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Dose-response effects of alcohol on biochemical markers of bone turnover in non-human primates: Effects of species, sex and age of onset of drinking

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ABSTRACT

Purpose: Alcohol consumption suppressed bone turnover in male non-human primates; however, it is unclear the extent to which this effect depends upon biological variables. Using archived plasma samples, we investigated whether sex, age of onset of alcohol intake, and species influence the effects of graded increases in alcohol consumption on bone turnover markers.

Methods: 91 male and female macaques (rhesus and cynomolgus), ranging in age from 4 years (adolescent) to 10 years (adult) were required to increase their consumption of ethanol in 30-day increments: 0 g/kg/day, followed by 0.5 g/kg/day, 1.0 g/kg/day, and, finally, 1.5 g/kg/day. Plasma osteocalcin (formation), plasma CTX (resorption) and osteocalcin to CTX ratio (turnover balance) were measured during these intervals to assess the dose-response effects of alcohol.

Results: We detected no relationship between dose and osteocalcin when all monkeys were combined, but there was a significant effect of sex (lower levels in females) and interactions between alcohol dose and sex (osteocalcin levels increased with dose in rhesus females). In contrast, we detected a negative linear dose-response relationship for ethanol and CTX. We did not detect a relationship between dose and osteocalcin to CTX ratio overall, but there was a significant positive relationship detected in females (no change in males). Increased age predicted lower biomarker levels for both osteocalcin and CTX. Species was a significant predictor for osteocalcin and the osteocalcin to CTX ratio in these models.

Conclusion: These findings indicate that age, sex, and species influence bone turnover and support the concept that factors beyond quantity of alcohol affect skeletal response to alcohol consumption.

1. Introduction

Chronic alcohol abuse in humans is associated with deleterious health outcomes, including poor skeletal health (Diamond et al., 1989; Ulhøi et al., 2017). However, the skeletal response is notably inconsistent as not all studies investigating chronic alcohol abuse report clinically relevant bone loss and/or increased fracture rate (González-Reimers et al., 2013; Paccou et al., 2015). This suggests additional factors act to influence the skeletal response to alcohol intake (Gaddini et al., 2016; Turner et al., 2021). Putative invariant intrinsic factors include genetic susceptibility and sex, and putative variant extrinsic factors include age at onset of abusive drinking and quantity of alcohol consumed. Notably, interplay between intrinsic and extrinsic factors could further influence the effect of alcohol consumption on skeletal health.

In humans, initiation of regular alcohol intake peaks during early adulthood (18–25 y of age) but typically begins during late adolescence (16–18 years of age) (Gaddini et al., 2016). Of particular relevance, 40–50% of peak bone mass is accrued during late adolescence and early adulthood (Matkovic et al., 1994). Regular alcohol consumption during this period may lead to retardation of bone accrual and reduce peak bone mass, although there is insufficient evidence to support a firm conclusion (LaBrie et al., 2018; Seo et al., 2015). Sex may be a modulating factor because the growth spurt in females typically occurs two

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years earlier than in males.

Animal models support the concept that age and quantity of alcohol consumed are important extrinsic factors influencing skeletal response to alcohol. In rodents, alcohol consumption impairs accrual of cortical bone during growth and leads to concentration-dependent cancellous bone loss in adults (Turner et al., 2001). In contrast, there is a paucity of human or animal studies designed to rigorously evaluate the respective roles of sex or genetics in modulating bone response to alcohol. As a consequence, their respective roles remain largely speculative.

We have shown that long duration (1 year) chronic voluntary alcohol consumption can result in local suppression of histomorphometric indices of cancellous and cortical bone turnover in male rhesus macaques (n = 21; mean age at initiation of alcohol consumption = 5.4 years), resulting in cancellous but not cortical bone loss (Gaddini et al., 2015; Kahler-Quesada et al., 2019). Importantly, biochemical markers of bone formation (e.g., osteocalcin) and bone resorption (e.g., C-terminal cross-linking telopeptide of type I collagen, CTX) present in blood, and often used as a minimally invasive approach to assess bone turnover in humans (Cabral et al., 2016; Kuo and Chen, 2017), support a net, alcohol-induced dose-dependent reduction in bone turnover in macaques (Sattgast et al., 2021).

The purpose of this investigation was to test the hypothesis that sex and age of onset of alcohol intake influence the effects of chronic alcohol consumption on bone turnover in non-human primates. To accomplish this, we measured biochemical markers of bone turnover in 91 male and female macaques ranging in age from 4 years (late adolescent) to 10 years (adult at peak bone mass) exposed to increasing amounts of alcohol. We also determined whether independent and interactive responses of sex and age of onset of drinking were strongly influenced by genetic background by comparing two closely related species (rhesus macaques and cynomolgus macaques).

2. Methods

2.1. Cohorts

The current study capitalizes on an ongoing multidisciplinary NIAAA-funded project investigating the neuroendocrine basis for ethanol addiction. All animals are subjected to schedule-induced polydipsia (SIP, please see below) followed by open access to ethanol. Archived tissue and extensive multi-organ bioinformatics from completed cohorts are available through the NIAAA-sponsored Monkey Alcohol Tissue Research Resource (MATRR) (www.matrr.com). MATRR serves as a database and analytics platform to collate monkey, cohort, and experimental studies, distribute banked tissues, and provide users with tools to explore ethanol effects across more than 150 non-human primates under similar protocols (Daunais et al., 2014). Animals used in the current study represent an aggregate of 91 rhesus monkeys (*Macaca mullata*, $N = 50$, *Macaca fascicularis*, $N = 41$) across 12 different cohorts, designated as cohorts *2*, *3*, *4*, *5*, *6a*, *6b*, *7a*, *7b*, *8*, *9*, *10*, *13*. Cohorts represent both male ($N = 67$) and female ($N = 24$) animals, and range in age from late adolescent, approximately 4 years of age, to adult, approximately 10 years of age at the beginning of the ethanol induction period. Table 1 displays cohort, sex, and the average age and weight at onset of induction. The 12 cohorts represent all monkeys in studies conducted between 2006 and 2014; all animals were included in the current analysis to maximize available sample size. Furthermore, all monkeys administered ethanol in each cohort were included to avoid any selection bias. Individuals from cohorts used in this study have a rich history of description across other studies ranging from predictive drinking behavior (Baker et al., 2017), age associated drinking behaviors (Helms et al., 2014), and effects of ethanol on bone morphology (Kahler-Quesada et al., 2019), immunological responses (Messaoudi et al., 2013), and genomic screening (Cervera-Juanes et al., 2017).

2.2. Monkeys

All animals involved in this study adhered to well-established self-administration protocols (Helms et al., 2014), and were housed at the Oregon National Primate Research Center (ONPRC). All animals were transitioned to individual cages three months prior to the onset of experimental protocols, and housed individually in a single room throughout the experimental protocol. All animals within the same cohort had visual and auditory access to other animals within the cohort and were socially paired for 1–2 h daily.

2.3. Schedule-induced polydipsia

While individual cohorts were initiated across a span of 8 years, results can be aggregated because all animals participated in the same rigorous, well-documented SIP protocol, described in Grant et al. (2008). Briefly, animals were induced to drink 4% ethanol w/v in water by delivering a 1-gram banana-flavored pellet every 5 min (Noyes, Lancaster, NH; Bio Serv, Flemington, NJ). Under this schedule of food delivery, high rates of fluid consumption can be maintained and preset amount of fluid ingested (therefore dose of ethanol) could be controlled by the computer interface. Following a 30-day water induction period, using SIP, the monkeys consumed 0.5 g/kg/day ethanol, followed by 1.0 g/kg/day, and, finally, 1.5 g/kg/day. Each ethanol dose was consumed for 30 consecutive days. The escalating volumes of ethanol ensured that each animal experienced elevated ethanol levels as measured by blood ethanol concentrations (BECs), and the volume equivalent to the 1.5 g/kg/day dose ensured that most animals experienced intoxication, as defined by a BEC of 80 mg/dl (Crabbe et al., 2011). Mean BECs for each ethanol dose (0.5, 1.0, and 1.5 g/kg/day) per cohort are presented in Table 1 and Fig. 1B. Blood samples for BEC measurements were collected approximately every 5 days. Banana-flavored pellets were delivered on a fixed interval schedule until the

Table 1
Animal cohort details. Mean BEC is shown ± standard error. Cyno = cynomolgus macaques; Rhesus = Rhesus macaques.

Cohort	Year	Species	Sex	N	Mean age (years)	Mean weight (kg)	Mean BEC (mg %)		
							0.5 g/kg/day	1.0 g/kg/day	1.5 g/kg/day
2	2006	Cyno	M	11	6.5	7.9	14.6 ± 3.1	46.5 ± 7.5	87.3 ± 10.5
3	2007	Cyno	F	10	6.7	3.8	9.8 ± 4.3	26.9 ± 10.7	44.2 ± 11.9
4	2008	Rhesus	M	10	8.4	9.8	26.9 ± 3.7	49.3 ± 5.9	68.4 ± 8.2
5	2009	Rhesus	M	8	5.8	9.3	18.0 ± 4.9	45.2 ± 9.9	65.6 ± 14.8
6a	2010	Rhesus	F	6	4.1	5.4	21.5 ± 5.6	68.8 ± 11.6	112.2 ± 16.0
6b	2012	Rhesus	F	5	5.8	6.5	20.3 ± 7.0	42.7 ± 13.4	78.8 ± 24.3
7a	2010	Rhesus	M	8	4.3	8.3	17.4 ± 5.5	43.2 ± 12.5	54.6 ± 20.4
7b	2011	Rhesus	M	5	5.9	8.6	14.0 ± 6.1	57.3 ± 15.5	121.4 ± 15.0
8	2011	Cyno	F	3	10.3	5.2	30.5 ± 13.8	45.9 ± 20.6	70.7 ± 32.9
9	2012	Cyno	M	8	6.0	7.9	6.1 ± 3.7	28.8 ± 9.5	34.0 ± 12.9
10	2013	Rhesus	M	8	5.4	8.3	19.9 ± 5.6	71.4 ± 11.1	114.7 ± 14.6
13	2014	Cyno	M	9	6.6	8.1	18.4 ± 3.4	55.1 ± 6.8	82.4 ± 12.2

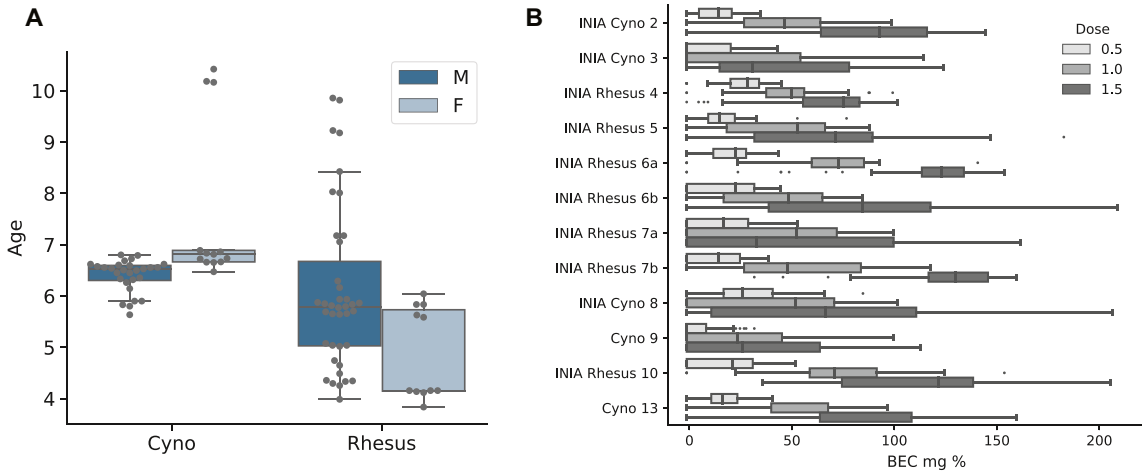

Fig. 1. There were significant differences in the age of the animals at the start of induction. (A) Age distributions are shown as boxplots, stratified by species and sex. Points for individual observations are shown. (B) Animals reach different levels of blood ethanol concentration (BEC) at each dose. Boxplot shows the distribution of BEC for each cohort at each dose during induction (0.5 g/kg/day, 1.0 g/kg/day, 1.5 g/kg/day). The 1.5 g/kg/day dose ensured that animals experienced BEC levels associated with intoxication (80 mg %). Blood samples for BEC were taken approximately every 5 days.

dose was consumed. Two hours after the cutoff volume was reached, the remaining pellets were available in the form of a meal (Grant et al., 2008; Vivian et al., 2001). Animals were weighed weekly during the course of the study. The number of food pellets was adjusted to maintain stable body weight.

2.4. Data collection

Food, ethanol and water intake data were collected through individual operant panels attached to cages. Ethanol and water volumes were measured by weight to an accuracy of 0.1 g (O'Haus Corporation, Parsippany, NJ). Data were cleansed for outlier events, artifacts, and equipment anomalies, and transferred to MATRR.

2.5. Collection of markers of bone turnover

All blood samples were collected within the first 3 h of the 11 hour light cycle as described (Jimenez et al., 2015). Plasma was stored at −80 °C until analysis. Osteocalcin and CTX were measured by the Endocrine Technologies Core at the ONPRC using a Roche Cobas e411 Automated Clinical Platform (Roche Diagnostics, Indianapolis, IN). The assay ranges of the osteocalcin and CTX assays were 0.5–300 ng/ml and 0.01–6.00 ng/ml, respectively. Intra-assay coefficient of variation (CV) for osteocalcin was 7.8% and intra-assay CV for CTX was 1.1%. Because all samples were measured the same day no inter-assay CV was calculated for these specimens. The ratio of osteocalcin to CTX was calculated as an index of overall bone turnover balance.

2.6. Power analysis

The post-hoc power analysis for the study was calculated using G*Power (v3.1.9.6). We calculated power for both a multiple regression and a repeated measures ANOVA with within-between interactions. We used a desired power of 0.8, an alpha of 0.05, and assumed a moderate effect size ($f^2 = 0.15$ or $f = 0.2$, respectively (Selya et al., 2012; Lenhard and Lenhard, n.d.)). We also conducted a sensitivity power analysis using both approaches, setting alpha at 0.05, and power at 0.8.

2.7. Regression analysis

We modeled the relationship between ethanol dose and markers of bone turnover during the induction period using generalized linear regression models. During induction, osteocalcin, CTX, and the ratio of osteocalcin to CTX were measured at four ethanol doses: 0 (baseline), 0.5 g/kg/day, 1.0 g/kg/day, and 1.5 g/kg/day. We used a multivariate linear regression to predict dose-response effects of ethanol on these markers, including species, sex, and age as additional variables of interest. Age was considered as a continuous variable in the regression models. As described (Sattgast et al., 2021), we used Akaike's information criteria (AIC) and likelihood ratio tests to choose a correlation matrix from one of the following: independent, compound symmetric, unstructured, autoregressive (AR) of order 1, and autoregressive moving average model (ARMA). Of the best performing models, we considered both homogeneous and heterogeneous variances across dose, selecting the final model using AIC and likelihood ratio tests. The final models for osteocalcin and CTX use heterogeneous variance and unstructured correlation matrices, and heterogeneous variance and ARMA(1,1) for the ratio of osteocalcin to CTX. We estimated the marginal means of linear trends for dose across sex and species. We consider significant differences at a p-value threshold of less than 0.05.

We performed the analysis using the nlme and emmeans package in R (version 3.4.3) and visualization with the Seaborn library for Python (Waskom et al., 2020; Pinheiro et al., 2021).

3. Results

There were significant age differences across groups after Bonferroni correction for multiple tests (Fig. 1; Table 2; $p = 0.002$). The median age for cynomolgus macaques in this analysis is 6.6 years, while the median age for rhesus macaques is 5.8 years. Cynomolgus males tended to be younger than females ($p = 0.06$), and rhesus males were significantly older than the females ($p = 0.008$).

When all monkeys were combined, we found no significant relationship between ethanol dose and plasma osteocalcin ($p = 0.2$; Fig. 2A, Table 3); however, there were significant effects of sex ($p = 1.0 \times 10^{-4}$), species ($p = 6.6 \times 10^{-3}$), and age ($p = 1.5 \times 10^{-6}$). Additionally, there were interactions between dose and sex ($p = 1.6 \times 10^{-2}$) and sex and

Table 2
Summary of age distribution stratified by species and sex.

Species	Sex	N	Mean (years)	SD	Median (years)
Cynomolgus	M	28	6.4	0.3	6.5
	F	13	7.5	1.6	6.8
Rhesus	M	39	6.1	1.6	5.8
	F	11	4.9	0.9	4.2

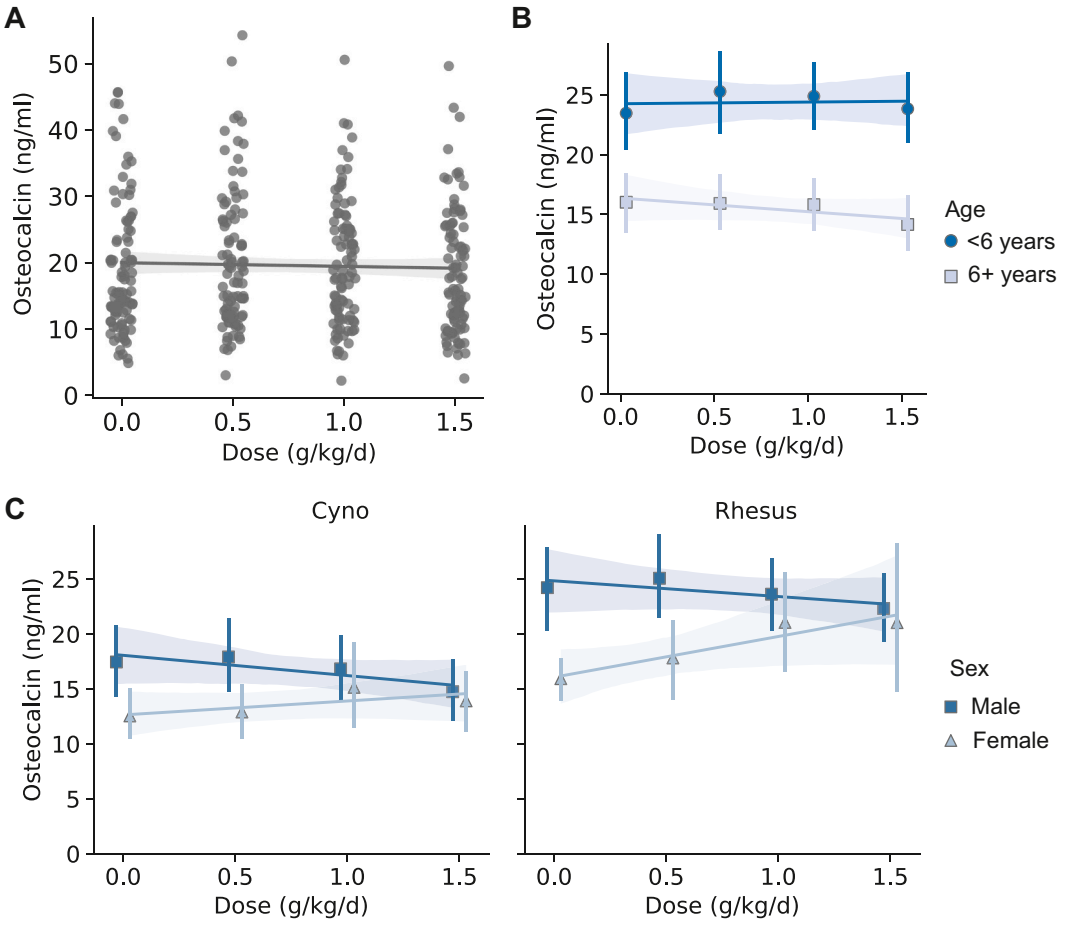

Fig. 2. We detected no significant overall dose-response relationship for osteocalcin levels. (A) The linear relationship between ethanol dose and osteocalcin levels. Each black point shows the observed value for an individual animal. Confidence bands are 95% bootstrapped confidence intervals. (B) The relationship between ethanol dose and osteocalcin stratified by age. Each point is the mean osteocalcin level for animals in the age group (>6 years or 6+ years) and dose. The age groups were chosen to visualize differences between late adolescent/young adult animals (<6 years old) and adult animals that have reached skeletal maturity (6+ years old); however, the regression considers age as a continuous variable. Confidence bands are 95% bootstrapped confidence intervals. (C) There were significant interaction effects for ethanol dose and sex, and sex and species on osteocalcin levels. The relationship for cynomolgus (left) versus rhesus (right) macaques are plotted with regression lines for males and females. The confidence bands are 95% bootstrapped confidence intervals.

species ($p = 1.7 \times 10^{-2}$). The relationship between age and osteocalcin is shown in Fig. 2B. The mean level of osteocalcin decreased 2.98 ng/ml for each unit (one year) increase in age (95% CI 1.78–4.18 ng/ml), showing that older animals had lower osteocalcin levels even at baseline. On average, females and cynomolgus macaques had lower osteocalcin levels when other variables were held constant, and the significant interaction effects in the model highlight differential dose-response effects between males and females. We observed a significant increase in osteocalcin levels in response to ethanol for rhesus females (3.4 ng/ml, $p = 0.04$), no change for cynomolgus females (0.9 ng/ml, $p = 0.6$), and weak tendencies for reduction for males of both species (rhesus: −1.2, $p = 0.2$ ng/ml, cynomolgus: −1.7 ng/ml, $p = 0.1$). These interactions are shown in Fig. 2C.

We observed a significant linear dose-response relationship for ethanol and CTX ($p = 2.7 \times 10^{-2}$; Fig. 3A, Table 3), and a significant decrease in CTX level with age ($p = 3.3 \times 10^{-3}$). The mean level of CTX decreased 0.12 ng/ml for each half unit (0.5 g/kg/d) increase in ethanol dose (95% CI 0.01–0.22 ng/ml). The relationship between age and CTX levels is shown in Fig. 3B, where the mean level of CTX decreased 0.10 ng/ml for each unit increase in age (95% CI 0.03–0.17 ng/ml). The interaction effects between dose, species, and sex were not significant in this model, suggesting that the relationship between ethanol and CTX levels is similar for individuals regardless of species or sex (Fig. 3C).

When all monkeys were combined, we found no significant relationship between ethanol dose and the osteocalcin to CTX ratio ($p = 0.41$; Fig. 4A, Table 3). Sex ($p = 3.6 \times 10^{-3}$), species ($p = 8.6 \times 10^{-4}$), and age ($p = 8.7 \times 10^{-4}$) were significant predictors. Age had a negative effect, where each unit increase in age decreased the mean value of the ratio by 1.42 (95% CI 0.61–2.23; Fig. 4B). Male rhesus macaques had higher values of the osteocalcin to CTX ratio than rhesus females with a significant interaction between sex and ethanol dose ($p = 1.4 \times 10^{-2}$), highlighting an increase in osteocalcin to CTX ratio for rhesus females with increasing ethanol (Fig. 4C). We observed a positive linear trend between osteocalcin to CTX ratio and ethanol dose for both rhesus and cynomolgus females (rhesus: 2.96, $p = 1.3 \times 10^{-3}$; cynomolgus: 1.79, $p = 3.4 \times 10^{-2}$; Fig. 4C). However, we did not observe a significant trend in the osteocalcin to CTX ratio in response to ethanol in males of either species (rhesus: 0.4, $p = 0.41$; cynomolgus: 0.2, $p = 0.71$; Fig. 4C).

Limited statistical power in this study due to sample size ($N = 91$) may impact our ability to detect significant interaction effects of ethanol dose with demographic variables on markers of bone turnover. From a post-hoc power analysis for linear multiple regression (alpha = 0.05) with eight predictors, including interaction effects, we determined that our study has a 70% probability (power = 0.70) to detect a medium effect ($f^2 \geq 0.15$), and a 12% probability (power = 0.12) to detect a small effect ($f^2 \geq 0.02$) (Selya et al., 2012). A sensitivity power analysis suggests that we are adequately powered (power = 0.80) to detect medium effects ($f^2 = 0.18$) with eight predictors. The results are similar when

Table 3
Effects of ethanol dose, species, sex and age of onset of drinking on biomarker levels. Categorical variables shown in reference to male rhesus macaques. Cyno = cynomolgus macaque.

Biomarker	Variable	Beta	Std dev.	T	p
Osteocalcin	Intercept	42.34	3.95	10.72	2.1×10^{-23}
	Dose	−1.13	0.88	−1.29	0.2
	Sex (F)	−11.87	3.01	−3.95	1.0×10^{-4}
	Species (cyno)	−5.80	2.12	−2.73	6.6×10^{-3}
	Age	−2.98	0.61	−4.89	1.5×10^{-6}
	Dose:Sex (F)	4.54	1.87	2.43	1.6×10^{-2}
	Dose:Species (cyno)	−0.56	1.36	−0.41	0.68
	Sex (F):Species (cyno)	10.41	4.33	2.40	1.7×10^{-2}
	Dose:Sex (F): Species (cyno)	−1.95	2.62	−0.75	0.46
CTX	Intercept	2.16	0.22	9.59	1.6×10^{-19}
	Dose	−0.12	0.05	−2.22	2.7×10^{-2}
	Sex (F)	−0.28	0.18	−1.57	0.12
	Species (cyno)	0.05	0.13	0.41	0.68
	Age	−0.10	0.03	−2.96	3.3×10^{-3}
	Dose:Sex (F)	0.04	0.12	0.31	0.76
	Dose:Species (cyno)	−0.07	0.08	−0.80	0.42
	Sex (F):Species (cyno)	0.35	0.26	1.36	0.18
	Dose:Sex (F): Species (cyno)	−0.03	0.16	−0.16	0.87
Osteocalcin: CTX	Intercept	25.0	2.64	9.46	4.5×10^{-19}
	Dose	0.40	0.49	0.83	0.41
	Sex (F)	−5.51	1.88	−2.93	3.6×10^{-3}
	Species (cyno)	−4.44	1.32	−3.36	8.6×10^{-4}
	Age	−1.38	0.41	−3.36	8.7×10^{-4}
	Dose:Sex (F)	2.57	1.04	2.48	1.4×10^{-2}
	Dose:Species (cyno)	−0.19	0.75	−0.25	0.80
	Sex (F):Species (cyno)	4.62	2.72	1.70	0.09
	Dose:Sex (F): Species (cyno)	−0.99	1.45	−0.68	0.49

considering a repeated measures ANOVA framework.

4. Discussion

We evaluated the dose-response effects of alcohol, covering a range that models moderate drinking (alcohol contributed 7% of total calories) to abusive drinking (alcohol contributed 21% of total calories), on biochemical markers of bone formation (osteocalcin) and bone resorption (CTX) in two closely related species of macaques. Additionally, we investigated whether the dose-response effects of alcohol on the biomarkers were influenced by sex, age of onset of alcohol consumption, or species. The results indicate that species, age, and sex influenced levels of biochemical markers of bone turnover, particularly osteocalcin. The results also indicate that alcohol altered biochemical markers in a dose-dependent manner that was influenced by sex and species but not by age.

Osteocalcin, CTX, and their ratio are often used in human studies as markers of global bone turnover and turnover balance, respectively (Cabral et al., 2016; Kuo and Chen, 2017; Qvist et al., 2002). We chose osteocalcin as a representative bone formation marker, in part, because it can be evaluated at the gene level in cell culture and animal models to obtain mechanistic insight, and we have been collecting osteocalcin data for alcohol studies for ~25 years, facilitating comparison across experiments. Similarly, we have used CTX as a marker for bone resorption in alcohol studies for over a decade. Osteocalcin, a hydroxyapatite-binding protein, is produced by osteoblasts and primarily deposited into bone matrix during bone formation. However, osteocalcin is also released into plasma in proportion to the formation process (Zoch et al., 2016). Osteoclast-mediated destruction of bone matrix results in the release of CTX into plasma in proportion to the rate of bone resorption (Cabral et al., 2016). The utility of these biomarkers for minimally invasive assessment of bone turnover in non-human primates is well established (Chen et al., 2000).

Combining all monkeys, we found (1) no change in osteocalcin levels with increasing alcohol consumption, (2) a decrease in CTX levels, and (3) no change in osteocalcin to CTX ratio. The significant interaction between ethanol dose and sex suggests that the influence of ethanol on osteocalcin levels differs between males (no change) and rhesus females (increase). No significant interaction between dose, sex, and species on CTX levels were detected, suggesting that the relationship between ethanol and CTX levels is similar for monkeys regardless of sex or species. Males had higher values of the osteocalcin to CTX ratio, and a significant positive interaction between sex and ethanol dose suggests that the increase in osteocalcin to CTX ratio was limited to females. Importantly, while species influenced the levels of markers of bone turnover and the response to alcohol, the direction of response is consistent across species, increasing the likelihood that the findings can be generalized to humans.

Based on earlier work, our failure to detect reduced osteocalcin levels following dose-response increases in alcohol consumption was initially a surprise. We reported that 12 months of chronic ethanol consumption in male rhesus macaques resulted in a strong trend for a reduction in osteocalcin and reduced cancellous bone formation (Kahler-Quesada et al., 2019). In another study, we observed dose-dependent reductions in osteocalcin levels in one cohort (cohort 13) of young adult (6.5–6.8 years old) male cynomolgus macaques (Sattgast et al., 2021). However, we have also shown evidence that the alcohol-induced suppression of initiation of intracortical bone remodeling precedes reduced bone formation (Gaddini et al., 2015). Specifically, chronic voluntary alcohol consumption resulted in decreased bone porosity with no change in surface-based indices of bone formation. Based on an anticipated large contribution of intracortical bone remodeling to biochemical markers of bone turnover in blood, relatively short duration alcohol studies in monkeys would be anticipated to result in net decreases in bone resorption associated with reduction in initiation of new osteons and not necessarily reduced bone formation. Furthermore, a sex difference was detected in the response; the model suggests osteocalcin levels increased with alcohol dose in rhesus females whereas there was no significant change in osteocalcin levels in males.

Linear bone growth occurs through endochondral ossification and in humans approaches completion in most girls by 16 years of age and in most boys by 18 years of age. However, bone continues to be added onto bone surfaces such that peak bone mass does not occur for another decade. A similar process occurs in cynomolgus and rhesus macaques. Linear growth ceases by ~5.5 years of age in cynomolgus females and ~6.5 years of age in cynomolgus males (Fukuda et al., 1978) and bone mass peaks at ~9 years of age (Chen et al., 2000; Jayo et al., 1994). Linear growth ceases by ~5.3 years of age in rhesus females and ~6.0 years of age in rhesus males (Cheverud, 1981) and bone mass peaks at ~10 years of age (Pope et al., 1989). During growth and skeletal maturation, formation exceeds resorption such that there is a net increase in bone mass. Cross sectional studies performed in female cynomolgus macaques demonstrate a dramatic decrease in levels of biochemical markers of bone formation, including osteocalcin, prior to peak bone mass with a much smaller diminution in markers of bone resorption, implying that reduced bone formation with age rather than

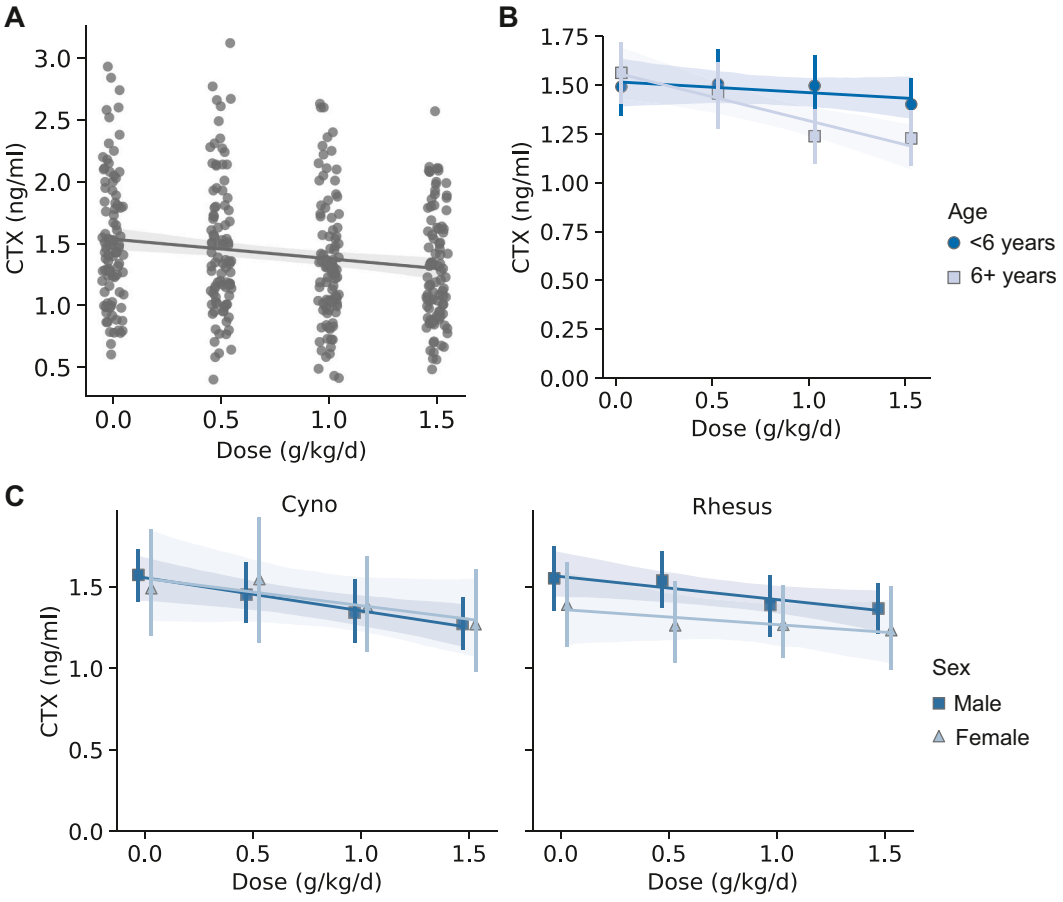

Fig. 3. We observed a significant overall negative dose-response relationship for CTX levels. (A) The linear relationship between ethanol dose and CTX levels. Each black point shows the observed value for an individual animal. Confidence bands are 95% bootstrapped confidence intervals. (B) The relationship between ethanol dose and CTX stratified by age (>6 years or 6+ years old). The age groups were chosen to visualize differences between late adolescent/young adult animals (<6 years old) and adult animals that have reached skeletal maturity (6+ years old). Each point is the mean CTX level for animals in a specific age group and dose; however, we note that the regression considers age as a continuous variable. Confidence bands are 95% bootstrapped confidence intervals. (C) No significant interaction effects for dose, sex, and species on CTX levels. The relationship for cynomolgus (left) versus rhesus (right) macaques is plotted with regression lines for males and females. The confidence bands are 95% bootstrapped confidence intervals.

increased bone resorption is responsible for achievement of bone turnover balance at skeletal maturity (Chen et al., 2000). While modeling – the process by which bone is added to or removed from preexisting bone surfaces resulting in changes in bone shape – is essential for establishing peak bone mass and normal bone architecture during growth, remodeling – a coupled process initiated by bone resorption and then followed by bone formation at the same location to maintain bone shape – predominates in adults to maintain bone quality through repair of fatigue damage generated during normal physical activity. Intracortical remodeling, the principal form of bone remodeling in large animals, results in a transient increase in porosity as resorption cavities are formed and then filled. In humans, one remodeling cycle requires 4–6 months to complete. As in humans, intracortical bone remodeling occurs in non-human primates. The duration of a remodeling cycle has not been established with certainty, but long duration (1 year) studies performed in male rhesus macaques (Gaddini et al., 2015) suggest that inhibition of initiation of bone remodeling by alcohol would result in a positive bone turnover balance observed in the current shorter duration studies in females. It should be noted that this positive relationship is anticipated to be transient; bone formation will slow as preexisting (to initiation of drinking) Haversian canals (osteons) mature. In support, higher levels of alcohol consumption for longer durations was shown to result in a negative cancellous bone remodeling balance in lumbar vertebra of male rhesus macaques, resulting in bone loss (Kahler-Quesada et al., 2019).

It is well established that regular alcohol consumption can suppress bone accrual in rodents, resulting in relative osteopenia (Gaddini et al., 2016). However, the magnitude of suppression appears to be strongly influenced by how alcohol is delivered. Specifically, continuous incorporation of alcohol into the diet led to suppression of bone formation whereas delivery by gavage to model binge drinking did not negatively impact bone formation (Hogan et al., 2001; Iwaniec and Turner, 2013; Sampson et al., 1999). Few studies have been performed in skeletally mature rodents to evaluate the effects of alcohol on bone remodeling (Gaddini et al., 2016). However, one study reported a dose-dependent suppression of cancellous bone remodeling where lower levels of alcohol, modeling moderate drinking, uniformly lowered bone formation and bone resorption. In contrast, levels modeling chronic alcohol abuse resulted in a disproportionate reduction in bone formation leading to cancellous bone loss (Turner et al., 2001). Few studies have been performed in large animals to date. However, we have shown that voluntary heavy alcohol consumption in rhesus macaques suppresses initiation of intracortical bone remodeling leading to a decrease in cortical porosity (Gaddini et al., 2015).

Our previous work highlights the effects of long-term exposure to alcohol on measures of skeletal health, including cortical porosity and osteon density (Gaddini et al., 2015; Kahler-Quesada et al., 2019). These effects were concordant with measurements of markers of bone turnover, and our current results are consistent with this work. Due to the nature of the protocol, we do not have access to bone architecture data from the induction period, however, we expect that changes in

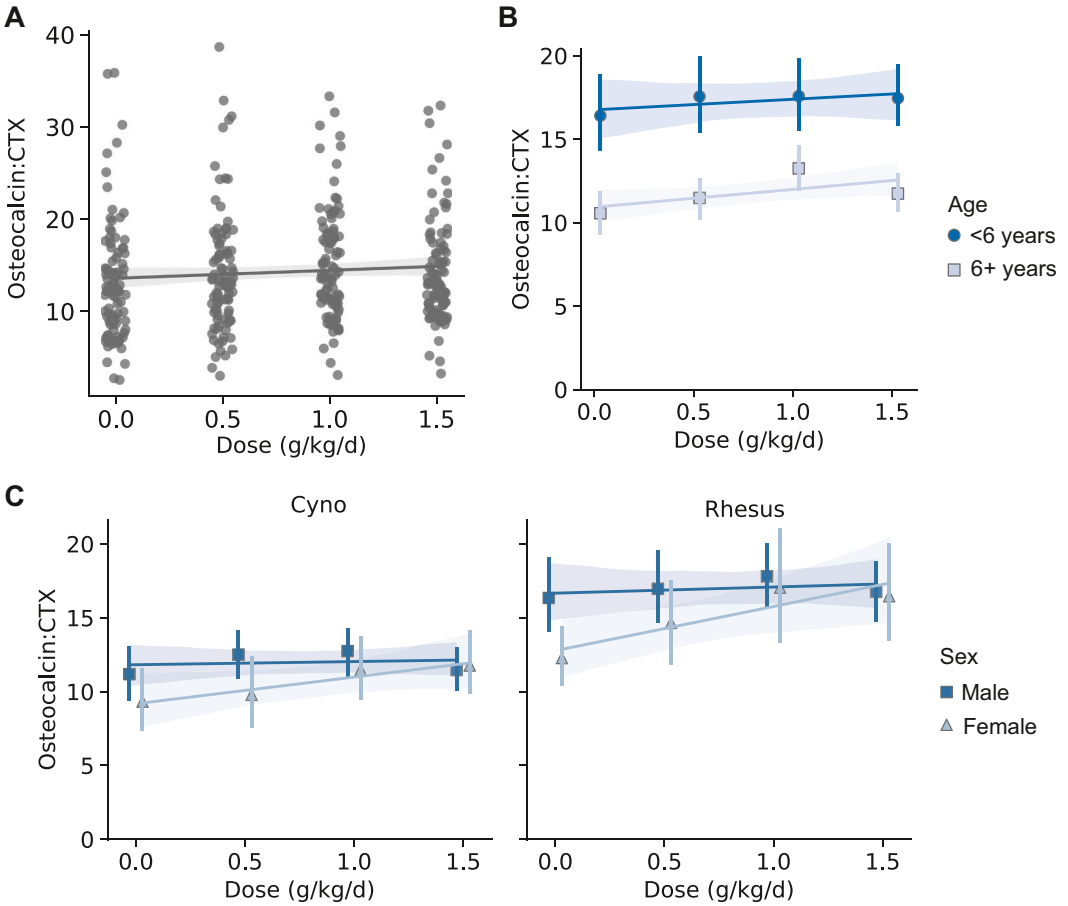

Fig. 4. We detected no overall dose-response relationship for the ratio of osteocalcin to CTX. (A) The linear relationship between ethanol dose and the osteocalcin to CTX ratio. Each black point shows the observed ratio for an individual animal. Confidence bands are 95% bootstrapped confidence intervals. (B) The relationship between ethanol dose and osteocalcin to CTX ratio (osteocalcin:CTX) stratified by age (>6 years or 6+ years). The age groups were chosen to visualize differences between late adolescent/young adult animals (<6 years old) and adult animals that have reached skeletal maturity (6+ years old). Each point is the mean osteocalcin to CTX ratio for animals in a specific age group and dose; however, the regression considers age as a continuous variable. Confidence bands are 95% bootstrapped confidence intervals. (C) There was a significant interaction effect for ethanol dose and sex on the osteocalcin to CTX ratio. The relationship for cynomolgus (left) versus rhesus (right) macaques are plotted with regression lines for males and females. The confidence bands are 95% bootstrapped confidence intervals.

biomarker levels are associated with changes in skeletal architecture over time. Regarding effect size, a 3.5% reduction in CTX in healthy postmenopausal women who drank moderately was associated with significantly higher total hip BMD (Marrone et al., 2012). This suggests that the effect sizes observed in this study (0.2 ng/ml or ~13% at the 1.5 g/kg/day level) are likely large enough to associate with meaningful skeletal changes.

As with other consortium studies, our analysis was constrained by the original experimental design. An additional potential limitation of the present study is that we only evaluated one biomarker for bone formation and one biomarker for bone resorption. However, a large cross-sectional study performed in female cynomolgus macaques evaluating biochemical markers of bone turnover reported similar age-related changes in osteocalcin, total alkaline phosphatase and bone specific alkaline phosphatase (Legrand et al., 2003). Although CTX was not measured in that study, we and others have found good agreement between circulating levels of CTX and other indices of bone resorption (Gaddini et al., 2015; Gingery et al., 2020). Other potential limitations include the influence of menstrual cycle in females, which was not evaluated, and circadian differences in levels of the biomarkers. The present studies were of relatively short duration; monkeys were required to increase their consumption of ethanol in 30-day increments. However, we and others have shown changes in blood levels in CTX and/or osteocalcin can occur in shorter intervals in response to alcohol or abstinence (Marrone et al., 2012; Sripanyakorn et al., 2009). Circadian changes were not evaluated, but blood was routinely collected at the same time of day in all animals. Plasma was stored at −80 °C for intervals ranging from 4 to 12 years prior to analysis. Studies have demonstrated no loss of immunoactivity of osteocalcin and CTX following 3 years of storage at temperatures ≤ 20 °C for CTX or − 70 °C for osteocalcin, but we cannot rule out that degradation occurred with longer duration storage (Qvist et al., 2004; Carlson et al., 2004).

In summary, cynomolgus and rhesus macaques experienced an alcohol-induced dose-dependent inhibition of plasma CTX levels, indicating a net reduction in bone resorption. The female monkeys experienced a corresponding increase in osteocalcin to CTX ratio, predicting a positive bone turnover balance. Sex and species, but not age, influenced the skeletal response to alcohol. Specifically, there were positive interactions between alcohol dose and osteocalcin to CTX ratio in females but not in males. Taken together, these findings support the concept that factors beyond quantity of alcohol determine the long duration skeletal response to alcohol consumption.

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Availability of data and material

All data is available through the Monkey Alcohol and Tissue Research Resource (MATRR).

Ethics approval

All authors attest to the appropriate application of ethical standards.

Consent to participate

All authors give their consent to participate.

Consent for publication

All authors give their consent for publication.

CRediT authorship contribution statement

Mary Lauren Benton: Methodology, Formal analysis, Visualization, Writing – original draft. **Vanessa A. Jimenez:** Investigation, Writing – review & editing. **Natali Newman:** Investigation, Writing – review & editing. **Steven W. Gonzales:** Investigation, Writing – review & editing. **Kathleen A. Grant:** Conceptualization, Writing – review & editing, Funding acquisition. **Russell T. Turner:** Conceptualization, Writing – original draft, Writing – review & editing. **Urszula T. Iwaniec:** Conceptualization, Writing – review & editing, Funding acquisition. **Erich J. Baker:** Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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