 2009; Delmas, Eastell, Garnero, Seibel, & Stepan, 2000; M. S. Lee et al., 1990; Wuster, 1993). In particular, the carboxylated form of osteocalcin (Gla-OCN or cOC) is a marker of bone formation that reflects the key role it plays in binding hydroxyapatite (bone mineral) and contributing to bone formation (Hauschka, Lian, Cole, & Gundberg, 1989).

Osteocalcin levels are used to identify individuals (usually women) at risk of osteoporosis and to monitor bone turnover in metabolic diseases such as thyroid disorders and growth hormone

deficiency. In industrialized populations, subadult osteocalcin levels rise and then fall in parallel with rapid bone formation during the adolescent growth spurt, before stabilizing in adulthood (Paldanius, Ivaska, Makitie, & Viljakainen, 2021). In adults, despite population variability, there is some evidence osteocalcin is higher in young adults, decreases at midlife, and then increases with age, particularly in postmenopausal women, making it a sensitive marker of osteoporosis risk (Diemar et al., 2020; Gundberg, Looker, Nieman, & Calvo, 2002; Hannemann et al., 2013; Smith et al., 2020). Since osteocalcin provides information about bone formation, it should be paired with a marker of bone resorption to distinguish between high net bone formation (in which formation outpaces resorption) and high bone turnover (in which resorption may outpace formation, leading to net bone loss). In addition to cOC, up to 20-30% of circulating osteocalcin in adults is in an undercarboxylated form (Glu-OCN or ucOC), which is not involved in bone mineralization. Intriguingly, ucOC is produced by insulin binding to osteoblast insulin receptors and improves glucose metabolism (reviewed in Riddle & Clemens, 2017). However, there are complex differences in ucOC function between humans and mouse models, and more work is needed to delineate the roles of the skeleton and ucOC in whole-body metabolism and energy allocation (Motyl, Guntur, Carvalho, & Rosen, 2017; Riddle & Clemens, 2017).

Circulating osteocalcin is lowest midday and peaks at night (Nielsen, Brixen, & Mosekilde, 1990), and has a short half-life (Farrugia & Melick, 1986), with the N-terminal fragment showing greater stability than the C-terminal fragment (A. J. Lee et al., 2000). For this reason, commercial assays usually target either the N-terminal fragment or the intact protein (Garnero, Grimaux, Seguin, & Delmas, 1994). There is conflicting information about the effect of feeding on OC levels, with one study in women showing a ~4% decrease in OC in the fed vs. fasted state (Clowes et al., 2002), but another study in men showing no effect of feeding on OC levels (Scott et al., 2012). OC also increases following exercise (Mohammad Rahimi, Niyazi, & Alaee, 2021), although the majority of studies do not report whether ucOC, cOC, or total OC was

measured, and short-term changes (e.g., pre- vs. post-exercise) likely reflect primarily ucOC. At room temperature, OC in plasma or serum degrades within 24 hours (Christensen et al., 2019), but our validation study showed that in DBS, osteocalcin was reasonably stable at room temperature for up to 28 days (Eick et al., 2020).

Looking ahead, the best target for future DBS validation as a bone formation marker is PINP, which (as noted above) is stable both before and after measurement. PINP measurement is readily available in clinical laboratories, and as an ELISA assay for research purposes, at a cost comparable to other standard ELISAs. This marker has been endorsed by the International Osteoporosis Foundation and International Federation of Clinical Chemistry and Laboratory Medicine for monitoring bone formation (Vasikaran et al., 2011), and the National Bone Health Alliance has issued recommendations for its use (Szulc et al., 2017).

Bone resorption

At the cellular level, bone resorption occurs when osteoclasts seal themselves to the bone surface and secrete acids and proteinases that dissolve the mineralized tissue and collagen, respectively (Salhotra et al., 2020). This process releases mineral (primarily calcium) and the byproducts of collagen degradation into the circulation.

Biomarkers of bone resorption from blood and urine samples. Commonly measured bone resorption markers include CTX (C-terminal telopeptide of type I collagen) and N-terminal telopeptide of type I collagen (NTX), as well as tartrate-resistant acid phosphatase 5b (TRACP5b, see below) (Table 1) (Szulc, 2018). CTX and NTX are parts of the type I collagen molecule that are cleaved by osteoclasts during bone resorption, such that the levels of these telopeptides are proportional to osteoclast activity, and can be measured in blood or urine as a proxy of collagen breakdown and thus of bone loss (Greenblatt et al., 2017). Two less frequently

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used markers, pyridinoline (PYD) and deoxypyridinoline (DPD), are collagen crosslinks that stabilize bone matrix; in one study, declines in PYD and DPD of 1 standard deviation were associated with a 1.8-2-fold increase in the probability of rapid bone loss (Ross & Knowlton, 1998).

Bone resorption biomarkers from DBS. We recently validated a DBS assay for TRACP5b by comparing values from matched plasma, venous DBS, and fingerstick DBS samples from 189 adults, and found linear relationships between plasma and DBS TRACP5b, with sample stability for up to one month at room temperature, and long term when frozen (Eick et al., 2019). TRACP5b is an enzyme secreted in proportion to osteoclast number (Halleen & Ranta, 2001; Halleen et al., 2002; Hannon et al., 2004). Two forms of TRACP circulate in human blood: TRACP5a and TRACP5b, the former closely associated with macrophages and the latter with osteoclasts. Blood levels of TRACP5b are correlated with the extent of active bone remodeling. For example, in postmenopausal women, the decrease in TRACP5b had greater sensitivity than decreases in CTX or PINP for tracking improvements in BMD in response to antiresorptive therapy for osteoporosis (Nenonen et al., 2005). TRACP5b is stable in serum for 2 days at room temperature, 3 days at 4°C, and months to years when frozen; it has low diurnal variability and is not influenced by feeding (Cavalier, Delanaye, & Moranne, 2013; Halleen et al., 2000; Hannon et al., 2004).

In future research, the best candidate for DBS validation as a bone resorption marker is CTX, which has been endorsed by the International(Cavalier et al., 2013; Halleen et al., 2000; Hannon et al., 2004) Osteoporosis Foundation and International Federation of Clinical Chemistry and Laboratory Medicine for tracking bone resorption (Vasikaran et al., 2011). CTX measurement is readily available in clinical laboratories, and as an ELISA assay for research

purposes, at a cost comparable to other standard ELISAs. As noted above for PINP, the National Bone Health Alliance has issued recommendations for its use (Szulc et al., 2017).

CHALLENGES AND OPPORTUNITIES

Incorporating BTMs into biological anthropology research introduces several challenges. Despite the high potential of BTMs as a data source, their promise has not been fully realized clinically at the individual level for several reasons, including a lack of established reference ranges, limited availability of standardized assays, and high intra- and inter-individual variability of these markers (e.g., by age and sex) (Vasikaran et al., 2021). As a result, although human studies at the cohort level are informative, BTMs alone are rarely used for clinical decision-making.

For BTMs to become more informative at the individual level, whether for clinical or anthropological purposes, expected ranges must be established for each marker by age, sex, and population, as well as patterns of diurnal and seasonal fluctuation. For example, CTX levels are influenced by food intake and time of day, but PINP levels are not (Vasikaran et al., 2021); PYD and DPD are higher in winter than in summer, when Vitamin D levels are lowest (Brown et al., 2009). More importantly, it is highly likely that the "normal" ranges of BTMs will vary across populations, as is seen for BMD, sex steroids, and other biological variables (Wiley, 2021). Expanding the biological toolkit to more fully incorporate BTMs, BMD, and other skeletal data will first require addressing the current data bias toward individuals from wealthy nations (Gurven & Lieberman, 2020).

The good news is that the availability of DBS makes collecting the necessary data to develop such reference ranges much more feasible. The standard DBS card has five 0.5" (12.7 mm) circles, and the average sample consists of 40-60 μL of blood. In our validation studies,

TRACP5b required two 6-mm diameter punches from a DBS, and OC required one 3-mm diameter punch (Eick et al., 2019; Eick et al., 2020). Thus, study design requires considering the required sample size of the markers to be measured in advance, and having participants fill the DBS circles as fully as possible. Another consideration is which marker to choose. If the goal is simply to measure the extent of bone formation and/or resorption, then it is reasonable to choose a marker based on factors such as cost and sample size. Specific markers allow hypothesis testing about particular aspects of bone formation or resorption. For example, if the hypothesis is that a given stimulus will increase or decrease mineral deposition, then OC (which reflects mineralization) would be more informative than BSAP (osteoblast activity) or PINP (collagen deposition). In general, given the complexity of bone physiology and the diversity of environmental factors that influence it, measuring several markers when possible will likely help us understand how particular environmental factors influence specific aspects of bone density and morphology.

Along with these challenges, BTMs offer new opportunities to expand research in biological anthropology as well as in public health. Two key areas are the global burdens of osteoporosis and osteoarthritis. Osteoporosis is exacerbated by the reduction in estrogen at menopause, which leads to increased osteoclast activity and rapid, significant bone loss. In industrialized societies, bone loss begins in perimenopause (-1.5-2% per year after age 40) and increases to 3% per year after menopause (Crandall et al., 2013). In men, bone mass declines more slowly, paralleling the gradual age-related decrease in testosterone levels seen in some populations (Alswat, 2017). The consequences of bone loss are significant: in industrialized populations, the lifetime risk of osteoporotic fracture is about 50% for women and 20% for men (Kanis et al., 2000). Hip fractures cause substantial morbidity, and the mortality rate is 20-25% in the first year and remains elevated for up to 5 years (Leibson, Tosteson, Gabriel, Ransom, & Melton, 2002). Osteoporosis is on the rise not only in wealthy nations, but also in traditional populations

transitioning to market economies, and the burden will only increase with the growing worldwide population of aging adults. As noted above, the vast majority of current data comes from wealthy nations, so population differences in the relationships among BMD, fracture, and physical activity level, risk factors and disease progression, and genetic predisposition are largely unknown (Madimenos, 2015).

Osteoarthritis is also common and increasing in humans, affecting an estimated 14% of US adults (Cisternas et al., 2016). The hallmark of this disease is progressive degeneration of articular cartilage, leading to painful bone-bone contact between adjoining joint surfaces (D. Chen et al., 2017), particularly in the hip, knee, and hands (Lawrence et al., 2008). Osteoarthritis is commonly attributed to age-related wear and tear and injuries (Berenbaum, Eymard, & Houard, 2013), but more recent studies have hypothesized that inactivity, obesity, and inflammation are more salient risk factors in industrialized populations (Berenbaum, Wallace, Lieberman, & Felson, 2018; Wallace et al., 2019; Wallace et al., 2017). As the incidence of metabolic diseases increases in transitioning economies, more work is needed to understand the prevalence, risk factors, rate of progression, and genetic predisposition to osteoarthritis outside of wealthy nations.

CONNECTING BONE TURNOVER TO BIOLOGICAL MECHANISMS

Perhaps the most exciting aspect of incorporating bone turnover markers into anthropological research is the potential for more precise time resolution. Osteoblasts and osteoclasts respond to stimuli in hours to days, but the resulting skeletal changes are not immediately detectable by imaging. Serial BTM measurements using DBS have the potential to allow more precise tracking of bone formation and resorption in response to changes in energy availability, physical activity level, and disease, and across key life history events such as the adolescent growth spurt, pregnancy and lactation, and the menopausal transition. Evidence from clinical studies in

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humans and from experimental studies in animal models supports the use of BTMs to monitor changes in skeletal activity.

SUBADULT BONE GROWTH

The most critical period for skeletal acquisition is the second decade of life, by the end of which the skeleton has acquired 60-80% of adult bone mineral content; the peripubertal interval is particularly essential, with 25-30% of adult BMC acquired in these 2-3 years alone (Bailey, 1997). Maximizing peak skeletal acquisition in adolescence is critical for reducing future osteoporosis risk, as by one estimate, increasing peak bone mass in young adulthood by 10% would reduce the risk of osteoporotic fracture during skeletal aging by 50% (Bonjour, Chevalley, Rizzoli, & Ferrari, 2007). Understanding the factors underlying adolescent skeletal acquisition is particularly important given the secular trends in growth and in reproductive maturation. Over recent decades, humans worldwide have become taller while also reaching reproductive maturity earlier. Since longitudinal growth ends shortly after puberty, these trends may have consequences for peak bone mass acquisition.

Many of the key questions during this life history stage relate to energy allocation between the skeleton and other tissues. For example, on an evolutionary timescale, natural selection may shape growth patterns to allow tradeoffs of energy allocation between the brain and the skeleton (Kuzawa et al., 2014), and between somatic growth and reproductive maturation; a longitudinal study of childhood bone acquisition using BTMs could help to delineate these patterns. Within an individual's lifetime, there is evidence of energetic tradeoffs between immune function and subadult growth, and this relationship is moderated by higher body fat (Garcia et al., 2020; Urlacher et al., 2018). BTMs would provide a way to monitor the effects of such external factors on bone health.

METABOLIC DISEASE

BTMs could also be used to investigate how obesity and metabolic dysfunction affect subadult skeletal acquisition. Both the metabolic syndrome and Type 2 diabetes mellitus are increasingly common in subadults in parallel with increases in childhood obesity (Lascar et al., 2018; Weihe & Weihrauch-Bluher, 2019), posing a substantial and growing public health burden (Viner, White, & Christie, 2017). From a skeletal perspective, both Type 1 and Type 2 diabetes are associated with increased fracture risk in adults (Shanbhogue, Mitchell, Rosen, & Bouxsein, 2016; Starup-Linde, Hygum, Harslof, & Langdahl, 2019). There is also evidence for impaired BMD acquisition in subadults with prediabetes (Pollock et al., 2011; Pollock et al., 2010), although the mechanisms are incompletely understood.

In terms of BTMs, in prepubertal children with metabolic syndrome, CTX levels were significantly higher in girls and trended higher in boys, but PINP levels were unchanged compared to controls, suggesting metabolic dysfunction was associated with lower net bone formation (Bilinski et al., 2022). In a recent meta-analysis of BTMs in patients with Type 1 or Type 2 diabetes, there was evidence for higher BSAP levels and lower OC and CTX levels, suggesting lower overall bone turnover in this population (Starup-Linde & Vestergaard, 2016). In contrast, in a mouse model of early onset Type 2 diabetes, PINP levels were lower compared to controls but CTX did not differ (Devlin et al., 2014). Thus one important contribution of BTMs is their ability to distinguish between decreased bone formation and increased bone resorption, both of which might lead to similarly low bone mass. In the above example, although the skeletal effects of diabetes appear similar in humans and in mice, BTMs showed that the underlying biology differs (decreased turnover vs. decreased osteoblast activity), such that mice may not be a good model for humans in this case.

MECHANICAL LOADING AND UNLOADING

In addition to genetically mediated bone growth, mechanotransduction above a bone's strain threshold can lead to higher bone mass (Pearson & Lieberman, 2004; Robling et al., 2019). Potential skeletal responses to mechanical loading include increasing external bone size (periosteal modeling), changing the bone's shape (cross-sectional geometry), increasing bone mineral density, and/or repairing damage without changing size or shape (Haversian remodeling) (Robling et al., 2019). Such responses vary by skeletal location and are not always clearly aligned with the locations of bone strain (Lieberman, Pearson, Polk, Demes, & Crompton, 2003; Wallace et al., 2014). Even more importantly, osteogenic responses to exercise-induced mechanical loading vary across ontogeny, and are greater around puberty than at any other part of life (Elhakeem, Heron, Tobias, & Lawlor, 2020; Jones, Priest, Hayes, Tichenor, & Nagel, 1977). For example, studies of young women playing racquet sports have shown that gains in bone mineral content are higher in girls who start before or around menarche vs. girls who start later (Kannus et al., 1995). High impact and/or atypical mechanical loads tend to be more osteogenic in humans (Kistler-Fischbacher, Weeks, & Beck, 2021a, 2021b; Nikander, Kannus, et al., 2010) and in animal models (Mustafy, Londono, Moldovan, & Villemure, 2019; Wallace, Kwaczala, Judex, Demes, & Carlson, 2013), particularly if broken into multiple bouts of fewer cycles rather than one extended bout (Robling, Hinant, Burr, & Turner, 2002). However, it is difficult to precisely delineate the timecourse and magnitude of osteogenic responses in order to determine which types of mechanical loading are most beneficial.

In older adults, osteogenic responses to mechanical loading are limited, but exercise can maintain or modestly increase bone mass (Nikander, Sievanen, et al., 2010). Given that exercise-induced changes in bone size and shape in older adults are subtle at best, BTMs can more precisely track osteogenic responses to exercise. In a recent meta-analysis, Smith et al. (2021) analyzed studies that measured BTM response to various exercise interventions in

middle-aged and older adults. The complex results showed that changes in BTMs depended not only on exercise intensity and modality (aerobic or resistance), but also on the sex and age of the subjects. Importantly, because the effects of exercise on bone size and shape in adults are subtle, BTMs picked up osteogenic responses at the cellular level that might not have been easily detected via bone imaging.

REPRODUCTION AND ENERGETICS

In pregnancy and lactation, DBS could be used to track the effects of parity, interbirth interval, and lactation on bone density and to relate these dynamics to maternal nutrition and activity level. Clinical and epidemiological studies have demonstrated a role for reproductive factors such as parity and lactation in shaping BMD in women (Bjornerem et al., 2017; Kovacs, 2016). However, findings among industrialized and subsistence populations have often been contradictory, and it is unclear the extent to which the unique reproductive ecology characteristics of wealthy nations (i.e., low fertility, limited breastfeeding, etc.) contribute to these inconsistent findings (Madimenos et al., 2012; Stieglitz et al., 2015). To resolve this issue, comprehensive studies of natural fertility, subsistence populations that include measurement of BMD, markers of bone turnover, and high resolution environmental/lifestyle data are needed. In addition, although it is well known that new fathers experience a decrease in testosterone (Gettler, McDade, Feranil, & Kuzawa, 2011), it is unknown whether there is an associated decrease in BMD, as seen in aging and hypogonadism (Rochira, 2020).

Another exciting potential application of DBS methods is developing a more detailed understanding of how changes in energy availability affect bone turnover. High pathogen load is correlated with lower childhood growth in the Shuar (Urlacher et al., 2018) and lower adult calcaneal BMD in the Tsimane (Stieglitz et al., 2016). However, most existing work in osteoimmunology is from clinical studies in high-income countries, and methodological

limitations have led to a paucity of human biology studies on bone-immune interactions or the effects of environmental factors and life history tradeoffs on this complex system. BTMs in DBS can be integrated into studies of pathogen load, which would help add the skeleton to discussions of ecoimmunology and provide a more holistic perspective on biological normalcy (Wiley, 2021).

Similar analyses could investigate the effects of weanling diarrhea, marasmus, and kwashiorkor on childhood skeletal acquisition. Energy availability is also affected by thermoregulation. Specifically, there is evidence of higher energy expenditure in cold-dwelling humans (Leonard et al., 2014; Levy et al., 2022; Ocobock et al., 2022; Snodgrass, Leonard, Tarskaia, & Schoeller, 2006) as well as an increase in individuals with lower BMD (Harper, Laughlin, & Mazess, 1984; Lazenby, 1997; Mazess & Mather, 1974; Mazess & Mather, 1975; Thompson & Gunness-Hey, 1981). DBS could be used to study the dynamics of cold exposure, temperature homeostasis, and skeletal dynamics.

POSTMENOPAUSAL BONE LOSS

Incorporating BTMs into studies of skeletal aging could improve our understanding of the etiology of osteoporosis, which is increasingly prevalent in wealthy nations (Sambrook & Cooper, 2006). A retrospective analysis of postmenopausal women found that fracture risk was approximately doubled for participants in the highest quartile of bone resorption markers (e.g. CTX) compared to participants in the remaining three quartiles, particularly when combined with low estradiol levels (Garnero, Sornay-Rendu, Claustrat, & Delmas, 2000). However, osteoporotic fractures are rarely seen in subsistence economies, despite these populations exhibiting similar age-related decreases in BMD (Madimenos et al., 2015; but see Stieglitz et al., 2015); longitudinal measurement of BTMs could help resolve this discrepancy.

Muscle loss is increasingly being recognized as a risk factor for impaired balance and falls, which contribute to osteoporotic fracture (Hirschfeld, Kinsella, & Duque, 2017; Yeung et al., 2019). One possibility is that higher physical activity levels in non-industrialized societies help maintain both bone and muscle mass and balance, but there could also be important differences in patterns of bone loss across societies that contribute to this phenomenon. Collection of DBS, particularly combined with calcaneal ultrasound, could be used to increase the amount of data available for non-industrialized populations at low/no risk for osteoporosis, potentially providing important insights into the etiology of osteoporosis in industrialized populations. Furthermore, BTMs could be used to investigate the skeletal effects of changing lifestyles as currently non-industrialized populations become increasingly market integrated.

EVOLUTIONARY TRENDS

Skeletal robusticity has declined steadily from *Homo erectus* to *Homo sapiens*, and has continued to decline within modern humans (Ruff et al., 2015; Ruff, Trinkaus, Walker, & Larsen, 1993). The decline in cortical bone area leads to lower compressive strength, while the decline in *J* (the polar moment of inertia) leads to lower strength in bending and torsion (Robling et al., 2019). Trabecular bone volume fraction has also decreased in recent modern humans compared to earlier modern humans, particularly in agriculturalists compared to foragers (Chirchir et al., 2015; Ryan & Shaw, 2015). These trends are often attributed to decreased mechanical loading (Macintosh, Davies, Pinhasi, & Stock, 2015; Ruff et al., 2015; Shaw & Stock, 2013). However, no population-based studies to date have prospectively tracked bone turnover in response to exercise, particularly in subadults when osteogenic responses to mechanical loading are greatest. Using a combination of accelerometer data to track mechanical loading and DBS to measure bone formation and resorption, it would be possible to measure skeletal responses to exercise over short time intervals, and to see how such responses change depending on factors such as diet, age, hormone levels, and energy

availability. Such studies would also provide insights into how the body's energetic constraints shape energy allocation to the skeleton versus other tissues, even in contemporary food environments in wealthy nations in which calorie availability is typically not a limiting factor (Pontzer, 2018; Pontzer et al., 2016). This approach would help to clarify the factors underlying gracilization and allow more accurate hypothesis testing about evolutionary trends in bone strength.

CONCLUSION

The skeleton is a critical data source for testing hypotheses in human biology, but incorporating bone imaging into studies of living humans is challenging due to cost and radiation exposure. Bone turnover markers obtained from validated assays conducted on DBS samples are a practical alternative, offering a minimally invasive, field-friendly technique to obtain quantitative measurements of bone formation and resorption at the cellular level. Integrating bone turnover markers with field-friendly measures of bone strength such as calcaneal ultrasonography will allow more robust studies of bone gain and loss in response to energy availability, exercise, disease burden, reproductive status, and aging. Further, it is our hope that this review will prompt researchers to apply available BTMs to research in biological anthropology and also to validate new DBS BTMs for use in future studies. These insights will not only address key questions in human biology but also provide valuable data for clinical and public health initiatives to maintain lifelong skeletal strength.

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2019. We thank the other presenters in the session and two anonymous reviewers of the current version for useful feedback.

AUTHOR CONTRIBUTIONS

Maureen J. Devlin: Conceptualization (equal); writing-original draft (lead); writing-review and editing (equal). **Geeta N. Eick**: Conceptualization (equal); writing-review and editing (supporting). **J. Josh Snodgrass**: Conceptualization (equal); writing-original draft (supporting); writing-review and editing (equal).

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this research.

ETHICS STATEMENT

Not applicable

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Table 1. Key bone turnover markers

Bone formation	Type	DBS available?	References
Osteocalcin	Bone matrix protein	Yes	(Hauschka et al., 1989; A. J. Lee et al., 2000)
PINP (N-terminal propeptide of type I collagen)	Collagen deposition marker	No	(Garnero et al., 2008; Gillett et al., 2021)
Bone-specific alkaline phosphatase	Osteoblast enzyme	No	(Singer et al., 2014)
Bone formation	**Type**		**References**
TRACP5b (tartrate-resistant acid phosphatase 5b)	Osteoclast enzyme	Yes	(Halleen & Ranta, 2001; Halleen et al., 2002; Hannon et al., 2004)
CTX (C-terminal telopeptide of type I Collagen) and NTX (N-terminal telopeptide of type I Collagen)	Collagen breakdown markers	No	(Greenblatt et al., 2017)
Pyridinoline (PYD) and Deoxypyridinoline (DPD)	Collagen crosslinks	No	(Ross & Knowlton, 1998)

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