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Measurement of 25-hydroxyvitamin $D_{2\&3}$ and 1, 25-dihydroxyvitamin $D_{2\&3}$ by Tandem Mass Spectrometry: A Primate Multispecies Comparison

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Abstract

Vitamin D metabolites are widely studied for their roles in bone health, immune functions and other potential physiologic roles in humans. However, the optimal blood levels of vitamin D metabolites are still unclear. Various methods for measuring vitamin D metabolites have been used and recently liquid chromatography tandem mass spectroscopy (LC-MS/MS) has been adopted as the gold standard for vitamin D metabolite measurement. Here we report the use of LC-MS/MS to measure 25-hydroxyvitamin D ($25(OH)D_{2\&3}$), and 1,25-dihydroxyvitamin D ($1,25(OH)_2D_{2\&3}$), in three laboratory nonhuman primate species: common marmoset (*Callithrix jacchus*), rhesus macaque (*Macaca mulatta*), and cynomolgus macaque (*Macaca fascicularis*), and compare them to humans using the same technique. The nonhuman primates showed blood levels for $25(OH)D_3$ and $1,25(OH)_2D_3$ significantly higher than human values with marmosets having the highest levels. Marmoset samples showed significantly more variability among individuals than those from macaques for both metabolites, but all three nonhuman primate species exhibited large variation within species for both $25(OH)D_{2\&3}$ and $1,25(OH)_2D_{2\&3}$. Marmoset females had significantly lower values than the males for $25(OH)D_3$, while rhesus males showed a significant decrease in $25(OH)D_3$ with age. The most striking finding is the variation within species for vitamin D levels even in laboratory primates that have a controlled diet, UV exposure, and in some cases, genetic constraints. Similar variation in 25(OH)D responses to a fixed dose of oral vitamin D supplementation has been reported in humans. We suggest that these species can provide primate models for examining the factors influencing variation in the levels of vitamin D necessary for human and nonhuman primate health.

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Keywords

marmoset; rhesus; cynomolgus; 25 hydroxyvitamin D; 1,25 dihydroxyvitamin D

INTRODUCTION

Vitamin D has multiple physiologic roles and affects multiple systems, organs, hormones and cells [Bikle, 2010]. Vitamin D comes in two forms: cholecalciferol is Vitamin D_3 and erogcalciferol is Vitamin D_2. Vitamin D_3 is synthesized in the skin of vertebrates during exposure to UVB rays from the sun and is converted to 25 hydroxyvitamin D_3 in the liver. It is the most common form found in primates and although the skin is the major route it is also found in fish, eggs, meat, dairy and poultry where it is absorbed in the intestinal tract. Vitamin D2 is found in fungi and some plants and is a lesser form than vitamin D_3. It also is converted to 25 hydroxyvitamin D_2 in the liver. Metabolite measurements have largely focused on circulating 25 hydroxyvitamin $D_{2\&3}$ (25[OH]$D_{2\&3}$) which is widely recognized as the measurement to define an individual's vitamin D status [Ross et al. 2011] and the bioactive form 1,25 dihydroxyvitamin $D_{2\&3}$ (1,25$[OH]_2D_{2\&3}$) which is a metabolite of 25[OH]$D_{2\&3}$. Measurement of these metabolites has dramatically increased due to implications of a vitamin D role in cancer prevention, neural function, immune modulation and effects on inflammation [Holick 2007].

How to define optimal vitamin D status is controversial and expert group recommendations are not congruent [Ross et al 2011; Holick et al. 2011]. Meta- and even mega-analyses, have failed to clarify optimal 25(OH)D levels [Hilger et al. 2014]. As an example, a report of 195 studies from 44 countries found no significant age or sex-related differences in 25(OH)D although there have been reports of significantly lower levels in aged people and may predict mortality [Lee et al., 2013]. Importantly, virtually all studies find substantial heterogeneity in 25(OH)D levels even in populations within limited geographical regions [Hilger et al. 2014]. Moreover, major between-individual variability exists in 25(OH)D response to oral vitamin D supplementation [Binkley et al. 2011; Aloia et al. 2008]. The factors causing this variability are not adequately defined.

Nonhuman primates are excellent translational models [Schnatz et al. 2012] that, given their similar physiology, may enhance understanding of human vitamin D metabolism. However, they have not been widely investigated for the study of vitamin D measurement and actions. Indeed, only a relatively small number of studies (using different measurement methods) have reported vitamin D metabolite levels in marmosets (*Callithrix jacchus*), rhesus (*Macaca mulatta*), and cynomolgus macaques (*Macaca fascicularis*). Reported values of 25(OH)$D_{2\&3}$ and 1,25$(OH)_2D_{2\&3}$ for nonhuman primates generally are higher than human values [Shinki et al. 1983]. Most New World primate levels reported are 2–10 fold higher than Old World monkeys (Adams et al., 1985; but see Crissey et a., for lower values in *Callimico goeldii*) probably due to high expression of intracellular vitamin D binding proteins and concurrent high baseline levels of 1,25$(OH)_2D_3$. Additionally New World monkeys cannot utilize vitamin D_2 and rely solely on vitamin D_3 [Marx et al. 1989].

Various methods exist to measure vitamin D metabolites including RIA (radioimmunoassay), ELISA (enzyme linked immunosorbent assay), HPLC-UV (high pressure liquid chromatography - ultraviolet detection) and LC/MS/MS (liquid chromatography - tandem mass spectrometry) [Binkley et al. 2009]. ELISA and RIA methods do not separately measure the two forms (D_2 and D_3) without prior extensive separation extractions. Automated immunoassays are the most common methodology in routine human clinical use. Multiple comparisons of the various methodologies with LC/MS/MS have been performed [Koivula et al. 2013]; as a generalization, there is reasonable agreement between methods, but greater variability with immune based approaches. International efforts are ongoing to standardize vitamin D metabolite measurements; this essential effort is being facilitated by the availability of standard reference materials from the National Institute of Standards and Technology (NIST) [Binkley and Sempos 2014; Sempos et al. 2012].

Vitamin D metabolite measurement in research and some clinical laboratories has largely shifted to LC-MS/MS methodology which allows simultaneous measurement of vitamin D_2 and D_3 metabolites and is capable of detecting the low (pg/mL) levels of $1,25(OH)_2D_{2\&3}$ [de la Hunty et al. 2010]. Mass spectrometry methods, while very sensitive, require derivatization to measure these low $1,25(OH)_2D_{2\&3}$ levels [Netzel et al. 2011; Hedman et al. 2014]. We recently reported on a LC-MS/MS method for $25(OH)D_{2\&3}$ used in our laboratory that requires a low sample volume of 50 µl with a 5 minute run time [Hedman et al. 2014]. Sample volume and method sensitivity are important when measuring vitamin D metabolites in nonhuman primates due to lower blood volume available for analysis.

Despite the advantages of LC-MS/MS, to our knowledge there is only one lab that has measured vitamin D metabolites using this methodology in nonhuman primates, [Schnatz et al. 2012] and that study evaluated only one species, the cynomolgus monkey. Here we present LC-MS/MS vitamin D metabolite data from three nonhuman primate research species: the rhesus monkey, the cynomolgus monkey, and the common marmoset, and compare human values obtained using the same methodology. We performed this study to: 1) establish normal values for these species under laboratory conditions using an LC-MS/MS methodology that is traceable to the NIST standards; 2) provide a comparison with human data, 3) use the state-of-the-art methodology and the same methods for all species, and 4) to evaluate the three nonhuman primates as models for disease related research.

METHODS

Subjects

Serum samples were collected as a one-time blood draw from representative monkeys of each species at the Wisconsin National Primate Research Center (WNPRC). We used 25 adult monkeys in good health from each species. All marmosets and rhesus macaques used in this study were born at the Wisconsin National Primate Research Center (WNPRC). The cynomolgus monkeys were originally from an outdoor, provisioned colony on the island of Mauritius where the very small founding population has reduced genetic differences among individuals [Burwitz et al., 2009], but all had lived at the WNPRC for 14 months prior to use. The cynomolgus monkeys (mean ± SD = 6.8 ± 0.8 years; range of 6.7 to 8 years) were

all males, while the rhesus and marmoset monkeys were both male and female adults. The rhesus group included 18 males and 7 females (mean ± SD = 7.83 ± 4.86 years; range of 3 to 25 years). The marmoset group consisted of 15 males and 10 females (mean ± SD 6.2 ± 1.79 years; range of 3 to 10 years). None of the rhesus females were pregnant at the time of the blood sampling, as this would alter their vitamin D metabolite concentration [Novakovic et al., 2009; Zhang et al., 2014]. However, half of the marmoset females were in very early pregnancy, prior to receiving estrumate (prostaglandin F2a analogue to end the luteal phase; Mobay Corp., Shawnee KS) to terminate the pregnancy. Mean levels of $25(OH)D_3$ were not different between females who were pregnant (256.4 ± 64.43, n=5) compared to non-pregnant females (289.32 ± 101.41, n=5; P = 0.79, t = 0.27, df = 8) and mean levels of $1,25(OH)_2D_3$ were not significantly different between females who were pregnant (864.0 ± 135.6, n=5) compared to non-pregnant females (755.8 ± 148.46, n=5; P = 0.79, t = 0.27, df = 8) so it is unlikely that the early pregnancies contributed to the relationship of the vitamin D metabolites. All blood-sampling procedures reported in this study were in a protocol reviewed and approved by the University of Wisconsin Graduate School Animal Care and Use Committee (ACUC). The research adhered to the American Society of Primatologists Principles for the Ethical Treatment of Non Human Primates.

All monkeys were housed indoors with controlled lighting conditions 12:12 hr light:dark) and temperature and humidity. Light was provided by fluorescent lamps (GE F32T8) that emit UV light in the spectrum 400–100 nm) with resin coating that attenuates UV transmission in the range 380–180 nm. The plastic lamp covers further attenuate UV transmission.

Diet and vitamin D supplements

Both macaque species received the same diet produced by Harlan (#2050, Teklad Global 20% Protein Primate Diet, Madison, WI). The diet contained 1% calcium and vitamin D_3 as 8,000 IU/kg or 200 ng/g cholecalciferol. The amount of diet provided to individual macaques was based on estimated energy needs (i.e., body condition scores, age and appetite) and ranged from approximately 65 to 170 grams/day. The diet was supplemented with fresh fruits and vegetables daily.

The marmosets were fed Mazuri Callitrichid High Fiber Diet (Mazuri 5MI6, Land O'Lakes, Arden Hills, MN) once per day in an amount that would provide on average 20 grams per animal in the group, as well as browse material daily (fruit, mealworms, vegetables). The marmoset diet contained 1.1% calcium and Vitamin D_3 as 5,690 IU/kg or 142 ng/g cholecalciferol. It was supplemented with a Vitamin D_3 as a premix (TD.10936.PWD, as 8 g:992 g maltodextrin) to bring the total concentration of D_3 in the diet to 13,000 IU/kg or approximately 26 IU per animal per day.

Vitamin D metabolite analyses

For between species comparisons, the samples from the three nonhuman primates were processed simultaneously and along with human samples and analyzed identically for comparison of the vitamin D metabolites. We used 20 human serum samples obtained from a reference laboratory (ARUP Laboratories, Salt Lake City, Utah, USA; http://

www.aruplab.com) and analyzed for their vitamin D metabolites: 25(OH)$D_{2\&3}$ and 1,25$(OH)_2D_{2\&3}$ along with the nonhuman primate samples. The ARUP samples were analyzed both by the University of Wisconsin Hospital Clinics and our Assay Services Laboratory at the WNPRC. These human samples have a known value to which we found matching results. Details have been published (Hedman et al., 2014). Acetonitrile was added to samples to be analyzed for 25(OH)$D_{2\&3}$ for protein precipitation according to the method developed by AB SCIEX (Foster City, CA). For the macaques and human samples, 50 μl of serum was combined with 100 μl acetonitrile containing 12 ng/ml of d6–25(OH)D_3 as the deuterated internal standard. For the marmoset, 10 μl of serum plus 40 μl of distilled water was added to the acetonitrile. The sample was vortexed, sonicated for 10 minutes, then centrifuged at 14,500 rpm for 5 minutes. An 100 μl aliquot was put into a minivial for LC/MS/MS analysis. Vitamin 25(OH) $D_{2\&3}$ calibrators were purchased from the National Institute of Standards and Technology (NIST 972A, Gaithersburg, MD) and quality control pools were purchased from Utak Laboratories (Valencia, CA). Calibrators and pools were extracted in an identical manner as the unknowns. Liquid chromatography separated the 25(OH) $D_{2\&3}$ on a Phenomenex Luna column of 3 μ C8(2), 100A (50 mm × 2.0 mm); gradient: A=0.1%formic acid in ultrapure water, B=acetonitrile with 1% formic acid; program %B at 0 minutes = 50%, 2.7 to 3.7 minutes = 98%, 3.8 min=50% on a Shimadzu Prominence (Addison, Il, USA) integrated PLC interfaced with an AB SCIEX (Foster City, CA) QTRAP 5500 Quadrupole – Linear Ion Trap Mass Spectrometer equipped with an ACPI source. The lowest level of detection was 0.5 ng/ml for both 25(OH)$D_{2\&3}$.

Aliquots of all the samples were then tested for 1,25$(OH)_2D_{2\&3}$ concentrations. The extraction procedure, derivatization and LC/MS/MS procedures followed our recently published method (Hedman et al., 2014). All samples from each species were analyzed at the same volume. 200 μl of serum was combined with 700 μl of deionized water and 20 μl of internal standard (50 pg, d6–1,25-$(OH)_2D_{2\&3}$) before double extracting with a dual column SPE (solid phase extraction) using Chromabond XTR (6 ml, 1 g, Nacherey-Nagel) and silica (6 cc, 500 mg, Waters, Milford, MA). Dried samples were derivatized with Amplifex Diene (AB SCIEX) by adding 30 μl of a 1.5 mg/ml solution, vortexed, centrifuged and incubated for 30 minutes. Deionized water was then added at 30 μl and the samples were transferred into injection vials for LC injection. Samples were eluted at a flow rate of 0.25 ml/min using a binary reversed phase gradient (Channel A = 0.1% formic acid in 18 MΩ/cm water, Channel B = methanol) as follows: 0 min, 2% B; 1 min, 2% B; 17.8 min, 65% B; 18 min, 100% B; 19.8 min, 100% B; 20 min, 2% B; 30 min, 2% B. Relevant MS/MS settings were: CAD gas at 6 psig; CUR at 20 psig; GS1 at 60 psig; GS2 at 30 psig; IS at 5000 V; EP at 10 V; and TEM at 600 °C. The limit of quantitation was 15.63 pg/ml. Intra-assay coefficient of variation, CV, for the 1,25$(OH)_2D_3$ was 5.79. Since all samples were run at the same time, there was not an inter-assay CV. As part of the NIST program our mass spectrometry results for 25(OH)$D_{2\&3}$ values show excellent placement.

Statistical analyses

The 25(OH)D_3 and 1,25$(OH)_2D_3$ data for the four species (marmoset, rhesus, cynomolgus and human), were determined to be normal by the D'Agostino & Pearson omnibus normality test. Since the vitamin D_2 metabolites were either very low or nondetectable, there

were no statistics performed for comparison between species. To determine if the values for the monkey species were different, we used One-Way Analysis of Variance, ANOVA. Post-hoc differences were analyzed by Tukey's Multiple Comparison Test. Bartlett's test for equal variances was used to determine the variability within each species for the vitamin Ds and to compare it with the other species. We used Pearson's r to test for correlation with a two-tailed P value. Unpaired t-tests, two-tailed, were used to compare marmoset samples analyzed by two different methods.

RESULTS

Mean vitamin D metabolite concentrations were compared to other previously published methods for the D_3 forms. The prior reports contrasted with our data as they only reported the D_3 form, or the two forms were not separated (Table I). Our present study indicated numerically higher levels of $25(OH)D_3$ than all but one study. In an attempt to validate the higher values obtained by LC-MS/MS, marmoset samples in this study were compared with other marmoset samples from the WNPRC colony analyzed by RIA $25(OH)D_3$, (Diasorin); $1,25(OH)_2D_3$, (WI NPRC in-house RIA). We found no significant difference between the RIA and LC-MS/MS for $25(OH)D_3$ ($t = 0.83$, df 68, $P = 0.41$) but did find significant differences for $1,25(OH)_2D_3$ where the LC-MS/MS method provided higher levels (LC-MS/MS, mean ± SEM = 726 ± 85; RIA = 464 ± 64; $t = 2.45$, df = 18, $P = 0.03$). For rhesus monkeys, our $25(OH)D_3$ results were similar to some of the mean values previously reported, although several other studies were substantially lower. The cynomolgus monkey values were similar to the rhesus monkeys.

ANOVA indicated that significant differences for $25(OH)D_3$ were found between the values for the 4 species ($F = 38$, df = 3,84, $P = 0.0001$, Fig. 1). Marmosets were significantly higher than all the other species ($P<0.05$) and rhesus and cynomolgus were significantly different from the human values ($p<0.05$). The marmoset showed a large range of concentration. Significant differences were also found between the species for $1,25(OH)_2D_3$ ($F = 84$, df = 3,88, $P = 0.0001$). Marmoset values were significantly higher than all the other species ($P<0.05$) and showed more variability. Additionally, both rhesus and cynomolgus had significantly higher values than human ($P<0.05$). Bartlett's test for variances indicated that for each comparison the marmoset showed significantly more variability than the other species for the vitamin D forms ($P<0.0001$). The frequency distribution of $25(OH)D_3$ levels is shown for all three species (Fig. 2).

Vitamin D_3 is the predominant form of vitamin D to supplement the diet in the nonhuman primates but Vitamin D_2 is found in some food products. There was almost no detectable $25(OH)D_2$ measured by our selective measurements for any of the nonhuman primates. The marmosets did not show any detectable $25(OH)D_2$ measurement. For humans, however, all samples had detectable levels of $25(OH)D_2$ but only as 1/100 of the levels of D_3. Conversely, with $1,25(OH)_2D_2$ both the rhesus and cynomolgus monkeys had detectable levels and had higher levels than humans (Fig. 3).

Marmosets and macaques were compared for sex differences in vitamin D metabolites (Fig. 4). Marmosets showed a significant sex difference in levels of $25(OH)D_3$ where females had

significantly lower levels than males (t = 2.5, df = 23,*P* = 0.02). There was a trend towards a significant difference in $1,25(OH)_2D_3$ for the marmosets but the trend was for higher $1,25(OH)_2D_3$ levels in females (t = 2.0, df = 21, *P* = 0.06). The rhesus monkeys did not reveal sex differences for $25(OH)D_3$ (t = 0.54, df = 23, *P* = 0.59) or for $1,25(OH)_2D_3$ (t =1.6, df = 22, *P* = 0.13).

We then assessed whether age had an association with vitamin D metabolite levels. Age as a factor was compared with both $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations to determine if there was a relationship that might explain some of the variance found in the vitamin D concentration. Age did not correlate with either $25(OH)D_3$ or $1,25(OH)_2D_3$ neither in the marmoset, nor for rhesus age with $25(OH)D_3$. However, rhesus age significantly correlated with $1,25(OH)_2D_3$ (r = −0.42, n = 24 *P* = 0.04) so that the older the rhesus monkeys the lower levels of $1,25(OH)_2D_3$. For the cynomolgus monkeys there was no association of $25(OH)D_3$ with age but a weak correlation of age and $1,25(OH)_2D_3$ (r = 0.39, n = 25, *P* = 0.05).

We examined the body weights of the monkeys at the time of the evaluation of the vitamin D levels and found no evidence of an association with any of the vitamin D metabolites for the weight of the marmoset, rhesus or cynomolgus monkeys.

Examination of the relationship between food allocation, an approximation of food intake, revealed a significant positive correlation with $25(OH)D_3$ (r = 0.44, *P* = 0.02) for rhesus macaques and marginally significant correlations with $1,25(OH)_2D_3$ and total $1,25(OH)_2D$ (r = 0.38 for both, *P* < 0.057) for cynomolgus macaques only.

DISCUSSION

Our data provides a comparison of vitamin D values within and between the three most commonly used laboratory nonhuman primate species and human subjects using the most sensitive and specific methods to date. With the use of LC/MS/MS we were able to make these comparisons for both of the most studied vitamin D metabolites and in both the D_2 and D_3 forms using the same methodology. Few nonhuman primate studies have values for D_2 in any species. The values we have from this method allowed us to validate the significant differences in vitamin D concentration between species and to document the large variations that occur within species, as it has been reported for humans (Hilger et al., 2014).

In order to establish our methodology we have used human samples that are part of a reference laboratory (ARUP, USA, http://www.aruplab.com) and validated the method with a sensitive RIA and with the most recent sensitive LC/MS/MS technique [Hedman et al. 2014]. Furthermore, our 25(OH)D assay is compliant with the NIST program. Our reported results for these samples reflect the results from other nonhuman primate sample methodologies. While we had similar values to other human studies, our mean for $25(OH)D_3$ was higher than reported for other studies [Shinki et al. 1983; Orgaz-Molina et al. 2013]. The human samples we used are selected for the range they provide in values, and therefore, do not necessarily reflect the normal range found for humans. However, we can reliably report the values we have for the nonhuman primate species and compare them to

reports in the literature that use different methodology. Our values for the marmoset, rhesus and cynomolgus monkeys were on the high end of reported values for both $25(OH)D_3$ and $1,25(OH)_2D_3$ but not out of the range of values reported by other studies. This may be due to the mass spectrometry method having the ability to ionize the vitamin D compounds so effectively that it picks up more of the analyte than immunoassays or simply due to inaccuracies of the prior methods. As expected, we found that vitamin D metabolite values for the marmoset were significantly higher than other primate species and that rhesus and cynomolgus monkeys had values significantly higher than humans. We discuss our findings below by species.

Marmosets

Most New World primates have been reported to have extremely high levels of circulating vitamin D_3 metabolites in both the $25(OH)D_3$ and $1,25(OH)_2D_3$ forms with levels two to 10 times higher than those found in Old World primates and humans [Adams et al., 2003; Gacad et al. 1992; Crissey et al. 1999 with the exception of Callimico]. Several species of New World primates have a high expression of competitive binding proteins intracellular that bind to the vitamin D metabolites and require elevated levels of both $1,25(OH)_2D_3$ for activation of cells and $25(OH)D_3$ as the substrate for $1,25(OH)_2D_3$ production [Adams et al. 2003; Liberman et al. 1985; Teixeira et al. 2012]. Our study also indicated that levels were higher for New World primates for both vitamin D metabolites. Additionally, since our method measured both D_2 and D_3 forms for the metabolites, we also demonstrated that marmoset D_2 was not measurable in our marmoset samples and therefore supports previous findings that D_2 is not absorbed and reflected in $1,25(OH)_2D$ [Marx et al. 1989].

Our data revealed high variability in vitamin D metabolite values for both $25(OH)D_3$ and $1,25(H)_2D_3$ forms. The levels we have found for $25(OH)D_3$ were generally higher than the levels in Jarcho et al., [2013] by around 40% but showed a wide range in values. Their study demonstrated that marmosets with significantly reduced $25(OH)D_3$ values were associated with a significantly reduced digestive efficiency; indicating a lack of the ability to absorb food in the digestive tract. They suggest that marmosets have a wide range of digestive abilities that are reflected in the $25(OH)D_3$ levels and that low $25(OH)D_3$ levels may indicate marmosets that are more likely to exhibit metabolic bone disease in the future [Jarcho et al. 2013]. Our data only revealed very low $25(OH)D_3$ in two samples out of 25 and these samples did not show the concordant low levels in $1,25(OH)_2D_3$. In fact no correlation between the levels of $25(OH)D_3$ and $1,25(OH)_2D_3$ was found, indicating that $25(OH)D_3$ do not always predict $1,25(OH)_2D_3$ levels from marmoset/=85 samples.

The marmosets have very high levels of vitamin D_3 added to their diet. The WNPRC Marmoset Colony provides 30% more vitamin D supplement than the SWNPRC colony when compared to the Jarcho et al. [2013] study. This may explain why our marmosets did not show concordant low levels in $25(OH)D_3$. Marmosets that are free-living in the wild and exposed to natural light have a range of $25(OH)D_3$ between 20.1–103.3 ng/ml (Teixeira et al. 2012) which was much lower than the range we found: 4–875 ng/ml. It is also possible that the WNPRC marmosets were not only absorbing their vitamin D from the supplement but also generating it in their skin as a result of some exposure to UV exposure from the

lighting in their rooms. Marmosets are well known for hanging from the top of their cage upside down, exposing their hairless abdomen to light, as they would hang from branches outdoors. Nonetheless, exposure to UV light under the conditions of our vivarium is not expected to be a major contributor to circulating vitamin D.

There could be a number of factors responsible for the wide variation found in the concentration in circulation, in particular, for $25(OH)D_3$. As mentioned previously, this variation could be due to the absorption problems that have been reported in marmosets or the lack of the $25(OH)D_3$ could be the cause of the lack of absorption [Jarcho et al. 2013]. Additionally, nonhuman primates, like other mammal species with hair/fur covering the majority of their bodies, do not have exposed skin visible for vitamin D absorption from UV radiation to initiate the cascade creating $25(OH)D_3$. However, as occurs with many mammals, it is likely that the vitamin D generated through the oily secretions deposited onto the fur can be obtained orally through grooming, thereby adding an additional source of $25(OH)D_3$ and the variability that occurs in both $25(OH)D_3$ and $1,25(OH)_2D_3$ [Carpenter and Zhao 1999].

The higher amount of vitamin D_3 in the diet of the WNPRC marmosets is reflected in the higher measurements of $25(OH)D_3$ and $1,25(OH)_2D_3$ measured by our LC/MS/MS methods. New methods are currently being developed that will allow us to measure the breakdown products of vitamin D (24-OH-D, 24,25-OH D) as well as circulating levels of colecalciferol. These further measurements would provide insights into what are appropriate levels for the marmoset and what levels might compromise the liver's ability to hydroxylate the vitamin.

Rhesus

Rhesus monkeys exhibited higher levels of both $25(OH)D_3$ and $1,25(OH)_2D_2$ than humans as have previously been reported [Shinki et al. 1983; Marx et al. 1989]. Notably, our values for $25(OH)D_3$ were most similar to the values reported for rhesus macaques living outdoors and free-range at Cayo Santiago, Puerto Rico [Vieth et al. 1987]. The Cayo Santiago rhesus monkeys have access to UV from the sunlight consistently throughout the day and continuously throughout the year while also having 8.0 IU/g of vitamin D_3 supplemented in their diet. If the methods can be reliably compared, then it would appear that the exposure to sunlight and vitamin D conversion in skin has not increased the levels of $25(OH)D_3$ in circulation over the dietary component. One possibility would be that the high levels of the dietary cholecalciferol inhibit skin conversion by UV. Alternatively, the dense hair covering the rhesus macaque body significantly lowers the skin's ability to absorb UV rays needed for vitamin D conversion.

Levels of $1,25(OH)_2D_3$ were also similar between the Cayo rhesus and our results. The WNPRC rhesus monkeys showed consistent results with little variation in $25(OH)D_3$ levels and more variation in the $1,25(OH)_2D_3$ levels, which may indicate the importance of keeping a consistent level of $25(OH)D_3$ circulating and ready for hydroxylase conversion as demanded.

A significant decrease in $1,25(OH)_2D_3$ levels with increased age was reported for the Cayo rhesus monkeys [Vieth et al. 1987]. While our study and the Cayo Santiago rhesus study did not show differences in $1,25(OH)_2D_3$ levels by sex, they both showed it by age. However, the Cayo Santiago study found only male age influenced the decline of $1,25(OH)_2D_3$ and our study did not see a sex difference in age effect. This could be due to the limited number of females that were in the study and lack of selection of rhesus monkeys to produce a continuum of ages. Aging in humans is associated with a significant decline in $25(OH)D_3$ in circulation(Resmini et al., 2013). There appears to be a reduction in the effective synthesis of vitamin D from the skin. A significant decrease in the amount of 7-dehydrocholesterol in the skin occurs with age in humans. Additionally, the conversion of UV light from the sun into pre-vitamin D3 is the main source of vitamin D as the amount of vitamin D_2 in the diet is minimal.

Both the rhesus and the cynomolgus monkey showed measurable levels of $1,25(OH)_2D_2$ without showing measurable levels of $25(OH)D_2$. This must indicate that the $25(OH)D_2$ was lower than the sensitivity of our 25(OH)D assay and therefore was not recorded. The source of the $1,25(OH)_2D_2$ would have to be from the diet. Although no vitamin D2 was added to the diet, brewers dried yeast is a component of the diet. Ergosterol is produced in yeast and can be absorbed and converted into $25(OH)D_2$ [Fryberg et al. 1972]. Vitamin D_2 is not as potent as vitamin D_3, and not as readily available [Cranney et al. 2007], although at the tissue level differences appear to be minor since $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ are comparable in binding the vitamin D receptor [Bikle 2010]. In nonhuman primates there is variability in how vitamin D2 is absorbed and available for use.

Cynomolgus

As expected, the cynomolgus monkeys were very similar to the rhesus monkeys in vitamin D metabolite levels. At the WNPRC the macaques were fed identical diets and housed in the same type housing. Cynomolgus monkeys in this study fell into a relatively narrow range of age, were males, were Mauritian derived, and therefore expected to be very genetically similar. Yet, Fig. 2 indicates the wide variability of $25(OH)D_3$ found in the males. One possibility would be if differences in intestinal health influenced the absorption in vitamin D as occurs in the marmosets [Jarcho et al. 2013]. Investigators using cynomolgus as models for human related vitamin D research have documented that variability exists even when all the monkeys are fed a controlled diet [Schnatz et al. 2012]. They hypothesized that the variability is largely dependent on genetics. However, if in a genetically constrained species, such as Mauritian cynomolgus macaques, there is still much variability, then this may be an environmental or epigenetic influence affecting the levels of $25(OH)D_3$. We did, however, find a significant correlation between vitamin D levels and individual food allocations, a rough approximation of food intake. While our cynomolgus monkeys were all males for this study, the fetoplacental unit during pregnancy is a major site of $1,25(OH)_2D$ production for both D_2 and D_3 [Markestad et al. 1984]. This exposure could influence the rate of synthesis in the offspring's dihydroxylated vitamin D through maturity.

CONCLUSIONS

While there were different levels of the two forms of vitamin D metabolites observed between the species, the most notable differences were within each species. Humans show considerable variation in their 25(OH)D, some of which may come from measurement techniques, but recently these have been greatly reduced. All three of the nonhuman primate species we examined also showed significant variation between individuals within a species. Since much of the differences in exposure to UV radiation has been eliminated in laboratory primates the individuals within each species should be getting equal amounts of dietary vitamin D. This indicates there has to be other variables affecting the vitamin D concentration.

Genetics could explain differences in laboratory cynomolgus monkeys with controlled dietary vitamin D, sun exposure, and menopausal status [Schnatz et al. 2012]. Yet more factors than genetics could be in play. Absorption of dietary vitamin D can be variable per individual within a species. Binding to chylomicrons absorbs Vitamin D within the gastrointestinal tract. It is then transported by the lymphatic system to the blood circulatory system where it binds with vitamin D binding protein and is transported to the liver for metabolism or to adipose tissue for storage. The rate and extent of 25(OH)D production is dependent upon vitamin D hydroxylase and its inactivation by cyctochromes P-450 CYP24, CYP3A4 and the vitamin D binding protein. Markers in the corresponding genes may contribute to the variations found within a species [Mallah et al. 2011].

Environmental influences might also cause the variability within a species for vitamin D metabolite differences. In utero exposure to mother's vitamin D levels may predispose an individual to levels that influence the absorption of dietary D or limit the rate of the hydroxylase needed to convert the 25(OH)D. Exposure to illness could influence the absorption ability of the intestinal tract as well. There are a number of factors that could explain the variability and these primate models offer the opportunity to examine these factors.

The rhesus macaque and the cynomolgus macaque would make ideal models for humans in studying the sources of the variability in vitamin D levels and how this influences the multitude of roles both 25(OH)D and $1,25(OH)_2D_3$ have in the body, from bone homeostasis, neuroprotection and endocrine responses to immune function. The marmosets show the most variability and are possibly the most sensitive to disruptions in vitamin D levels. Determining the cause of the extremely low levels of 25(OH)D that can exist would greatly benefit the health of marmosets. Additionally, marmosets could be models for *in utero* and epigenetic effects on vitamin D variability due to their twinning and short generation time.

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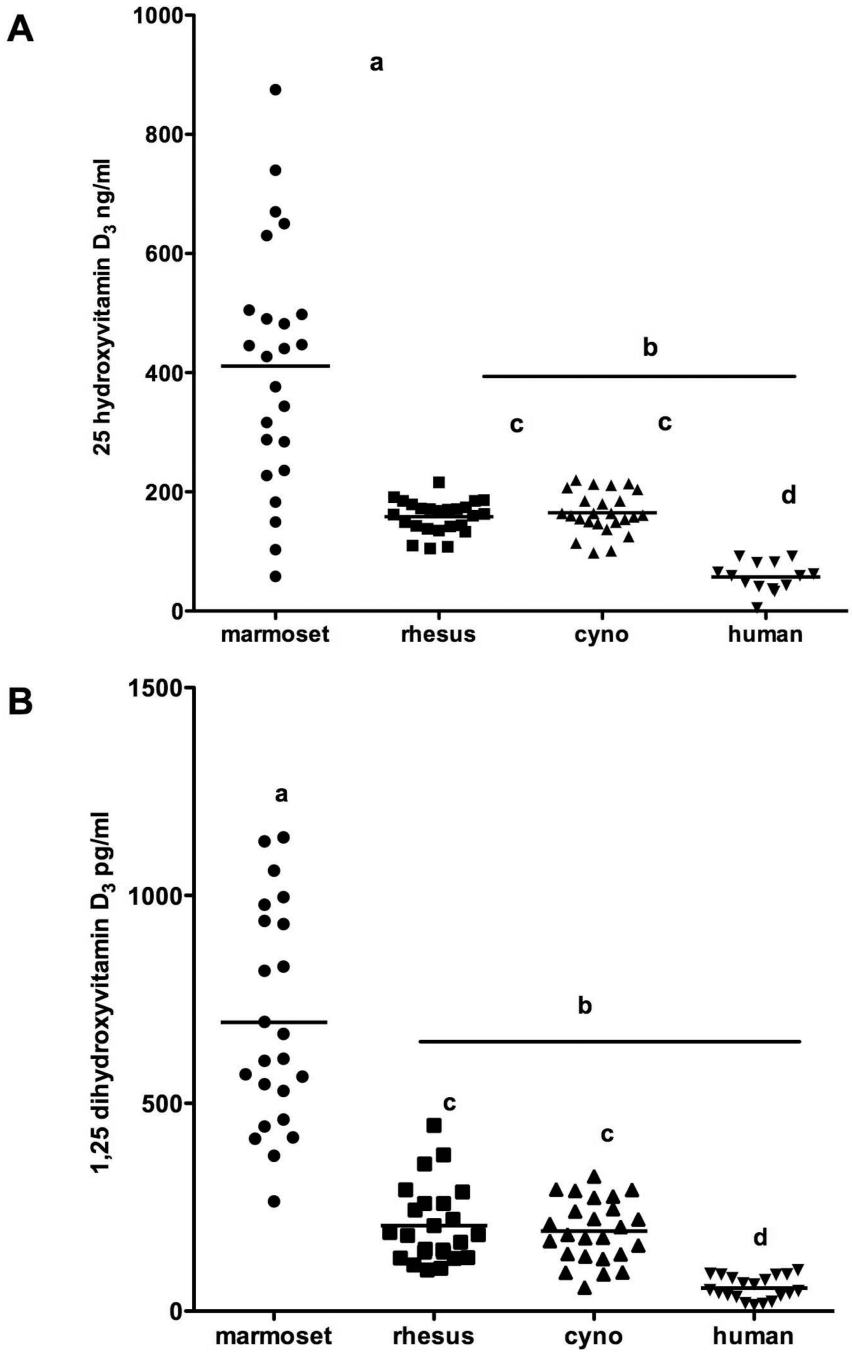

Fig 1.

Comparison of vitamin D levels between the species including humans

A. 25 hydroxyvitamin D_3, B) 1,25 dihydroxyvitamin D_3,for four primate species. Lines indicate medium for the samples of each species. Significance is indicated by: **a** is significantly different to **b, c** is significantly different to **d**.

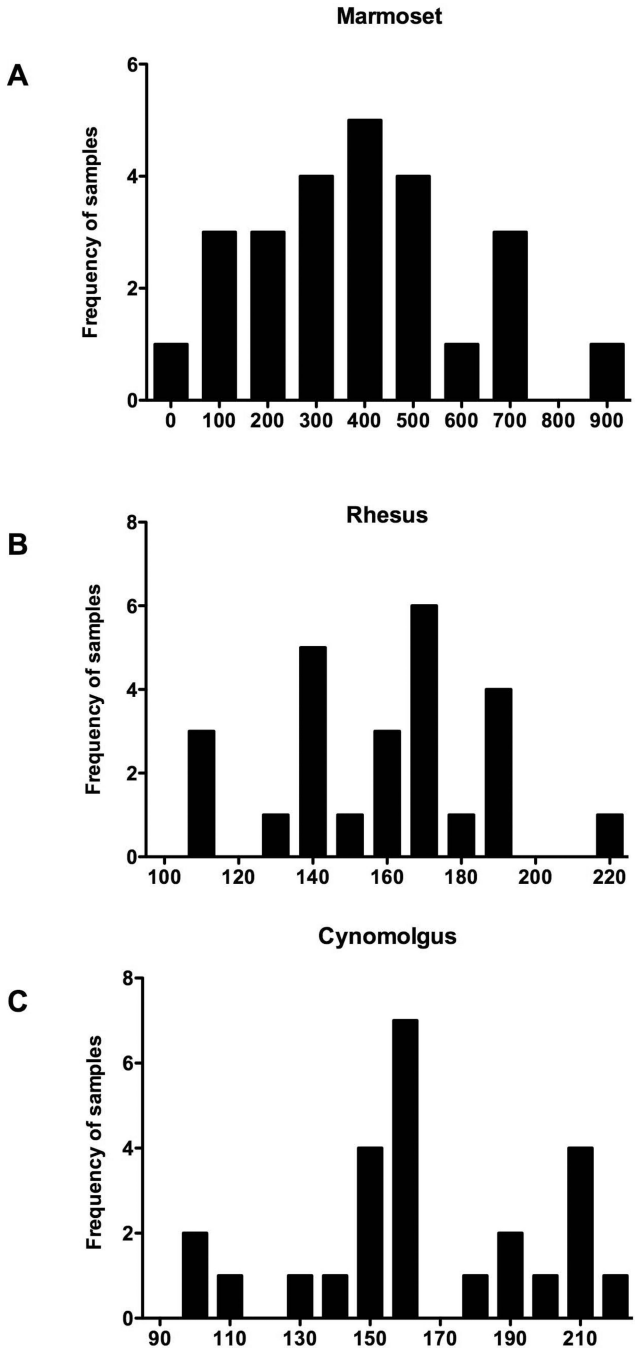

Fig 2.

A histogram of the frequency distribution of samples with 25 hydroxyvitamin D_3 values for the three nonhuman primate species indicating the variability found in the vitamin D levels: A) common marmoset, B) rhesus macaque, C) cynomolgus macaque.

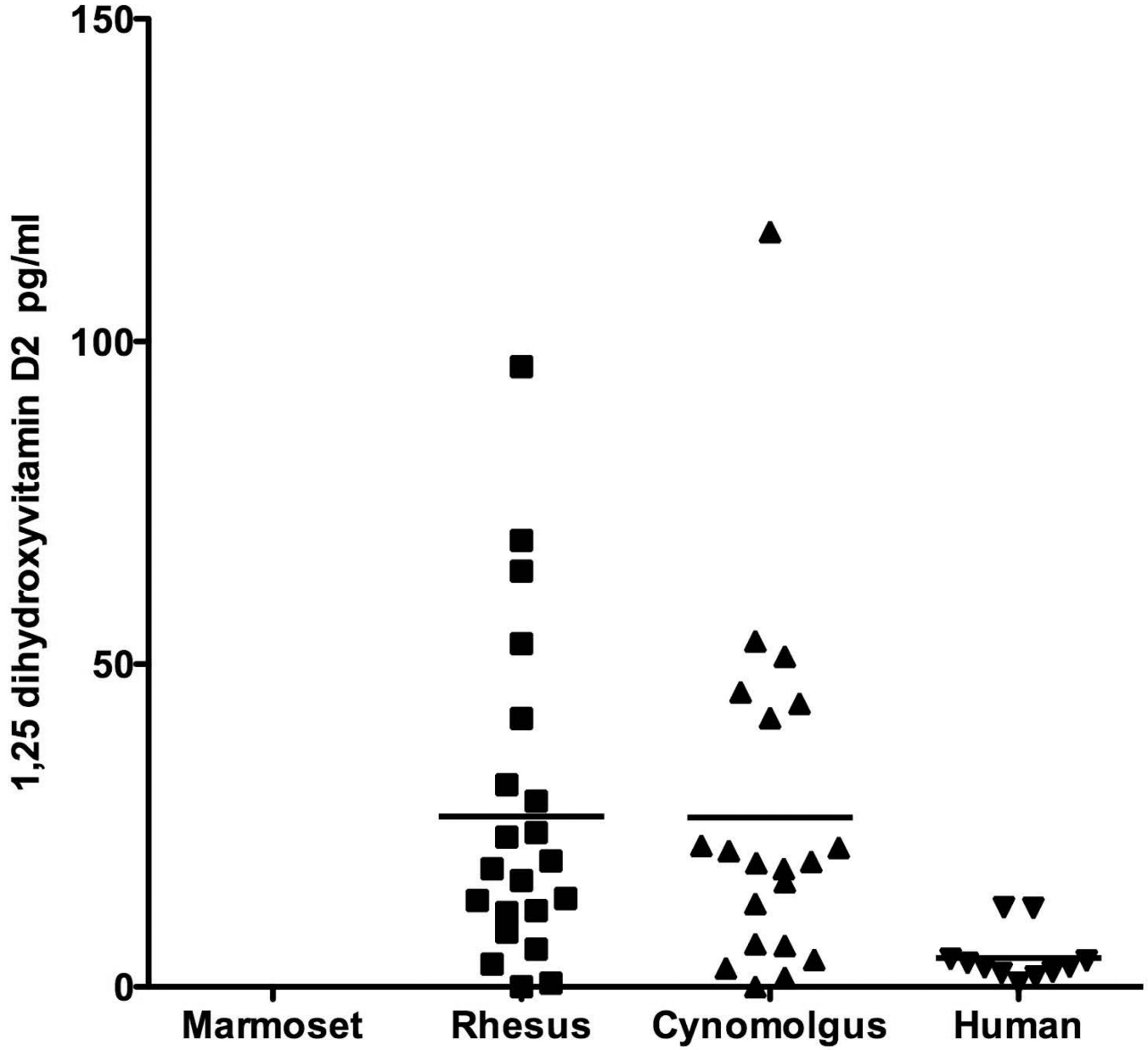

Fig 3.

Comparison of the amount of 1,25 dihydroxyvitamin D_2 found in the serum samples of the four species: marmoset, rhesus, cynomolgus and human. Horizontal lines indicate the mean for samples of each species. Marmosets did not show any D2 measurement while the macaques showed a higher range of variation than did humans.

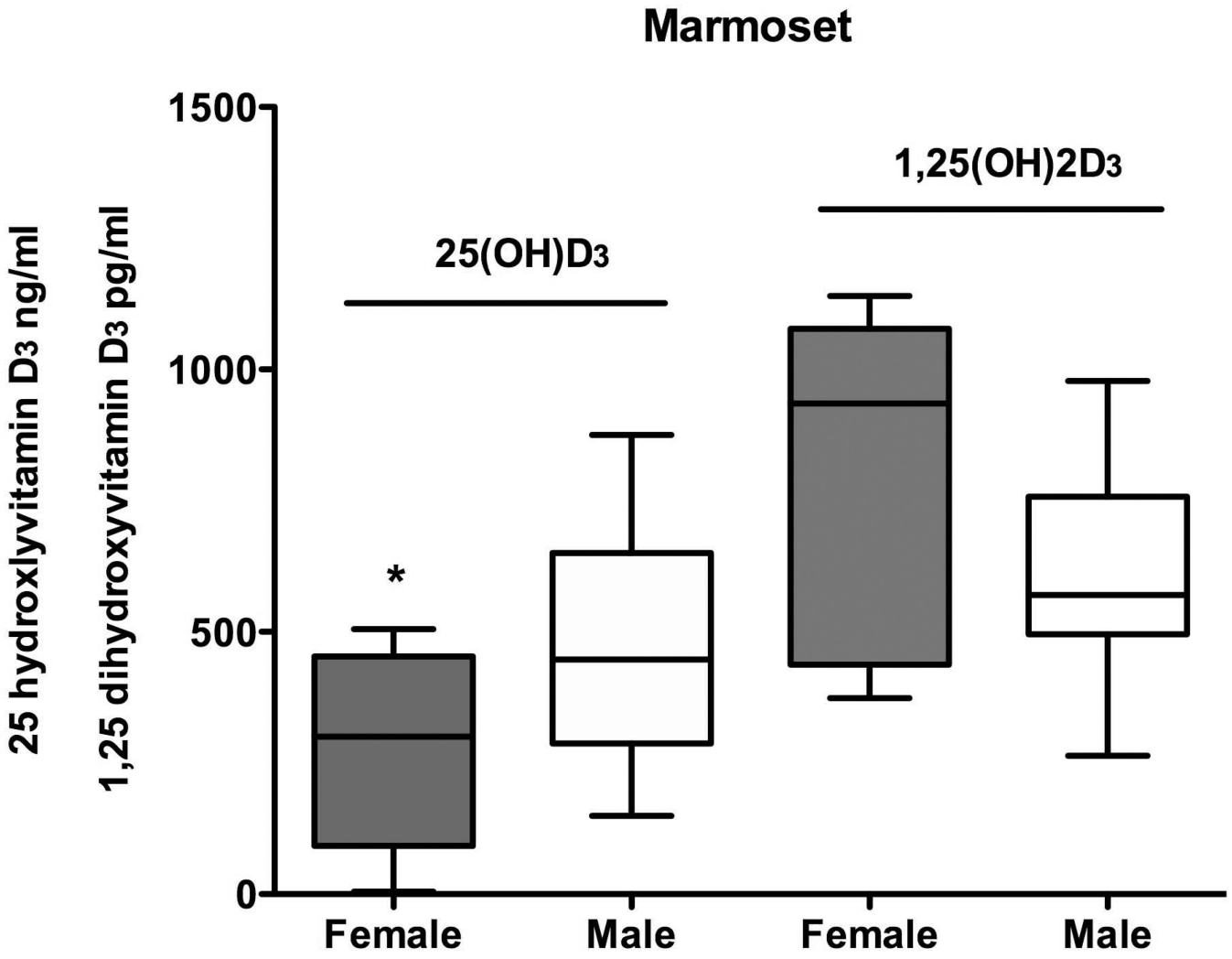

Fig 4.

Comparison of values for male and female marmosets for 25 hydroxyvitamin D_3 and 1,25 dihydroxyvitamin D_3. Female levels were significantly lower than males for 25 hydroxyvitmain D_3.

TABLE I

Comparisons of mean 25-hydroxyvitamin D ($25(OH)D_3$) and 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) concentrations from this study with concentrations from other methods and facilities for four primate species; Common marmoset (*Callithrix jacchus*); rhesus monkey (*Macaca mullata*), cynomolgus monkey (*Macaca fascicularis)* and human (*Homo sapiens*).

$25(OH)D_3$ ng/ml	$1,25(OH)2D_3$ pg/ml	Method	Reference
Marmoset			
394.81±43.5 (25)	694.78±52.3 (25)	LC/MS/MS	this study
94.51±30.5 (7)	361.5±60.6 (7)	HPLC/RIA	Shinki et al., 1983
474±96 (17)	481±82 (17)	HPLC/RIA	Yamaguchi et al., 1986
61.7±20.8 (15)	-	RIA	Teixeira et al., 2012*
119±46.2 (7)**	-	RIA	Jarcho et al., 2013
Rhesus			
154.84±5.5 (25)	205.91±18.5 (24)	LC/MS/MS	this study
50±5.1 (6)	100+10 (6)	HPLC/RIA	Shinki et al., 1983
50±4 (5)	95±17 (5)	HPLC/RIA	Yamaguchi et al., 1986
198.36±62.25 (20)	169.62±34.6 (20)	HPLC/RIA	Vieth et al., 1987
114.8±8.6 (11)	-	RIA	Colman et al., 1999
10–30*** (44)	-	RIA	Black et al., 2001
144.23±24.0 (6)	-	HPLC/UV or RIA	Marx et al., 1989
Cynomolgus			
164.81±6.9 (25)	192.76±14.9	LC/MS/MS	this study
48.9+16.6 (74)	-	LC/MS/MS	Schnatz et al., 2012
96.15±16.0 (6)	-	HPLC/UV or RIA	Marx et al., 1989
Human			
57.01±6.6 (14)	55.26±6.14 (20)	LC/MS/MS	this lab
20±2.5 (6)	45±3.1 (6)	HPLC/RIA	Shinki et al., 1983
30.38±9.9 (61)	-	RIA	Orgaz-Molina et al., 2013

Numbers are mean ± standard error (N)

* *Callithrix pencillata*, a closely related marmoset species

** Based on Study 1

*** range, including males and females