



Between-subject and within-subject variability in measures of biochemical markers of bone turnover in cynomolgus and rhesus macaques

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ABSTRACT

Development of optimal bone mass during early adulthood is determined by the balance between bone formation and resorption. The utility of minimally invasive biomarkers for monitoring bone turnover balance in maturing non-human primates has received limited attention. This study evaluated the biological variation of osteocalcin (a marker of bone formation), carboxyterminal cross-linking telopeptide of type 1 collagen (CTX, a marker of bone resorption), and the ratio of osteocalcin to CTX (reflecting bone turnover balance), in 136 rhesus and cynomolgus macaques aged 3.8–11.6 years. In a subsample of the animals ($n = 28$), blood samples were collected at monthly intervals over 4 months. Between-subject analysis revealed that there were no sex or species differences for CTX. Osteocalcin and the ratio of osteocalcin to CTX were higher in males than in females, and in rhesus macaques than in cynomolgus macaques. There were no changes in osteocalcin, CTX, or the ratio of osteocalcin to CTX across 4 months for any of the groups. In contrast, there was considerable within-subject variation in osteocalcin and CTX concentrations. However, differences in values exhibited no discernible pattern, suggesting that within-subject variation can be reduced by averaging repeat measurements. In summary, the data provide reference values for male and female rhesus and cynomolgus macaques and support the utility of osteocalcin and CTX as biomarkers to monitor bone turnover at the population level.

1. Introduction

Late adolescence to early adulthood is a key period for the accrual of bone mass. Following the completion of skeletal growth, bone mass continues to accumulate until peak bone mass has been achieved by approximately 30 years of age in humans (Baxter-Jones et al., 2011). Maximizing peak bone mass is important for reducing the long-term risk of fragility fracture and the development of osteoporosis in later life (Bonjour et al., 2009). Peak bone mass is largely determined by genetic factors, but its achievement is influenced by environmental factors, such as exercise, diet, and smoking (Valimaki et al., 1994; Mcguigan et al., 2002).

The skeleton is maintained over time through a cyclic process of osteoclastic bone resorption coupled with osteoblastic bone formation, with formation predominating during periods of growth and bone accrual (Allen and Burr, 2013). Biochemical markers offer a non-

invasive index for evaluating global bone turnover and turnover balance (Shetty et al., 2016). Two commonly used markers include osteocalcin, a marker of bone formation, and carboxyterminal cross-linking telopeptide of type 1 collagen (CTX), a marker of bone resorption (Cabral et al., 2016). Osteocalcin is the most abundant non-collagenous protein in bone matrix and is primarily produced by osteoblasts. It is a well-described marker of bone formation and turnover with a controversial and somewhat contested role as a hormone (Manolagas, 2020). CTX is a degradation product of bone matrix collagen that is released during bone resorption. The ratio of osteocalcin to CTX can be used as an index of bone turnover balance. Bone turnover markers are considered useful for identifying patients at high risk of bone loss and fracture (Leeming et al., 2006), understanding bone remodeling (Cavalier et al., 2016), and monitoring progression and treatment of metabolic bone disorders (Greenblatt et al., 2017).

Non-human primates are an effective large animal model for

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studying bone metabolism due to their phylogenetic and physiologic similarity to humans (Black and Lane, 2002; Phillips et al., 2014); notably, unlike rodents, commonly used as small animal models, non-human primates and humans maintain cortical bone quality through intracortical bone remodeling. This is significant because 80% of the human skeleton is comprised of cortical bone. Two closely related species that are used to model human physiology, including bone physiology, are rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*). Others have measured biochemical markers of bone turnover in both species (Brommage, 2001; Colman et al., 2012; Gaddini et al., 2015; Andersen et al., 2018), and have reported that markers are useful for monitoring bone formation and resorption in the ovariectomized cynomolgus macaque model of osteoporosis (Legrand et al., 2003). However, few studies have focused on the interval preceding the achievement of peak bone mass accrual.

Biochemical markers are subject to considerable biological variation that are not fully understood but include diurnal fluctuations and seasonal changes. An understanding of normal biological variation of bone turnover markers is critical in interpreting significance of changes in these values in individuals. In humans, within-subject variability can be substantial, for example, ranging from 7 to 27% for osteocalcin (Looker et al., 2000). To our knowledge, the normal biological variation of osteocalcin and CTX has not been closely evaluated in late adolescent or young adult macaques.

In this study, we investigated age (late adolescence to adulthood), sex, and species differences in osteocalcin, CTX, and the ratio of osteocalcin to CTX in rhesus and cynomolgus macaques. The goals of this analysis were to 1) establish reference values for the biomarkers, and 2) identify potential differences due to age, sex, and species in levels of the biomarkers. In addition, we evaluated plasma from a subset of animals to determine the biological variation in levels of osteocalcin, CTX, and the ratio of osteocalcin to CTX; specifically, osteocalcin and CTX were sampled monthly over a 4-month interval. The goals of this additional analysis were to 1) characterize within-subject as well as between-subject variability in the biomarkers, and 2) evaluate the utility of the biomarkers in monitoring bone turnover at the individual and population levels.

2. Materials and methods

2.1. Study population

The experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee at the Oregon National Primate Research Center (ONPRC) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study population was comprised of 63 male rhesus macaques (4.0–9.8 years), 17 female rhesus macaques (3.7–6.1 years), 34 male cynomolgus macaques (5.6–6.7 years), and 22 female cynomolgus macaques

(6.4–11.6 years). The animals were pooled from 13 cohorts of monkeys housed at the ONPRC (Beaverton, OR) between 2006 and 2016. The monkeys were enrolled in studies investigating the effects of long-term ethanol self-administration (Daunais et al., 2014). As such, sample size and age range for the current study is constrained by the original study design. Blood samples were collected in all animals at baseline (no ethanol consumption) and from control monkeys (no ethanol consumption) an additional three times approximately every 30 days (N = 11 male and 3 female rhesus macaques and 6 male and 8 female cynomolgus macaques). Monkeys were housed individually under controlled temperature (20–22 °C), humidity (65%), and an 11-h light cycle in a room allowing visual, auditory, and olfactory contact with other monkeys. Diets consisted of 1 g banana-flavored pellets (63% carbohydrate, 4% fat, and 22% protein; PJ Noyes, Lancaster, NH).

2.2. Biomarker measurements

Blood samples were collected from the femoral vein of unfasted monkeys. Plasma was stored at –80 °C until analysis. Osteocalcin and CTX were measured. Assays were performed by the Endocrine Technologies Core at the ONPRC using a Roche Cobas e411 Automated Clinical Platform (Roche Diagnostics, Indianapolis, IN). The assay ranges were 0.5 to 300 ng/ml for osteocalcin and 0.01 to 6.00 ng/ml for CTX. Intra-assay coefficients of variance (CV) were 7.8% for osteocalcin and 1.1% for CTX. No inter-assay variation is reported because all of the assays were performed at the same time. The use of the osteocalcin and CTX assays in nonhuman primates was validated by the Endocrine Technologies Core at the ONPRC. The ratio of osteocalcin to CTX was calculated as a surrogate measure of global bone turnover balance.

2.3. Statistical analysis

Sample means and 95% confidence intervals were calculated for each biomarker, with *t*-tests used for pairwise comparisons. Linear regression was used to estimate the association between mean values of each biomarker and age for four groups: 1) male cynomolgus macaques, 2) female cynomolgus macaques, 3) male rhesus macaques, and 4) female rhesus macaques. Because there was relatively wide variation in age across species and sex, mean values of each biomarker were also compared across sex for subgroups where there was overlap in age: rhesus macaques aged 3.7–6.1 years (N = 52 male and 17 female), and cynomolgus macaques aged 6.0–7.0 years (N = 34 male and 22 female).

For the repeated measures data, sample means and 95% confidence intervals were calculated for each biomarker among all animals across all four timepoints. Linear mixed models with compound symmetric covariance matrices were used to compare different mean structures for concentrations of osteocalcin, CTX, and their ratio over time, including 1) constant mean concentration across time, 2) different mean concentration for each time point, and 3) linear mean trend to determine

Table 1

Age and plasma concentrations of biochemical markers of all monkeys at a single time point.

	Rhesus macaque			Cynomolgus macaque			Rhesus vs. cynomolgus	
	Male (N = 63)	Female (N = 17)	P*	Male (N = 34)	Female (N = 22)	P*	P (male) [†]	P (female) [‡]
Age (years)	5.7 (95% CI: 5.3, 6.0)	4.9 (95% CI: 4.4, 5.3)	0.027	6.4 (95% CI: 6.3, 6.5)	8.3 (95% CI: 7.5, 9.2)	<0.0001	0.015	<0.0001
Osteocalcin (ng/ml)	27.6 (95% CI: 24.6, 30.7)	18.7 (95% CI: 14.7, 22.7)	0.005	18.0 (95% CI: 14.8, 21.3)	12.0 (95% CI: 10.1, 14.0)	0.007	<0.0001	0.002
CTX (ng/ml)	1.65 (95% CI: 1.50, 1.80)	1.46 (95% CI: 1.22, 1.70)	0.237	1.53 (95% CI: 1.38, 1.68)	1.38 (95% CI: 1.15, 1.61)	0.244	0.407	0.624
Osteocalcin:CTX ratio	17.5 (95% CI: 15.7, 19.2)	13.2 (95% CI: 10.8, 15.6)	0.021	11.9 (95% CI: 10.1, 13.8)	9.2 (95% CI: 7.7, 10.8)	0.042	0.0004	0.004

Data presented are means and 95% confidence intervals (CI).

* p-Value for comparison of males vs. females within each species (e.g., male vs. female rhesus macaques; male vs. female cynomolgus macaques).

[†] p-Value for comparison of male rhesus macaques vs. male cynomolgus macaques.

[‡] p-Value for comparison of female rhesus macaques vs. female cynomolgus macaques.

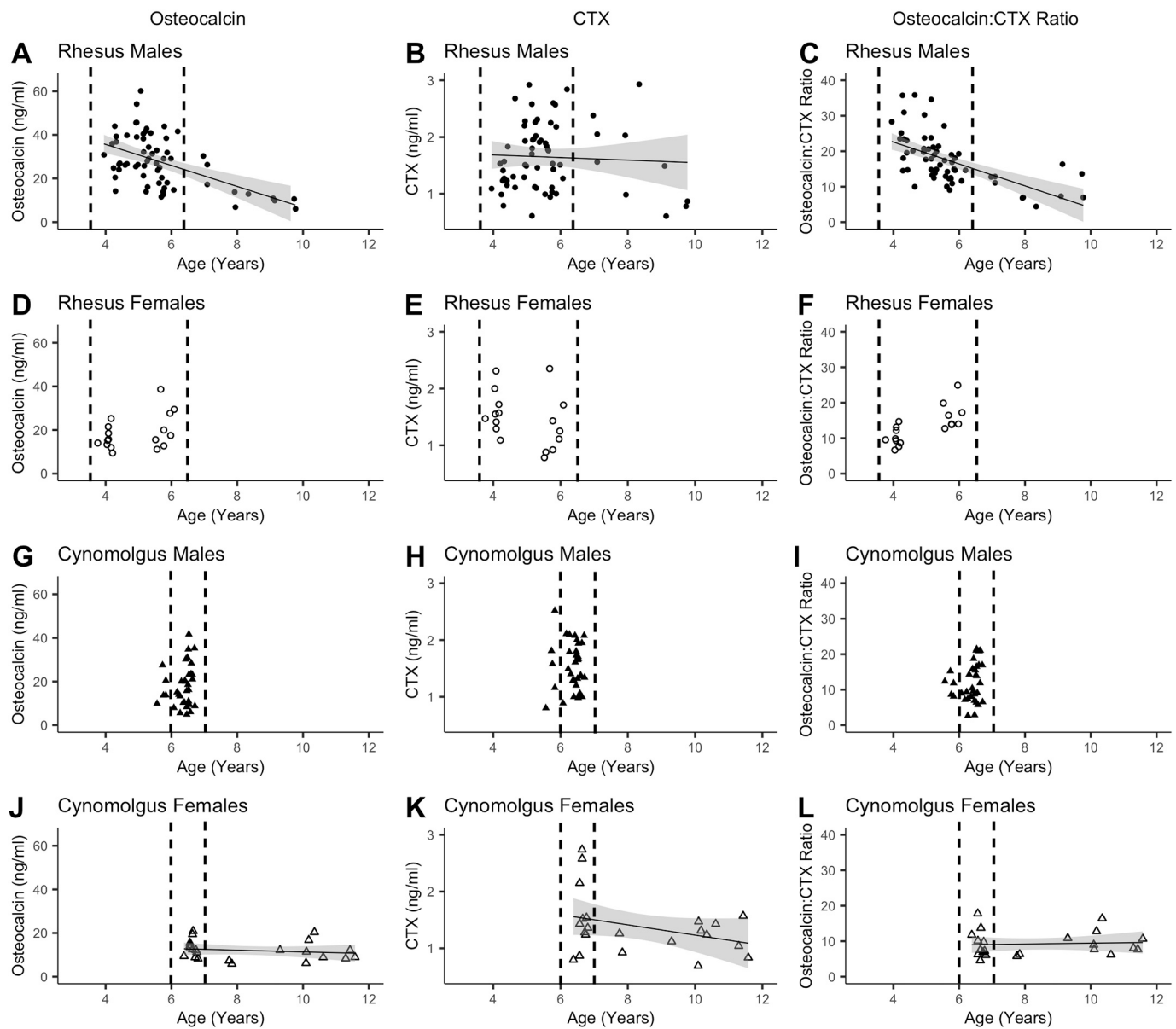


Fig. 1. Blood levels of osteocalcin, CTX, and the ratio of osteocalcin to CTX among male rhesus macaques (A–C, respectively, $n = 63$), female rhesus macaques (D–F, respectively, $n = 17$), male cynomolgus macaques (G–I, respectively, $n = 34$), and female cynomolgus macaques (J–L, respectively, $n = 22$). The biomarkers were measured once for each animal and each point represents a single measurement for a given animal. Linear regression lines were fit for male rhesus macaques and female cynomolgus macaques. Mean values of each biomarker were compared across sex for subgroups where there was overlap in age (as demarcated with dotted lines): 1) rhesus macaques aged 3.7–6.1 years ($N = 52$ male and 17 female), and 2) cynomolgus macaques aged 6–7 years ($N = 30$ male and 11 female).

whether concentrations increased or decreased over time in a straight-line fashion.

Measures of biological variation for the repeated measures data were estimated, namely the within-subject coefficient of variation (CV_I), within-subject correlation (intraclass correlation coefficient, ICC), within-subject variance, and between-subject variance. Separate ICCs were calculated for each biomarker for each of the four sex/species groups. A perfectly reproducible biomarker would have an ICC value of 1.0 (Thomas et al., 2009), while an ICC value of at least 0.60 is considered acceptable reproducibility (Chinn, 1991), and values ranging between 0.41 and 0.60 suggest moderate agreement (McGraw and Wong, 1996).

Model assumptions for t -tests, linear regression, and linear mixed models were assessed using quantile-quantile plots and the Anderson-Darling test of normality, and Levene's test of homogeneity of variance. Osteocalcin and the ratio of osteocalcin to CTX were log-

transformed for analysis because their distributions violated assumptions of normality; CTX was not transformed.

Data analysis was performed using R version 3.6.1. Measures of biomarker variation and correlation (between-subject variability, within-subject variability, and ICC) were estimated by fitting one-way random effects models to the repeated measures data using Stata version 15.1.

3. Results

3.1. Cross-sectional measurements

The mean ages and plasma concentrations of biomarkers at a single time point for each of the four groups of animals are shown in Table 1. Mean age was higher in males than in females for rhesus macaques (5.7 vs. 4.9 years, $p = 0.027$) and lower in males than in females for

Table 2
Age and plasma concentrations of biochemical markers in a subset of monkeys collected over four monthly intervals.

	Rhesus macaque			Cynomolgus macaque			Rhesus vs. cynomolgus	
	Male (N = 11)	Female (N = 3)	P*	Male (N = 6)	Female (N = 8)	P*	P (male) [†]	P (female) [‡]
Age (years)	5.1 (95% CI: 4.9, 5.2)	4.3 (95% CI: 4.3, 4.4)	<0.0001	6.7 (95% CI: 6.7, 6.8)	10.2 (95% CI: 9.7, 10.7)	<0.0001	<0.0001	<0.0001
Osteocalcin (ng/ml)	37.1 (95% CI: 34.1, 40.2)	19.5 (95% CI: 14.6, 24.4)	<0.0001	20.7 (95% CI: 16.8, 24.6)	10.5 (95% CI: 8.9, 12.2)	<0.0001	<0.0001	<0.0001
CTX (ng/ml)	1.69 (95% CI: 1.56, 1.82)	1.49 (95% CI: 1.27, 1.71)	0.153	1.38 (95% CI: 1.23, 1.53)	1.02 (95% CI: 0.91, 1.13)	<0.0001	0.005	<0.0001
Osteocalcin:CTX ratio	22.7 (95% CI: 21.0, 24.5)	12.8 (95% CI: 10.8, 14.8)	<0.0001	15.0 (95% CI: 12.5, 17.4)	10.9 (95% CI: 9.0, 12.9)	0.009	<0.0001	0.265

Data presented are means and 95% confidence intervals (CI).

* p-Value for comparison of males vs. females within each species (e.g., male vs. female rhesus macaques; male vs. female cynomolgus macaques).

[†] p-Value for comparison of male rhesus macaques vs. male cynomolgus macaques.

[‡] p-Value for comparison of female rhesus macaques vs. female cynomolgus macaques.

cynomolgus macaques (6.4 vs. 8.3 years, $p < 0.0001$). Mean age was lower in rhesus macaques than cynomolgus macaques for males (5.7 vs. 6.4 years, $p = 0.015$) and females (4.9 vs. 8.3 years, $p < 0.0001$). Mean plasma osteocalcin levels were higher in males than in females for rhesus macaques (27.6 vs. 18.7 ng/ml, $p = 0.005$) and cynomolgus macaques (18.0 vs. 12.0 ng/ml, $p = 0.007$). When species were compared, osteocalcin was higher in rhesus macaques for both males and females ($p <$

0.0001 and $p = 0.002$, respectively). No statistically significant differences were observed in plasma CTX levels between sexes or species. The ratio of osteocalcin to CTX was higher in males than in females for both rhesus macaques (17.5 vs. 13.2, $p = 0.021$) and cynomolgus macaques (11.9 vs. 9.2, $p = 0.042$). When species were compared, the ratio of osteocalcin to CTX was higher in rhesus macaques than cynomolgus macaques for both males and females ($p = 0.0004$ and $p = 0.004$,

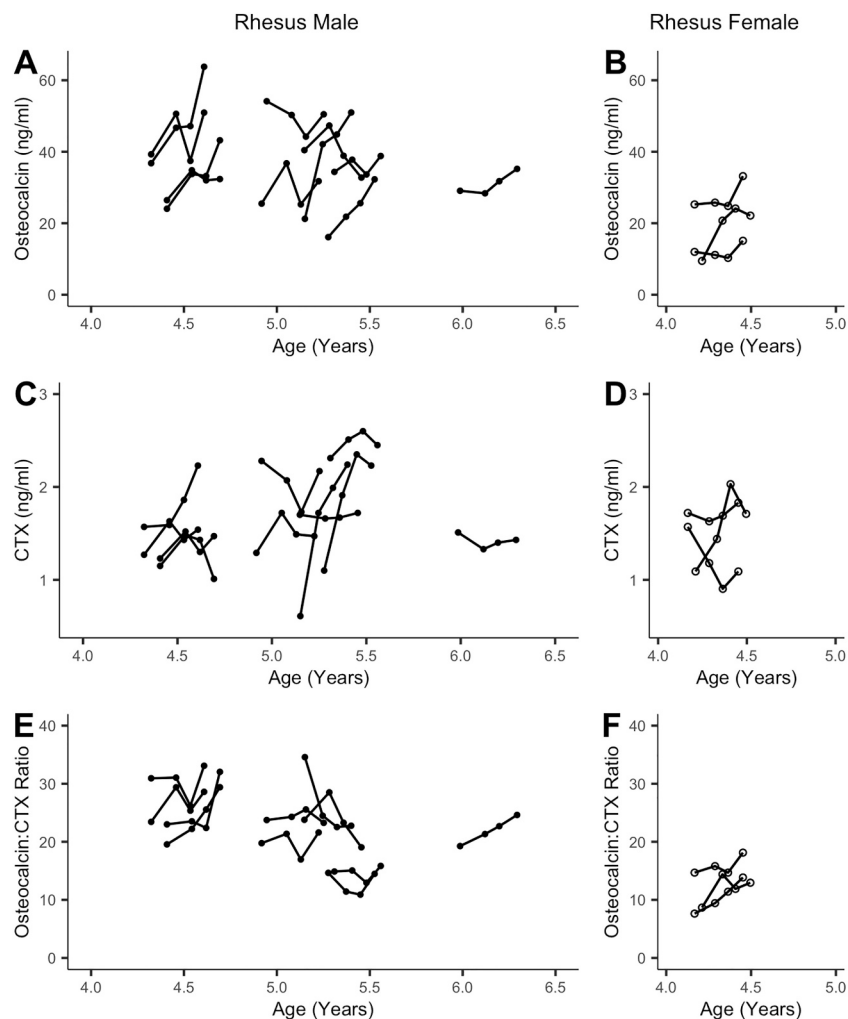


Fig. 2. Longitudinal levels of osteocalcin (A–B), CTX (C–D), and the ratio of osteocalcin to CTX (E–F) over 4 monthly intervals in male (n = 11) and female (n = 3) rhesus macaques. Data are presented by age (years) at time of sample collection. There is considerable variability in biomarker values for individual monkeys over each timepoint and there was no discernible pattern in this variability.

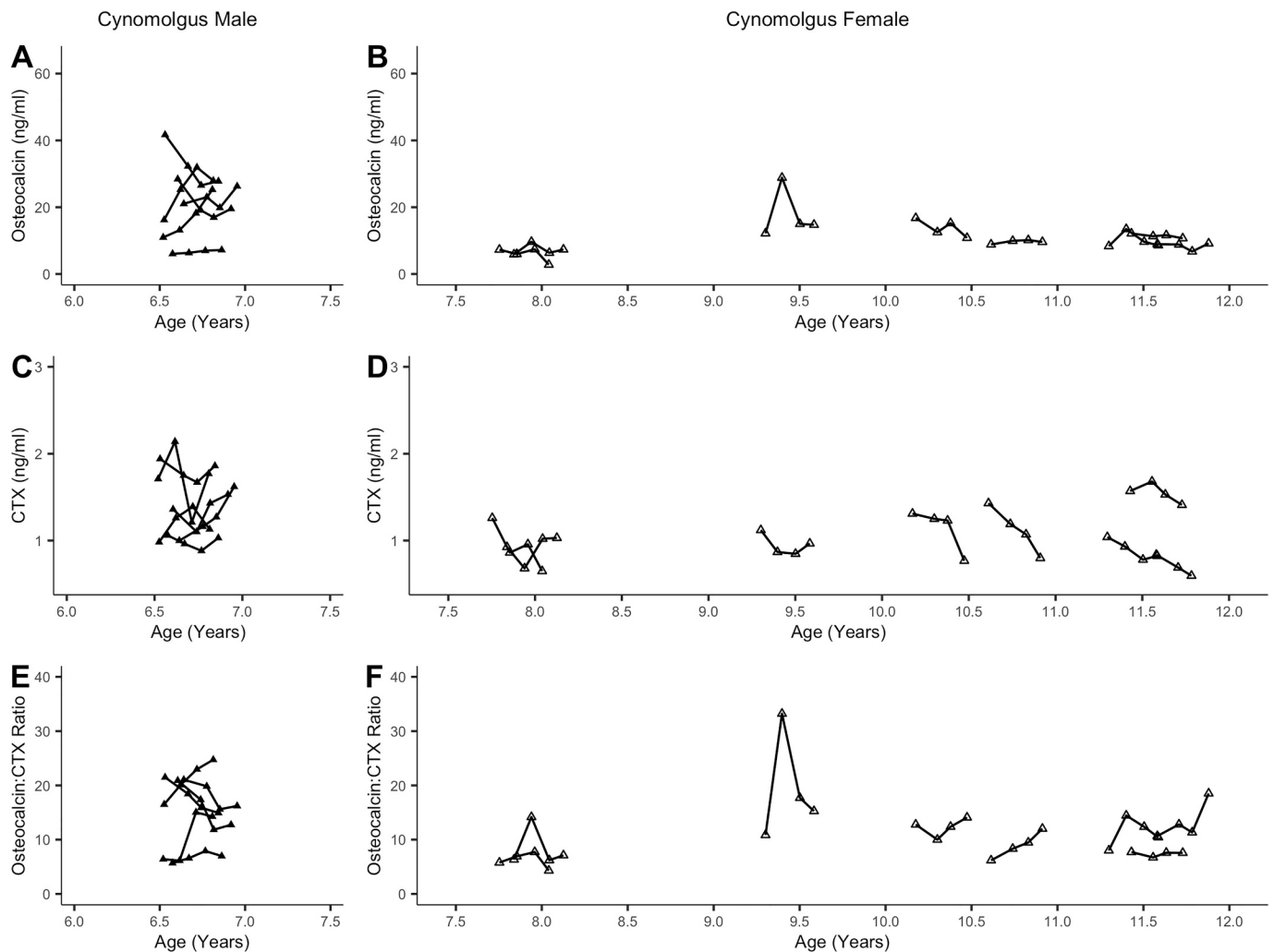


Fig. 3. Longitudinal levels of osteocalcin (A–B), CTX (C–D), and the ratio of osteocalcin to CTX (E–F) over 4 monthly intervals in male ($n = 6$) and female ($n = 8$) cynomolgus macaques. Data are presented by age (years) at time of sample collection. There is considerable variability in biomarker values for individual monkeys over each timepoint and there was no discernible pattern in this variability.

respectively).

Concentrations of osteocalcin and CTX, and the ratio of osteocalcin to CTX across all subjects as functions of species, sex and age are shown in Fig. 1. There was considerable spread in the age ranges for male rhesus macaques (4.0–9.8 years) and female cynomolgus macaques (6.4–11.6 years), and narrower age ranges for female rhesus macaques (3.7–6.1 years) and male cynomolgus macaques (5.6–6.7 years). The wider age ranges for male rhesus macaques and female cynomolgus macaques made it possible to evaluate age differences in levels of osteocalcin, CTX, and the ratio of osteocalcin to CTX for these two groups using regression analyses. For the male rhesus macaques ranging in age from 4.0 to 9.8 years old, there was an inverse relationship between age and level of osteocalcin ($p < 0.0001$), no relationship between age and CTX, and an inverse relationship between age and the ratio of osteocalcin to CTX ($p < 0.0001$). For female cynomolgus macaques ranging in age from 6.4 to 11.6 years old, no relationships between age and level of osteocalcin, CTX, and osteocalcin to CTX ratio were noted.

Because of significant age differences across sex and species, subgroups were compared where there was overlap in age: rhesus macaques aged 3.7–6.1 years (Fig. 1A–F) and cynomolgus macaques aged 6.0–7.0 years (Fig. 1G–L). Mean osteocalcin was 11.2 ± 2.9 ng/ml higher in male rhesus macaques than in females ($p < 0.0001$) in the age range of 3.7–6.1 and 6.0 ± 2.1 ng/ml higher in male cynomolgus macaques than in females ($p = 0.007$) in the age range 6.0–7.0. No significant

differences in mean CTX were observed between males and females within the age ranges measured for either species. The mean ratio of osteocalcin to CTX was 5.8 ± 1.7 higher in male rhesus macaques than in females ($p = 0.001$) and 2.7 ± 1.3 higher in male cynomolgus macaques than in females ($p = 0.042$) in the respective age ranges evaluated.

3.2. Longitudinal measurements

Standard descriptive statistics, along with within- and between-subject analyses, were performed in a subsample of animals over four monthly intervals. The mean ages and biomarker concentrations across the four months are shown in Table 2. Overall, similar trends were observed for the longitudinal measurements as for the cross-sectional measurements. Mean age was higher in males than in females for rhesus macaques (5.1 vs. 4.3 years, $p < 0.0001$) and lower in males than in females for cynomolgus macaques (6.7 vs. 10.2 years, $p < 0.0001$). Mean plasma osteocalcin levels were higher in males than in females for rhesus macaques (37.1 vs. 19.5 ng/ml, $p < 0.0001$) and cynomolgus macaques (20.7 vs. 10.5 ng/ml, $p < 0.0001$). When species were compared, osteocalcin was higher in rhesus macaques for both males and females ($p < 0.0001$). Mean plasma CTX levels were higher in males than in females for cynomolgus macaques (1.38 vs. 1.02 ng/ml, p

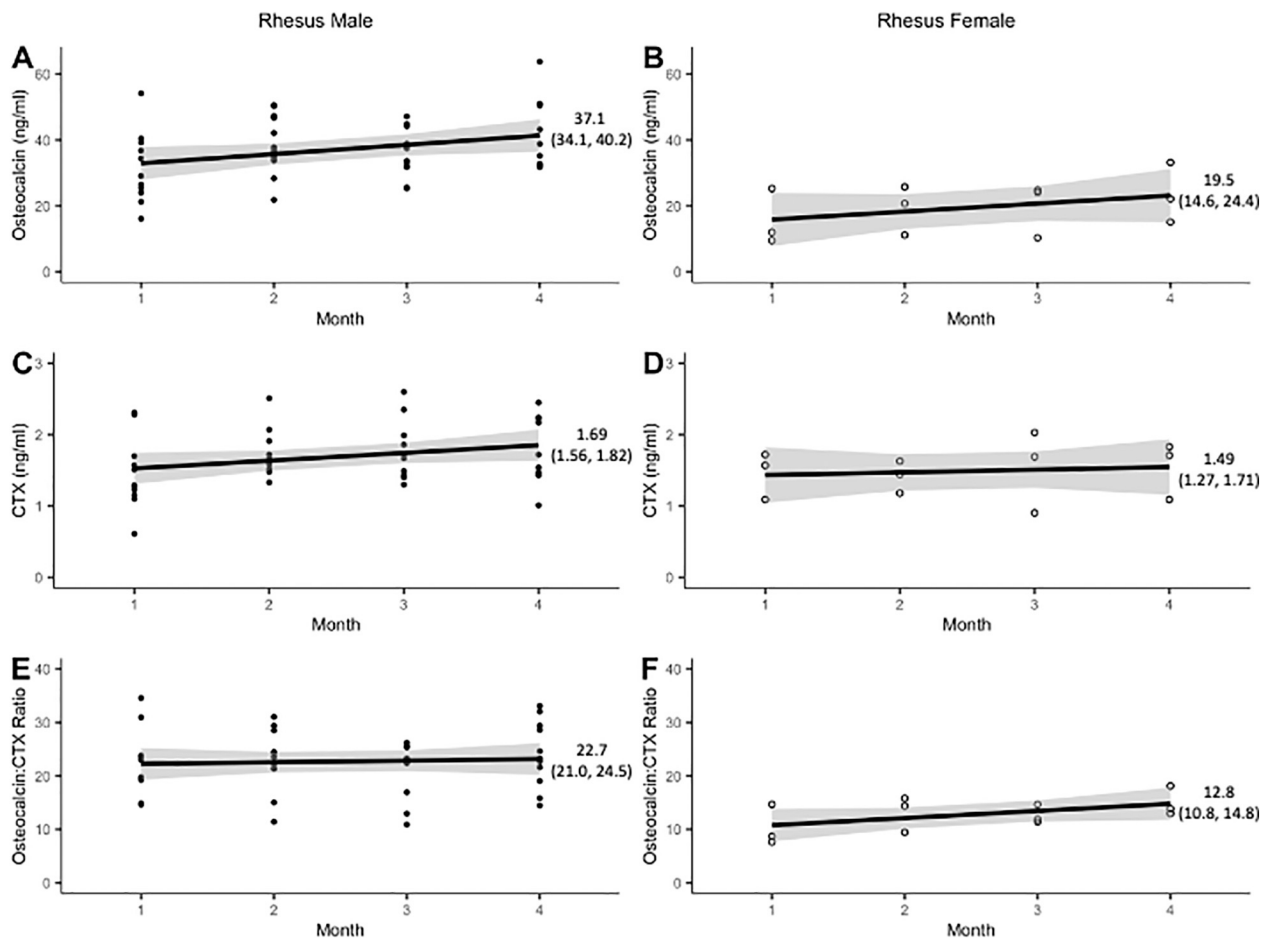


Fig. 4. Longitudinal levels of osteocalcin (A–B), CTX (C–D), and the ratio of osteocalcin to CTX (E–F) over 4 monthly intervals in male ($n = 11$) and female ($n = 3$) rhesus macaques. Data are presented by month of measurement and means were estimated using linear mixed models with 95% confidence intervals. The slope of each of the regression lines was nonsignificant, suggesting that the mean values of each biomarker did not change over the 4-month period at the population level. The mean and 95% confidence intervals for each regression line are included to the right of each graph.

< 0.0001). No statistically significant differences were observed in plasma CTX levels between male and female rhesus macaques or between species. The ratio of osteocalcin to CTX was higher in males than in females for both rhesus macaques (22.7 vs. 12.8, $p < 0.0001$) and cynomolgus macaques (15.0 vs. 10.9, $p = 0.009$). When species were compared, the ratio of osteocalcin to CTX was higher in rhesus macaques for males ($p < 0.0001$) but not females.

Concentrations of osteocalcin and CTX, and the ratio of osteocalcin to CTX across all subjects by age at time of sample collection for rhesus and cynomolgus macaques are shown in Figs. 2 and 3, respectively. For all four groups, male and female rhesus macaques and male and female cynomolgus macaques, there was considerable variability in biomarker values for individual monkeys over each four-month period, but there was no discernible pattern in this variability.

Estimated means from linear mixed models evaluating concentrations of osteocalcin and CTX, and the ratio of osteocalcin to CTX over the four times of sample collection for rhesus and cynomolgus macaques are shown in Figs. 4 and 5, respectively. No statistically significant associations between sequence of sample collection and mean osteocalcin, CTX, or ratio of osteocalcin to CTX were observed for any of the four groups.

Table 3 presents estimates of CV_I , ICC, within-subject variance, and between-subject variance for osteocalcin, CTX, and the ratio of osteocalcin to CTX stratified by group (male and female rhesus macaques; male and female cynomolgus macaques). For osteocalcin, male rhesus macaques had a CV_I range of 8.2–53.3% and an ICC of 0.19 (95% CI:

0.03–0.45) and female rhesus macaques had a CV_I range of 14.6–34.5% and an ICC of 0.54 (95% CI: 0.09–0.93). Male cynomolgus macaques had a CV_I range of 8.1–37.6% and an ICC of 0.81 (95% CI: 0.53–0.95); and female cynomolgus macaques had a CV_I range of 5.3–42.6% and an ICC of 0.59 (95% CI: 0.28–0.86). Within-subject variance was higher than between-subject variance for male rhesus macaques (0.40 vs. 0.19), similar for female rhesus macaques (0.28 vs. 0.30), and lower for male cynomolgus macaques (0.24 vs. 0.48), and female cynomolgus macaques (0.25 vs. 0.31).

For CTX, male rhesus macaques had a CV_I range of 1.6–46.0% and an ICC of 0.16 (95% CI: 0.02–0.56) and female rhesus macaques had a CV_I range of 4.9–25.5% and an ICC of 0.27 (95% CI: 0.01–0.89). Male cynomolgus macaques had a CV_I range of 6.6–22.4% and an ICC of 0.58 (95% CI: 0.23–0.88); and female cynomolgus macaques had a CV_I range of 7.2–27.3% and an ICC of 0.59 (95% CI: 0.27–0.85). Within-subject variance was higher than between-subject variance for male rhesus macaques (0.42 vs. 0.18) and female rhesus macaques (0.29 vs. 0.17), and similar for male cynomolgus macaques (0.22 vs. 0.26) and female cynomolgus macaques (0.19 vs. 0.23).

For the ratio of osteocalcin to CTX, male rhesus macaques had a CV_I range of 4.1–40.3% and an ICC of 0.55 (95% CI: 0.29–0.41) and female rhesus macaques had a CV_I range of 10.3–25.3% and an ICC of 0.41 (95% CI: 0.07–0.85). Male cynomolgus macaques had a CV_I range of 13.5–46.4% and an ICC of 0.67 (95% CI: 0.33–0.91); and female cynomolgus macaques had a CV_I range of 6.1–50.5% and an ICC of 0.51 (95% CI: 0.20–0.82). Between-subject variance was similar to within-

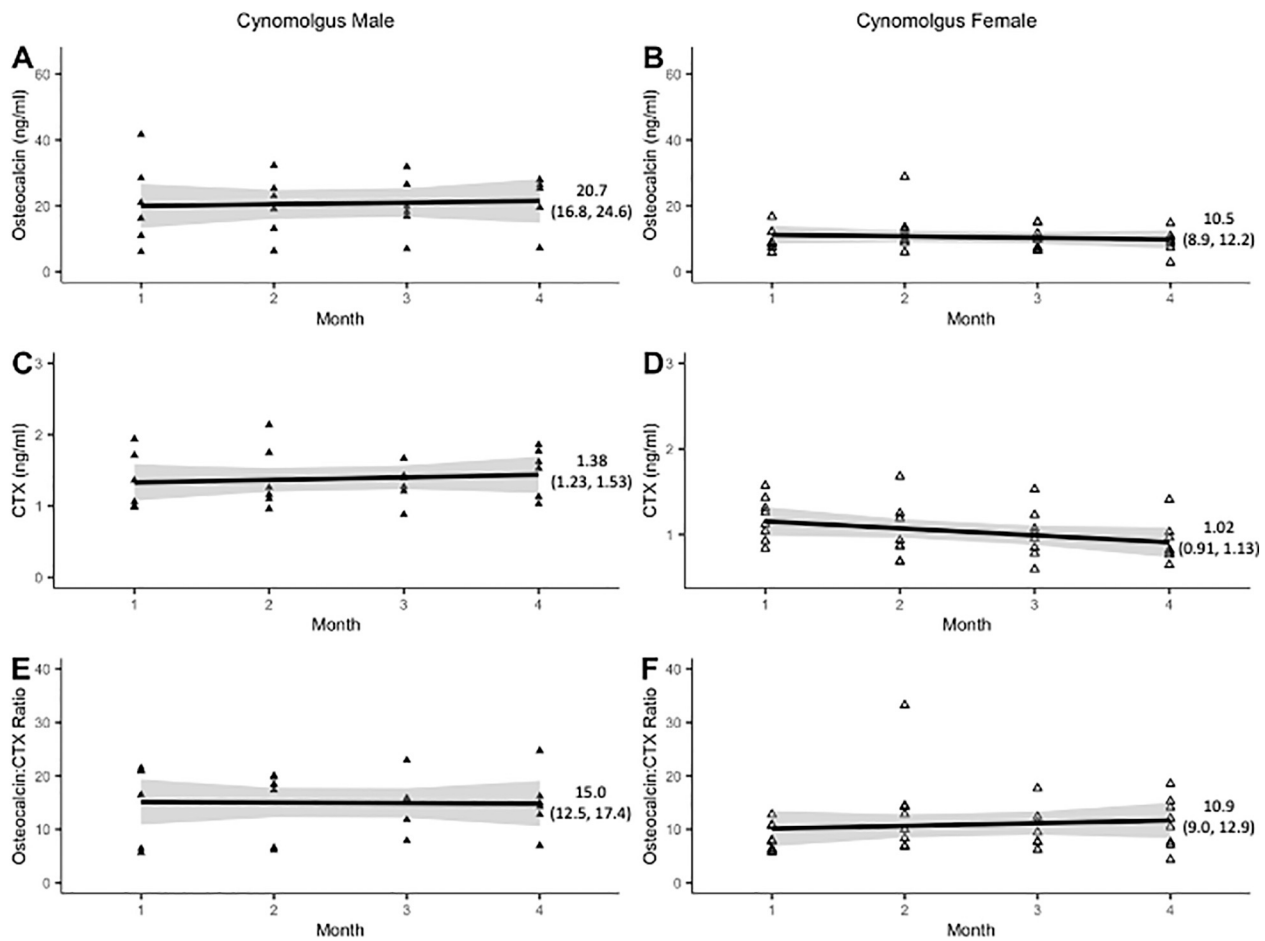


Fig. 5. Longitudinal levels of osteocalcin (A–B), CTX (C–D), and the ratio of osteocalcin to CTX (E–F) over 4 monthly intervals in male ($n = 6$) and female ($n = 8$) cynomolgus macaques. Data are presented by month of measurement and means were estimated using linear mixed models with 95% confidence intervals. The slope of each of the regression lines was nonsignificant, suggesting that the mean values of each biomarker did not change over the 4-month period at the population level. The mean and 95% confidence intervals for each regression line are included to the right of each graph.

subject variance for male rhesus macaques (0.22 vs. 0.25), lower in female rhesus macaques (0.20 vs. 0.15), higher for male cynomolgus macaques (0.26 vs. 0.38), and the same in female cynomolgus macaques (0.29).

4. Discussion

In this study, we estimated the biological variation in osteocalcin and CTX as markers of global bone formation and resorption, respectively, as well as the ratio of osteocalcin to CTX, as an index of bone turnover balance, in late adolescent and young adult male and female rhesus and cynomolgus macaques. The results of this study provide bone turnover biomarker reference values for both species of macaques during the critical interval preceding peak bone mass. The study also identifies strengths and weaknesses in routine use of osteocalcin and CTX to monitor bone turnover at the individual and population levels.

Because there were significant differences in age across sex and species, subgroup analyses were conducted where there was age overlap in order to investigate sex differences in rhesus macaques aged 3.7–6.1 years and cynomolgus macaques aged 6.0–7.0 years. For both species, osteocalcin was higher in males than in females, indicating higher rates of bone matrix synthesis. This is in agreement with literature in humans which have reported higher osteocalcin in males (Vanderschueren et al., 1990; del Pino et al., 1991). There were no observed differences in CTX levels between males and females of either species, suggesting no major sex differences in bone resorption. Given the sex differences for

osteocalcin, but not CTX, it would be anticipated that males have a higher positive bone turnover balance compared to females.

Monthly repeat measurements over four months revealed large variation in osteocalcin and CTX levels in individual monkeys. The magnitude of variation exceeded intra-assay variation indicating that this represents biological variation and will unlikely be decreased by increasing number of replicate samples assayed. Biological variation may occur due to cyclical variations (e.g., diurnal/circadian, hormonal) or changes over the lifespan associated with aging. The magnitude of variation during late stages of skeletal maturation is assessed in the present study using the within-subject coefficient of variation (CV_1) (Looker et al., 2000; Thomas et al., 2009). There were wide ranges in CV_1 values for osteocalcin, CTX, and the ratio of osteocalcin to CTX for individual animals throughout the four sampling time points. This high intra-individual variability supports previous findings in older cynomolgus macaques (Legrand et al., 2003), and suggests that this intrinsic variability is an important factor in interpreting longitudinal bone marker data.

Reproducibility, or the extent to which the same value is obtained for a given animal following a repeat measurement, is another important characteristic to consider. In the present study, at least moderate agreement (≥ 0.41) was met for the averages of the measurements of osteocalcin, CTX, and the ratio of osteocalcin to CTX in all but two instances. For osteocalcin, male cynomolgus macaques had acceptable reproducibility (≥ 0.60); female rhesus macaques and cynomolgus macaques had moderate agreement; and male rhesus macaques fell under

Table 3
Biological variance in male and female rhesus and cynomolgus macaques over four months.

	Rhesus macaque		Cynomolgus macaque	
	Male (N = 11)	Female (N = 3)	Male (N = 6)	Female (N = 8)
Osteocalcin (ng/ml)				
CV ₁ (%)	8.2 to 53.3	14.6 to 34.5	8.1 to 37.6	5.3 to 42.6
ICC	0.19 (95% CI: 0.03, 0.45)	0.54 (95% CI: 0.09, 0.93)	0.81 (95% CI: 0.53, 0.95)	0.59 (95% CI: 0.28, 0.86)
Within-subject variance	0.40 (95% CI: 0.31, 0.50)	0.28 (95% CI: 0.18, 0.44)	0.24 (95% CI: 0.17, 0.33)	0.25 (95% CI: 0.19, 0.34)
Between-subject variance	0.19 (95% CI: 0.08, 0.45)	0.30 (95% CI: 0.11, 0.80)	0.48 (95% CI: 0.27, 0.88)	0.31 (95% CI: 0.17, 0.54)
CTX (ng/ml)				
CV ₁ (%)	1.6 to 46.0	4.9 to 25.5	6.6 to 22.4	7.2 to 27.3
ICC	0.16 (95% CI: 0.02, 0.56)	0.27 (95% CI: 0.01, 0.89)	0.58 (95% CI: 0.23, 0.88)	0.59 (95% CI: 0.27, 0.85)
Within-subject variance	0.42 (95% CI: 0.33, 0.53)	0.29 (95% CI: 0.18, 0.45)	0.22 (95% CI: 0.16, 0.31)	0.19 (95% CI: 0.14, 0.25)
Between-subject variance	0.18 (95% CI: 0.07, 0.48)	0.17 (95% CI: 0.04, 0.69)	0.26 (95% CI: 0.14, 0.52)	0.23 (95% CI: 0.13, 0.40)
Osteocalcin:CTX ratio				
CV ₁ (%)	4.1 to 40.3	10.3 to 25.3	13.5 to 46.4	6.1 to 50.5
ICC	0.55 (95% CI: 0.29, 0.41)	0.41 (95% CI: 0.07, 0.85)	0.67 (95% CI: 0.33, 0.91)	0.51 (95% CI: 0.20, 0.82)
Within-subject variance	0.22 (95% CI: 0.18, 0.28)	0.20 (95% CI: 0.13, 0.32)	0.26 (95% CI: 0.19, 0.36)	0.29 (95% CI: 0.16, 0.54)
Between-subject variance	0.25 (95% CI: 0.15, 0.41)	0.15 (95% CI: 0.04, 0.49)	0.38 (95% CI: 0.20, 0.71)	0.29 (95% CI: 0.22, 0.38)

Data presented are means and 95% confidence intervals (CI).

the threshold for acceptability. For CTX, male and female cynomolgus macaques had moderate agreement, while male and female rhesus macaques fell under the threshold for acceptability. Finally, for the ratio of osteocalcin to CTX, male cynomolgus macaques had acceptable reproducibility, while male and female rhesus and female cynomolgus macaques had moderate agreement. Therefore, the use of repeated measures of markers may provide a better estimate of bone turnover status than relying on a single measurement. This is supported by the stability of values at the population level measured over 4 months.

Concentrations of osteocalcin and CTX oscillate over a circadian rhythm. In humans, osteocalcin levels are lowest in the morning and highest in the evening (Gundberg et al., 1985), while CTX concentrations are lowest in the afternoon and peak in early morning (Qvist et al., 2002). Given the potential for diurnal variation in biomarker concentrations, the timing of blood sampling is important for the comparison across samples. The blood samples for the present study were collected between 1800 and 1900 h, just before the end of the 11 h light cycle. This consistency allows for samples collected across time and individuals to be appropriately compared.

There are several limitations to our study. First, while osteocalcin and CTX were the two bone turnover markers assessed in the present study, there are other markers that are routinely evaluated. However, another study found that changes in levels of osteocalcin and CTX were similar to other biomarkers of bone turnover in cynomolgus macaques (Legrand et al., 2003). Second, bone mass and density were not measured, nor was bone turnover evaluated at the local level. While these measurements are desirable, the measurement of biomarkers is less invasive. In support of the use of turnover markers in monitoring

bone status, one study reported a correlation between increased levels of the bone resorption marker cross-linked N-telopeptide and reduced BMD (Legrand et al., 2003). Third, the monkeys were unfasted at the time of sample collection, which may have contributed to the variation in CTX measurements (Qvist et al., 2002). Fourth, this was a retrospective analysis of multiple cohorts of monkeys. To circumvent differences in age distribution among groups, subgroup analyses were performed on age-matched subjects. Finally, the stage of the menstrual cycle was not taken into account for the timing of sample collection in females. Although the menstrual cycle may influence bone turnover (Hotchkiss and Brommage, 2000), there was no statistically significant difference in marker variability between the males and females.

5. Conclusions

In summary, our results show that similar to humans, there is considerable within-subject variation in the concentrations of osteocalcin and CTX, suggesting that biochemical markers in blood are not ideal for monitoring an individual's bone turnover status over time. However, the overall stability of these markers across subjects during a 4-month time interval, supports the conclusion that they have practical utility for evaluating bone turnover at the population level. Additionally, similar patterns emerged between rhesus and cynomolgus macaques, suggesting that there is no advantage or disadvantage in using either of these commonly used species.

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Conceptualization: KG

Data Collection: VJ, NN

Data analysis: LS and AB

Drafting manuscript: LS

Revising manuscript content: LS, AB, VJ, NN, KG, RT, and UI

Approving final version: LS, AB, VJ, NN, KG, RT, and UI

UI takes responsibility for the integrity of the data

Declaration of competing interest

The authors report no declarations of interest.

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