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Validation of an enzyme-linked immunoassay assay for osteocalcin, a marker of bone formation, in dried blood spots

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Abstract

Objectives: Investigating factors that contribute to bone loss and accretion across populations in remote settings is challenging, particularly where diagnostic tools are scarce. To mitigate this challenge, we describe validation of a commercial ELISA assay to measure osteocalcin, a biomarker of bone formation, from dried blood spots (DBS).

Methods: We validated the Osteocalcin Human SimpleStep ELISA kit from Abcam (ab1951214) using 158 matched plasma and DBS samples. Passing-Bablok regression analysis assessed the relationships between plasma and DBS osteocalcin concentrations. Dilutional linearity and spike and recovery experiments determined if the DBS matrix interfered with osteocalcin measurement, and intra- and inter-assay coefficients of variation (CVs) were calculated. Limit of detection, analyte stability, and specific forms of osteocalcin measured by the kit were also investigated.

Results: Mean plasma osteocalcin value was 218.2 ng/mL (range 64.6-618.1 ng/mL). Linear relationships existed between plasma and DBS concentrations of osteocalcin, with no apparent bias in plasma vs DBS concentrations. There was no apparent interference of the DBS matrix with measurement of osteocalcin in DBS. Intra-assay CV for DBS was ~8%, while average inter-assay CV was 14.8%. Limit of detection was 0.34 ng/mL. Osteocalcin concentrations were stable in DBS stored at −28°C and room temperature, but not those stored at 37°C. This ELISA kit detects total osteocalcin.

Conclusions: Osteocalcin, a bone formation biomarker, can be measured from DBS. Combined with a previously validated DBS assay for TRACP-5b, a bone resorption biomarker, these assays have the potential to help researchers disentangle the many factors contributing to bone strength.

1 | INTRODUCTION

Bone is a highly vascularized, metabolically active, and dynamic organ that undergoes processes of formation and resorption throughout the lifespan. This remodeling ability involves the resorption of older microdamaged bone by osteoclasts and deposition of new bone tissue by osteoblasts. During typical subadult growth, bone formation outpaces resorption whereas throughout adulthood and particularly during skeletal aging, disproportionately

greater osteoclast activity relative to osteoblast activity may occur, contributing to compromised bone mineral density (BMD; grams of mineral per area or volume) and bone quality (a collective term that includes micro-architecture, bone turnover, damage accumulation, and mineralization) (Bartl & Frisch, 2009; Grynpas, 2003). Severely compromised bone strength, due to low BMD and microarchitectural deterioration of bone tissue, may be clinically defined as osteoporosis (Kanis et al., 2008; Singh, Kumar, & Lal, 2015), a debilitating, systemic condition associated with increased risk of fractures, disability, and chronic pain (Siris et al., 2014).

Many endogenous and exogenous factors contribute to bone quality and thus bone strength, including those widely recognized in chronic disease etiology more broadly: nutrition, hormonal status, physical activity level, genetics, and reproductive patterns, to name a few (Madimenos, 2015). For anthropologists investigating how heterogeneous ecological conditions shape human variability in bone accretion and loss within developmental stages across the life course, unraveling the complex interaction of influencing factors is a critical, albeit daunting task (Agarwal & Glencross, 2010; Madimenos, Liebert, Cepon-Robins, Snodgrass, & Sugiyama, 2015). Furthermore, for field researchers working with small-scale, remote populations, high cost, and infrastructural limitations preclude the availability of "gold-standard" bone imaging instruments like dual-energy x-ray absorptiometry (DEXA) that would otherwise be preferable for addressing these research questions. This lack of access largely explains the paucity of data available from non-industrialized, non-Western populations, although this is starting to be remedied. Portable quantitative ultrasound devices have facilitated research into the effects of developmental and lifestyle factors on BMD, specifically among subsistence-based populations like the Shuar forager-horticulturalists of Amazonian Ecuador (Madimenos, Snodgrass, Liebert, Cepon, & Sugiyama, 2012), rural nonindigenous Colonos of Ecuador (Madimenos et al., 2011; Madimenos et al., 2012), and the forager-horticulturalist Tsimane of Bolivia (Stieglitz et al., 2015; Stieglitz, Trumble, Kaplan, & Gurven, 2017). Despite the insights these studies have provided (Madimenos et al., 2020), BMD is simply one surrogate measure of bone strength and moreover, reflects the combined contributions of formation and resorption processes. Disentangling these distinct processes outside clinical settings is invaluable for enhancing current understanding of the relative contributions and interactions of life-stage and lifestyle factors to bone health more broadly (Agarwal & Glencross, 2010).

Measures of bone resorption and formation (ie, bone turnover) through biomarker analyses offer an opportunity to extricate the processes contributing to bone strength. Serum biomarkers of bone resorption include collagen degradation products such as hydroxyproline, cross-linked telopeptides of type I collagen, and osteoclast enzymes such as tartrate-resistant acid phosphatase 5b (TRACP-5b). Total alkaline phosphatase, bone alkaline phosphatase, and osteocalcin are protein biomarkers that have been used as markers of bone formation (Eastell & Hannon, 2008). Recently, we validated a dried blood spot (DBS)-based assay of one marker of bone resorption, namely TRACP-5b (Eick et al., 2019); however, until now, no validated assay for measuring a marker of bone formation in DBS samples has been reported. Because DBS assays facilitate measurement of biomarkers from populations in remote, field-based settings often characterized by limited diagnostic equipment, the development of such an assay has both clinical and non-clinical (eg, anthropological) value.

Osteocalcin, which is synthesized by osteoblasts, odontoblasts, and hypertrophic chondrocytes, is the major noncollagenous protein found in the bone matrix (Kuo & Chen, 2017); circulating osteocalcin represents the total fraction of osteocalcin not bound to the hydroxyapatite matrix of bone. Intact osteocalcin is a 49 amino acid molecule with three glutamic acid residues (amino acid positions 17, 21, 24) that can be carboxylated by γ-glutayml carboxylase using vitamin K as a co-factor. Osteocalcin, with all three of the glutamic acid residues carboxylated, is referred to as carboxylated osteocalcin (cOC), vs the uncarboxylated (no γ-carboxyglutamic acid residues) or undercarboxylated forms (1 or 2 γ-carboxyglutamic acid residues). Total osteocalcin (tOC) is the sum of the carboxylated and uncarboxylated forms of osteocalcin (Lee, Hodges, & Eastell, 2000). The carboxylated form of osteocalcin has a high affinity for calcium and hydroxyapatite, and this is the major form involved in bone formation. In contrast, the uncarboxylated and undercarboxylated forms appear to act as hormones involved in glucose metabolism (Booth, Centi, Smith, & Gundberg, 2013; Zoch, Clemens, & Riddle, 2016). Serum concentrations of tOC are generally proportional to osteoblast activity, and thus, tOC is an established proxy measure of bone formation (Booth et al., 2013; Zoch et al., 2016).

In addition to variation in the extent of carboxylation of osteocalcin, five different fragments of osteocalcin may be circulating in addition to the intact fragment: an N-terminal fragment (aa 1-19), mid fragment (aa 20-43), and C-terminal fragment (aa 44-49), as well as an N-terminal mid form (aa 1-43) and a mid-C terminal form (aa 20-49). The intact 49-aa molecule and N-terminal mid fragment are the most abundant circulating forms (Garnero, Grimaux, Seguin, & Delmas, 1994), and are the forms targeted by most currently available osteocalcin ELISA assays.

Clinically, osteocalcin levels are used to monitor bone turnover in metabolic diseases such as osteoporosis and growth hormone deficiency (Brown et al., 1984; Lee et al., 2000). High serum osteocalcin levels are a marker of either rapid bone formation, as seen in adolescence, or increased bone turnover, which results in a decrease in mineralization and therefore binding substrate for osteocalcin, as seen in osteoporosis (Boivin, Chavassieux, Santora, Yates, & Meunier, 2000; Szulc & Bauer, 2013). Low osteocalcin levels are associated with decreased bone turnover (Brown et al., 1984; Szulc & Bauer, 2013). In clinical practice, osteocalcin levels are measured in serum obtained from venous blood draws; however, while collection and processing of venous blood is feasible under some field circumstances (Gurven et al., 2017), the logistical, shipping, and biosafety advantages as well as low cost and analyte stability afforded by use of DBS have made them the biosampling method of choice for many biological anthropologists working with remote or underserved populations.

DBS cards are created by collecting a small amount (40-60 μL) of fingerprick blood on filter paper cards specifically manufactured for this purpose. After collection of blood drops, the cards are allowed to air-dry for 3 to 4 hours, and can then be packaged in a resealable storage bag with desiccant packets. It may then be possible to ship the DBS samples to their destination at ambient temperatures, as some biomarkers have been found to be very stable in DBS. Alternatively, the samples can be frozen for less stable biomarkers. When frozen at −20°C to −80°C in frost-free freezers, they can remain viable for years, although there is variability across different biomarkers of interest.

Here we describe the systematic validation of a commercially available ELISA to measure levels of osteocalcin in DBS.

2 | METHODS

2.1 | ELISA kit

We validated Abcam's Osteocalcin Human SimpleStep ELISA kit for use with DBS (ab195214). This ELISA kit detects intact osteocalcin and was developed for quantitative measurement of osteocalcin in human serum, plasma, and serum-free cell culture supernatants. The manufacturer-reported sensitivity of this kit is 0.1 ng/mL with a measurement range of 0.16 to 10 ng/mL osteocalcin. Preliminary experiments revealed that a single 3-mm diameter punch from a DBS eluted in 100 μL of diluent provided with the kit provided sufficient signal (data not shown).

2.2 | Validation samples

One hundred fifty-eight matched fingerprick DBS (fDBS), venous blood DBS (vDBS), and plasma samples collected from a convenience sample of adults in Eugene/Springfield, Oregon between November 2014 and February 2015 were used to validate the osteocalcin ELISA following an established protocol (Eick, Kowal, Barrett, Thiele, & Snodgrass, 2017). Ninety-one participants were female, with an age range of 18 to 72 years, median age of 26 years, and average age of 31.6 years. Age range of the 67 male participants was 18 to 80 years, median age was 28 years, and mean age was 31.6 years, indicating no major skew in age between male and female participants. All samples were collected between 5 PM and 9 PM in the evening. This collection was approved by the Committee for the Protection of Human Subjects, University of Oregon (#7062016.007) and all participants provided informed consent.

2.3 | Measurement of osteocalcin in E2V2 samples

Osteocalcin level was assessed in 158 plasma samples stored at −80°C (M:F 67:91). Plasma samples were diluted 1/100. A histogram was plotted of these plasma concentrations using Graphpad Prism software. Osteocalcin concentration ranged from 64.6 to 69.1 ng/mL in these 158 samples, with a median value of 196.6 ng/mL and mean value of 218.2 ng/mL. Fifty individuals with plasma osteocalcin values spanning the range of concentrations measured in the 158 samples (64.6-619.1 ng/mL) were identified. Levels of osteocalcin were then measured in matched fDBS and vDBS samples from these 50 individuals (M:F 16:34).

2.4 | Plasma/DBS comparisons

To assess if plasma, fDBS, and vDBS osteocalcin values were normally distributed, the D'Agostino and Pearson omnibus normality test was used. While the fDBS and vDBS values were normally distributed, the plasma values were not, therefore Passing-Bablok and Bland-Altman comparisons were done after $\log_{10}$ transforming the osteocalcin values, as this normalized the distributions of all osteocalcin values. Passing-Bablok regression analysis was used to assess relationships between osteocalcin values of the matched samples, as this analysis assumes uncertainty in both sets of values being compared. Bias was evaluated by Bland-Altman analyses (Bland & Altman, 1986). DBS values were converted into plasma-equivalent

values using the Passing-Bablok regression equations, as DBS dilution values cannot be determined (amount of plasma in one 3-mm punch will differ from sample to sample depending on the hematocrit and size of the dried blood spot in addition to location of DBS from which the spot was punched). The difference between plasma-equivalent fDBS or vDBS values and plasma values was plotted against the average of these values, and Bland-Altman plots were examined for bias and the number of samples that fell outside the 95% confidence intervals.

2.5 | Linearity of dilution

To assess the dilutional linearity of this assay for DBS samples, six vDBS samples with high osteocalcin concentrations were serially diluted 1:2, 1:4, 1:8, and 1:16 and the values obtained for the diluted samples (multiplied by the dilution factor) were compared to those measured for the undiluted sample.

2.6 | Spike and recovery

To assess if the DBS sample matrix contained any factors that interfere with detection of osteocalcin levels, we performed a spike and recovery experiment using two DBS samples with low intrinsic osteocalcin concentrations. In more detail, six punches from vDBS samples of two individuals were eluted in 700 μL assay buffer, and then the same volume of diluted osteocalcin standard provided with the ELISA kit was added to three 150 μL aliquots of eluted sample to achieve final osteocalcin concentrations of 5, 2.5, and 1.25 ng/mL. Sample diluent tubes with the same amounts of osteocalcin added as were added to the DBS eluates served as comparators to assess percentage recovery.

2.7 | Precision: Intra- and inter-assay coefficients of variation

To determine intra-assay variation, duplicate punches from all 50 vDBS and 50 fDBS samples were each eluted in 100 μL sample diluent, 50 μL of each duplicate eluate was loaded in a well, and then the coefficient of variation (CV) for each duplicate set of wells was calculated. To calculate inter-assay variation, two duplicate punches from a high osteocalcin vDBS sample and low osteocalcin vDBS sample were run on each plate ($n = 6$) and the coefficient of variation of the mean value of the high and low controls on each plate was calculated.

2.8 | Limit of detection

The limit of detection of the assay in our laboratory was determined by calculating the mean of 28 wells containing the zero standards (assay buffer only), adding two SD to this value, and then extrapolating the corresponding concentration of this from the four-parameter logistic curve for that plate.

2.9 | Analyte stability

Separate 3-mm punches taken from six vDBS samples from different individuals were stored at −28°C, room temperature, and 37°C for 2, 7, 14, and 28 days to determine the stability of osteocalcin concentrations to various storage conditions. Osteocalcin levels in these samples were expressed as a percentage of the osteocalcin concentration in matched samples stored at −80°C that were thawed only for this assay. To assess the impact of the number of freeze-thaw cycles on osteocalcin concentrations, vDBS samples from the same six individuals were allowed to thaw at room temperature for 2 hours 2, 4, 8, or 12 times and were refrozen after each thawing session at −28°C. All samples were thawed a final time for the assay for a total number of freeze-thaw cycles of 3, 5, 9, and 13, respectively. Osteocalcin levels in these samples were expressed as a percentage of the osteocalcin concentration in matched samples stored at −80°C that were thawed only once.

2.10 | Form of osteocalcin measured

To assess the specificity of the ELISA kit for carboxylated osteocalcin (cOC), we diluted cOC from Anaspec (cat. # AS-22829; [Gla17,21,24]-Osteocalcin (1-49)) to the same concentrations as used for the osteocalcin standard provided with the kit. If the antibodies used in the kit were specific for cOC, we expected the values obtained for the Anaspec dilutions to be roughly similar to those of the standard. Lower values for the Anaspec standard dilutions than the kit standard dilutions would indicate that the antibodies provided with the kit detect both carboxylated and un(der) carboxylated forms of osteocalcin, that is, total osteocalcin.

3 | RESULTS

3.1 | Measurement of osteocalcin in E2V2 samples

Among 158 individuals for whom plasma osteocalcin values were measured, the mean plasma osteocalcin

concentration was 218.2 ng/mL, the median osteocalcin concentration was 196.6 ng/mL, and osteocalcin values ranged from 64.6 to 619.1 ng/mL. Osteocalcin plasma values were not significantly different between males and females (Mann Whitney *U* test, $P = .06$, two-tailed). Due to the temporary increase in osteocalcin levels documented among females in their sixth decade and the generally higher osteocalcin values in postmenopausal women (Vanderschueren, Gevers, Raymaekers, Devos, & Dequeker, 1990), an additional analysis excluded females ≥50 years. In a comparison of males vs females <50 years, osteocalcin values were significantly higher in males (Mann Whitney *U* test, $P = .02$, two-tailed). In addition, osteocalcin plasma values were negatively correlated with age in males (n = 67, Spearman's correlation coefficient = −0.55, $P < .01$) but not in females (n = 91, Spearman's correlation coefficient = −0.15, $P = .16$). However, when only females <50 years were analyzed, osteocalcin levels were significantly inversely related to age (n = 79, Spearman's correlation coefficient = −0.34, $P < .01$). fDBS values (n = 50) ranged from 0.43 to 6.03 ng/mL, with mean and median values of 2.53 and 2.49 ng/mL, respectively. Venous DBS (vDBS) values (n = 49 after excluding one sample with an osteocalcin concentration below the limit of detection of the assay) had osteocalcin values ranging from 0.36 to 4.25 ng/mL, with mean and median values of 1.92 and 1.85, respectively.

3.2 | Plasma/DBS comparisons

There was a linear relationship between all pairwise comparisons of matched sample types (Figure 1; Passing Bablok regression equations reported on each graph). There was no obvious pattern of bias for the fDBS and vDBS vs plasma comparisons (Figure 2A,B), indicating that plasma osteocalcin levels are not systematically different from fingerprick osteocalcin values. There did appear to be a bias in the plot of fDBS vs vDBS values, with fDBS values generally higher than those of the matched vDBS sample (Figure 2C). However, when the vDBS and fDBS values were converted to their plasma-equivalent values and compared, no obvious bias was observed in the Bland-Altman plot (Figure 2D). It is unclear what to attribute the higher fDBS than vDBS values to; we previously observed slightly higher levels of TRACP-5b in vDBS than fDBS, and attributed this to the generally smaller diameters of the fDBS than vDBS and correspondingly lower analyte concentrations (Eick et al., 2017; George & Moat, 2016), but we observed the opposite pattern here.

3.3 | Linearity of dilution

Dilutional linearity results are presented in Table 1. None of the 1:16 dilution values is shown as these were below the limit of detection of the assay, while two of the 1:8 diluted samples also had values below the limits of detection. Recovery ranged from 85.6% to 116.3%, with an average recovery of 99.3%, indicating dilutional linearity of this assay for DBS in the dilution range of 1:2 to 1:4, and possibly as high as 1:8 in some participants with higher concentrations.

3.4 | Spike and recovery

Recovery values after spiking ranged from 96% to 102% (Table 2), indicating excellent recovery of osteocalcin in the sample and the absence of interfering factors.

3.5 | Precision: Intra- and inter-assay coefficients of variation

The CV for duplicate punches from 50 fDBS samples was 8.25%, and for duplicate punches from 50 vDBS punches

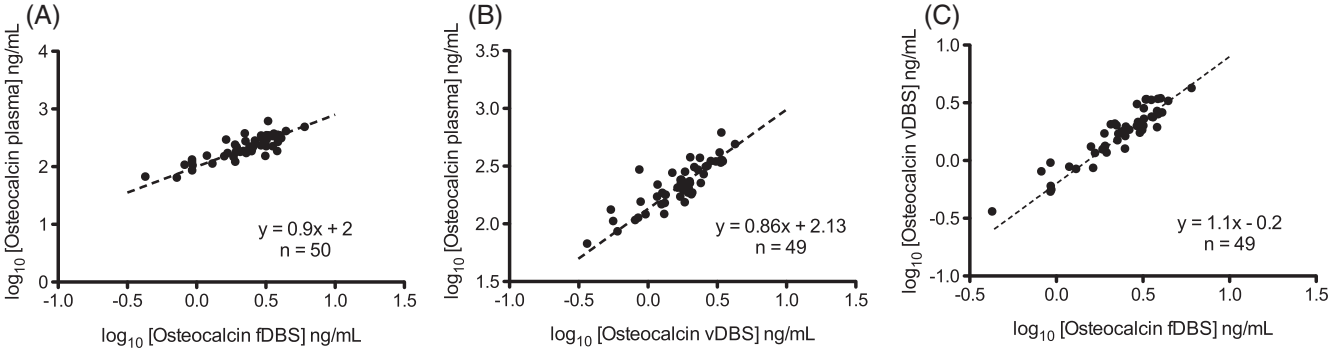

FIGURE 1 Passing-Bablok plots showing the relationship between matched, A, fingerprick DBS (fDBS) and plasma osteocalcin levels, B, venous DBS (vDBS) and plasma osteocalcin levels, and, C, fDBS and vDBS osteocalcin levels. The number of samples analyzed and the Passing-Bablok regression equations are indicated. Note all osteocalcin values were $\log_{10}$-transformed prior to analyses

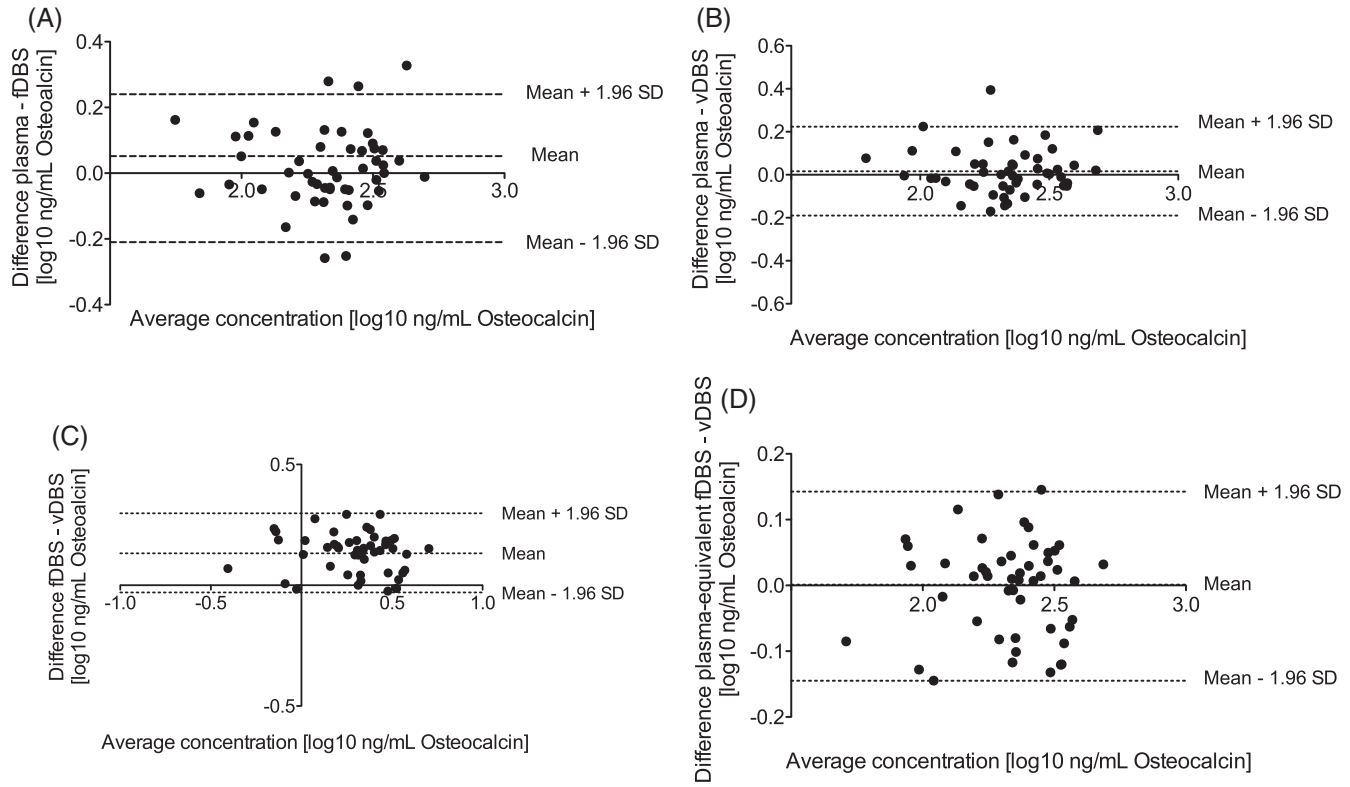

FIGURE 2 Bland–Altman plots of the difference in, A, plasma and plasma-converted fingerprick DBS (fDBS) osteocalcin values vs the average of these values, B, plasma and plasma-converted venous DBS (vDBS) osteocalcin values vs the average of these values, C, unconverted fDBS and vDBS osteocalcin values vs the average of these values, and, D, plasma-converted fDBS and vDBS values vs the average of these values. Note all osteocalcin values were $\log_{10}$-transformed prior to analyses

TABLE 1 Dilutional linearity of Abcam's osteocalcin ELISA (ab195214) for dried blood spots

		Dilution factor			
DBS sample		**Undiluted**	**2**	**4**	**8**
1	ng/mL	3.18	1.68	0.77	0.46
	% Expected value	**100.00**	**105.45**	**97.32**	**116.34**
2	ng/mL	3.09	1.61	0.76	nd
	% Expected value	**100.00**	**103.95**	**98.38**	nd
3	ng/mL	3.01	1.56	0.83	0.34
	% Expected value	**100.00**	**103.29**	**110.40**	**89.27**
4	ng/mL	2.96	1.35	0.80	< LOD
	% Expected value	**100.00**	**91.11**	**107.68**	-
5	ng/mL	2.67	1.14	0.64	< LOD
	% Expected value	**100.00**	**85.58**	**96.22**	-
6	ng/mL	3.17	1.44	0.80	0.37
	% Expected value	**100.00**	**90.75**	**100.47**	**93.15**

Note: < LOD indicates a value under the limit of detection (0.34 ng/mL) of the assay. Percentages are indicated in bold.
Abbreviation: nd, not determined.

was 8.40%. Note that this value encompasses both well-to-well variation and position-based differences in analyte concentrations in a dried blood spot. The inter-assay CV for one high and one low DBS control was 14.8% (14.6% for the high control and 15.0% for the low control). Note that this reflects inter-assay variability, position-based differences in analyte concentrations in DBS, and in addition, variation in the number of freeze-thaw cycles the DBS

TABLE 2 Spike & recovery results of Abcam's osteocalcin ELISA (ab195214) for dried blood spots

	Measured concentrations (ng/mL)			
	Neat	**+ 5 ng/mL OC**	**+2.5 ng/mL OC**	**+1.25 ng/mL OC**
DBS sample 1	0.34	4.979	2.736	1.511
Expected		4.95	2.848	1.519
Observed/expected (%)		**101%**	**96%**	**99%**
DBS sample 2	0.425	4.94	2.815	1.643
Expected		5.035	2.933	1.604
Observed/expected (%)		**98%**	**96%**	**102%**

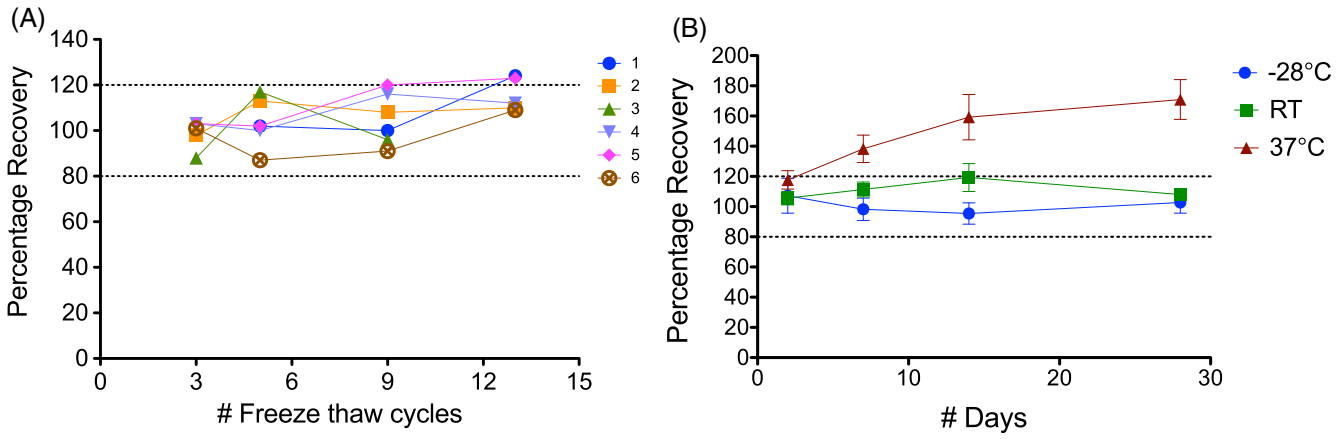

FIGURE 3 A, Stability of osteocalcin concentrations in response to different storage temperatures. Venous DBS (vDBS) samples from six individuals were stored at −28°C, at room temperature (RT, ~22°C), and at 37°C for 28 days. On day 2, 7, 14, and 28 of storage, samples were transferred to −80°C. Osteocalcin concentrations in these samples were compared to those in samples stored at −80°C after collection and thawed only once for this experiment. The average percentage recovery for all six samples at the various timepoints for the three different storage temperatures is shown. Error bars are standard errors of the mean. Dotted lines indicate 80% and 120% recovery. B, Stability of osteocalcin concentrations in response to different numbers of freeze-thaw cycles. vDBS samples from six individuals were stored at −28°C and then allowed to thaw at RT for 2 hr 2, 4, 8, or 12 times (each on separate days) after which they were returned to the −28°C freezer. These samples were then thawed one final time to assay osteocalcin concentrations, for a total number of freeze-thaw cycles of 3, 5, 8, 9, and 13, respectively. Osteocalcin concentrations in these samples were compared to those in samples stored at −80°C after collected and thawed only once for this experiment. Dotted lines indicate 80% and 120% recovery

cards were subjected to (the same DBS cards were used for all plates). For comparison, intra- and inter-assay CVs for the liquid standards were also calculated, and were 4.8% and 5.1%, respectively.

3.6 | Limit of detection

The limit of detection of this assay in our laboratory was 0.34 ng/mL, which was slightly higher than that reported by the manufacturer (0.10 ng/mL), as expected.

3.7 | Analyte stability

Osteocalcin concentrations appeared to be fairly stable in samples stored at −28°C and at room temperature for up to 28 days, with percentage recovery between 80% and 120% (Figure 3A). In contrast, a steady increase in osteocalcin level was seen in all samples stored at 37°C for longer than 2 days, implying degradation of osteocalcin into fragments immunoreactive with the antibodies provided with the ELISA kit. The percentage recovery after 3 to 13 freeze-thaw cycles ranged from 87% to 124%. No clear trend was apparent in all samples other than an apparent increase in osteocalcin concentration in all five samples available for this time point after 13 freeze thaw cycles relative to the matching samples that had only undergone a single thaw cycle (Figure 3B).

3.8 | Form of osteocalcin measured

Osteocalcin values for the Anaspec standard were roughly 18% of those of the osteocalcin standard provided with the kit, indicating that this kit measures total osteocalcin (both carboxylated and uncarboxylated forms).

4 | DISCUSSION

Here we demonstrated here that a commercial ELISA developed to measure osteocalcin concentrations in plasma, serum, and cell culture supernatant can be used to measure osteocalcin concentrations in DBS based on a systematic investigation using matched plasma, fDBS, and vDBS samples. Levels of osteocalcin ranged widely among individuals from 0.43 to 6.03 ng/mL in fDBS and 64.6 to 619.1 ng/mL in plasma. Previous studies have reported age- and sex-related differences in osteocalcin levels (Atalay, Elci, Kayadibi, Onder, & Aka, 2012; Hannemann et al., 2013; Vanderschueren et al., 1990). Consistent with these previous studies, we found an inverse relationship between osteocalcin concentration and age in all males and females in their second to fifth decades of life (Bao et al., 2013; Napoli et al., 2013; Vanderschueren et al., 1990). The lack of a significant relationship between osteocalcin concentration and age in our entire female sample was likely because osteocalcin concentrations have been shown to be higher in postmenopausal than premenopausal women (Atalay et al., 2012) and to increase temporarily in females in their sixth decade of life (Vanderschueren et al., 1990); when we removed females 50 years and older from our analysis, the expected inverse relationship with age was observed. Osteocalcin levels were slightly higher in males than females (mean: 229.3 vs 208.4 ng/mL, respectively) but this difference was not statistically significant. This is likely due to the inclusion of females in their sixth decade of life in the male/female comparison as these older females tend to have higher osteocalcin levels; when females 50 years or older were removed from this comparison, plasma osteocalcin values were significantly higher in males than females, as documented in previous studies (Del Pino et al., 1991; Napoli et al., 2013; Vanderschueren et al., 1990). The insert for the Abcam kit reports (graphically) a range of serum osteocalcin values for 10 male donors of 135 to 280 ng/mL, with an average value of 268.5 ng/mL. This is similar to the range of osteocalcin values measured in the plasma samples or our male participants (mean: 228.3 ng/mL, range: 105.9-490.8 ng/mL, n = 67). Comparing these values with reference intervals, however, is not appropriate because of the variability of platforms and reagents used to measure osteocalcin, differences in what forms of circulating osteocalcin are recognized by different antibodies, and the lack of certified reference standard material for calibration. Nevertheless, our results affirm that osteocalcin levels measured using this kit will be internally consistent.

The linear relationship between plasma and DBS osteocalcin concentrations, and the negligible bias in plasma-converted DBS vs plasma osteocalcin levels indicate that there is no systematic difference between osteocalcin measurements from fingerprick blood and/or that spotted on DBS cards vs plasma obtained from whole blood. Note, however, that although there was a linear relationship between most DBS and plasma samples, not all points fell exactly on the Passing-Bablok regression lines, suggesting some variability in DBS vs plasma concentrations of osteocalcin. The DBS matrix does not interfere with detection of osteocalcin as indicated by the linearity of dilution and spike and recovery results. In an earlier study, absence of dilutional linearity for serum samples (recovery values ranging from 200% to 700% for 1:8 dilution of serum) was found for several osteocalcin assays due to the presence of circulating osteocalcin fragments (Colford, Sailer, & Langman, 1997). In the current study, the variation in osteocalcin concentration between punches from duplicate blood spots run in wells on the same plate of around 8% was higher than that measured for plasma samples, but this result is reasonable given that this value encompasses well-well variation in the osteocalcin concentration in addition to position-based differences in analyte concentrations within a DBS. The inter-assay CV of 14.8% for high and low DBS controls is at the upper end of the range (< 15%) recommended by several ELISA manufacturers (https://docs.abcam.com/pdf/kits/elisa-guide.pdf, http://www.enzolifesciences.com/science-center/technotes/2018/january/cv-in-elisa-how-to-reduce-them-and-why-they-re-important/); as mentioned previously, we attribute this to the additional variability contributed by punching from different locations of the same DBS or punching from a different DBS due to differences in the distribution of analytes in DBS. Taken together with the low inter-assay CV obtained for liquid standards of 5.1%, we consider the reproducibility of this assay to be acceptable. Very few of the DBS we assayed had osteocalcin levels below the level of detection we measured for this assay (0.34 ng/mL), indicating that it is feasible to use it for population-level assays of osteocalcin from DBS, at least in the population our samples were drawn from (predominantly European ancestry). Consistent with some other DBS analytes (eg, TRACP-5b [Eick et al., 2019], immunoreactive trypsinogen [Li et al., 2006], ferritin and the transferrin receptor [Cook, Flowers, & Skikne, 1998]), osteocalcin levels were fairly stable at −28°C and at room temperature for up to 28 days, but appeared to degrade at 37°C to produce fragments that were immunoreactive with the antibodies used by the manufacturer (ie, there was an apparent increase in osteocalcin levels over time at this elevated temperature). Osteocalcin levels also tended to increase with the number of freeze-thaw cycles, but with variation among samples. After 13 freeze-thaw cycles, all samples measured had a higher osteocalcin concentration than at baseline, suggesting that it would be best to minimize the number of freeze-thaw

cycles the DBS cards are exposed to prior to assaying osteocalcin concentration.

The apparent stability of osteocalcin in DBS at room temperature is noteworthy, as osteocalcin has been shown to be unstable in vitro both at room temperature and at 4°C (Banfi & Daverio, 1994; Garnero et al., 1994). This suggests that the filter paper matrix protected against degradation, as has been demonstrated previously for other analytes such as TRACP-5b, hydrolases, and esterases (Eick et al., 2017; Freeman et al., 2018). Finally, the results we obtained for the carboxylated osteocalcin standard from Anaspec suggest that the Abcam assay targets both carboxylated and undercarboxylated forms of full-length osteocalcin (ie, total osteocalcin; no information is provided by the kit manufacturer regarding the specificity of the kit for carboxylated or uncarboxlyated osteocalcin), indicating that the osteocalcin values obtained using this kit can be used as a biomarker of bone formation.

This validation study has several limitations. First, osteocalcin levels in plasma samples were assayed using the same Abcam kit used to validate the DBS samples, rather than using a reference measurement. However, no gold standard method has yet been established for the measurement of osteocalcin in plasma or serum samples. This is largely due to the heterogeneity in both carboxylation levels of circulating osteocalcin and osteocalcin fragment size. Different assays vary in their ability to recognize the numerous fragments of osteocalcin, and specificity for one fragment vs another is, in many cases, not specified or evaluated by the manufacturer. Furthermore, even in cases where the specificity for a particular osteocalcin fragment or fragments has been reported by the manufacturer, this has been shown not to necessarily be accurate (Colford et al., 1997). Another limitation is that oral contraceptive use, hormone replacement therapy, diabetes mellitus, and body mass index <18 or >30 kg/m^2 can all affect (decrease) osteocalcin levels, but were not accounted for in our analyses. There are also diurnal and seasonal variations in osteocalcin levels, as well as menstrual cycle variations that may have affected the measured concentrations of osteocalcin (Lee et al., 2000). However, concordance between our findings and those reported in previous studies, such as the negative correlation between osteocalcin level and age, suggests that the effects of these factors were negligible, although diurnal variation was somewhat controlled for by collecting all samples between 5 PM and 9 PM A final limitation of this study is our focus on validating a commercially available ELISA kit. Commercially available ELISAs can unfortunately be withdrawn from the market at any time for a variety of reasons, which largely negates the substantial amount of work that may have gone into validating these ELISAs for other types of samples, such as DBS, than the ELISA was developed for. We originally sought to develop an in-house ELISA for osteocalcin, and after several months, we had developed an ELISA that could reliably measure osteocalcin concentrations in plasma samples; however, while these values showed a strong correlation with those measured using a commercially available ELISA, they were uniformly lower, and when this in-house ELISA was applied to DBS, osteocalcin concentrations for almost all samples were below the quantitation limit of the ELISA. We attributed this lack of sensitivity to the fact that antibody pairs with sufficiently high binding affinities for detecting low concentrations of proteins, as are present in DBS, are not readily available or are preferably used by their manufacturers in commercial ELISA kits and are not available for purchase. This highlights just one of the many constraints faced when developing an in-house ELISA.

5 | CONCLUSIONS

The development of DBS assays for both TRACP-5b, a marker of bone resorption, and now osteocalcin, a marker of bone formation, expands the toolkit available for biological anthropologists, particularly those working in remote field settings. These assays have potential to help researchers disentangle the complex parameters related to bone strength across heterogenous populations and pursue a more refined investigation of the factors contributing to and the tradeoffs between bone resorption and formation across and within life-history stages.

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