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Identification of a mechanism for increased cardiovascular risk among individuals with low vitamin D concentrations.

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Running Title: Cardiovascular Risk with Low Vitamin D

Précis:

Low concentrations of vitamin D_3 were associated with lower HDL-C concentrations and a worsening lipid profile when monkeys were fed a moderately atherogenic diet.

Abstract:

Objective: To investigate plasma concentrations of vitamin D and the association with plasma lipid profiles. **Methods:** Plasma vitamin D_3 and lipid concentrations were measured in 119 female cynomolgus monkeys (premenopausal n=49; ovariectomized n=70) consuming 1,000 IU/day of vitamin D_3. In a subset of the ovariectomized monkeys (n=23), Vitamin D_3 was re-measured after 6 months. Concentrations of vitamin D_3 were analyzed as a continuous variable and were divided at the median into higher (High, ≥48 ng/mL) *vs*. lower (Low, <48 ng/mL) groupings. **Results:** Among the 119 monkeys, the range of vitamin D_3 concentrations was 24.0 to 95.2 ng/mL (mean ± SD = 48.5 ± 12.7 ng/mL). Plasma vitamin D_3 concentration was positively associated with high density lipoprotein cholesterol (HDL-C) (p=0.003). Monkeys in the High vitamin D_3 group had a significantly greater plasma HDL-C concentration (57.9 mg/dL) than those in the Low vitamin D_3 group (47.1 mg/dL, p=0.001). Although the difference was not significant (p=0.120), monkeys in the High Vitamin D_3 group had a decreased total plasma cholesterol (TPC)/HDL-C ratio compared with the low Vitamin D_3 group (5.4 and 6.2, respectively), potentially putting them at lower risk for atherosclerosis development. **Conclusions:** Given that the monkeys all consumed a diet replete in vitamin D_3, it appears that individual differences in vitamin D absorption or metabolism may have determined whether the monkeys had high or low concentrations of vitamin D_3. Lower vitamin D_3 was associated with a more atherogenic lipid profile, a major risk factor for progressing to coronary artery atherosclerosis in monkeys and human beings.

Key Words: Cardiovascular / Coronary Heart Disease; Vitamin D Concentrations; High Density Lipoprotein; Lipid Profiles; Menopause

Introduction:

It has been known for many years that vitamin D is necessary in maintaining bone health by promoting intestinal absorption of calcium and phosphorus.[1] Vitamin D also is essential for the prevention of rickets[2] and osteoporosis.[3] For these reasons, the prevalence of vitamin D deficiency is an important health issue. According to the Third National Health and Nutrition Examination Survey (NHANES III), vitamin D deficiency is present in 25% to 57% of American adults.[4] Additional studies indicate similar deficiencies in children, adolescents, and elderly populations.[5,6] In the past, a deficiency has been defined as having a plasma 25(OH)D level below 15 ng/mL. A higher risk of deficiency is posed to individuals of darker skin pigmentation and individuals who live at higher latitudes due to decreased sun exposure.[7] Current data suggest that plasma 25(OH)D concentrations should be >30 ng/mL, and ideally 36-40 ng/mL for improved health outcomes.[8] Achieving these levels may require increasing daily vitamin D intake to 600-800 IU per day[8-10] as opposed to previous dietary standards which suggested 400 IU per day for individuals younger than 70 and 600 IU for individuals 70 or older.[11]

The results of more recent studies have suggested that additional benefits of vitamin D include improved overall health and chronic disease prevention. Vitamin D has been associated with the prevention of rectal cancer,[12] prostate cancer,[13] and breast cancer.[14] In addition to cancer prevention, Vitamin D has links to prevention of autoimmune diseases, such as multiple sclerosis[15] and rheumatoid arthritis,[16] along with type I[17] and type II diabetes.[18]

Coronary heart disease (CHD) also has been linked to a deficiency in Vitamin D. Well-documented risk factors for CHD include tobacco use, hypercholesterolemia, hypertension, obesity, diabetes mellitus, gender specific age, and a family history of CHD.[19,20] The findings of a recent study showed an inverse relationship between low plasma 25(OH)D concentrations (<15

ng/mL) and first-time cardiovascular events with an increased hazard ratio of 1.6 to 2.1.[21] The results of other studies have shown a higher incidence of hypertension and CHD in higher latitudes suggesting that those with lower exposure to sunlight, and hence lower vitamin D concentrations, may have a higher risk of heart disease.[22,23] CHD,[24] myocardial infarction,[25] sudden cardiac death,[26] stroke,[27] peripheral arterial disease,[28] greater carotid intima-media thickness,[29] and hypertension[30] all have been associated with low plasma 25(OH)D levels.

Despite an extensive search, we were able to find only a few articles analyzing the relationship between vitamin D and plasma cholesterol. The results of several older studies suggested higher vitamin D concentrations were associated with worsening cholesterol parameters[31,32], while a few newer studies have suggested improved parameters.[21,22] One report found that higher concentrations of vitamin D may be associated with a lower total plasma cholesterol (TPC) to high density lipoprotein cholesterol (HDL-C) ratio (TPC/HDL-C).[21] The results of another investigation showed lower triglyceride (TG) concentrations in those with higher vitamin D measurements.[33] We identified two reports suggesting an association between higher vitamin D concentrations and increased HDL-C[34,35] as well as a lower prevalence of metabolic syndrome[34], major determinants of CHD risk. However, these studies showing cholesterol improvement have potential confounding factors including compliance, recall bias, variations in ethnicity and other factors associated with population-based studies.[21,33] For these reasons, new suggestions that vitamin D supplementation is mostly unnecessary[9,10], along with the Institute of Medicine's challenge to continue targeted research related to vitamin D[10], the objective of this research was to investigate the relationship between vitamin D and plasma lipid concentrations. More specifically, we sought to evaluate whether, and to what extent, low plasma

concentrations of vitamin D are associated with a more atherogenic plasma lipid profile in a cohort of female cynomolgus monkeys.

Methods:

Animals and Diets:

The study utilized a total of 119 female cynomolgus monkeys (*Macaca fascicularis*) which were imported from Institute Pertanian Bogor in West Java, Indonesia (the Indonesian Primate Center, *i.e.*, the Pusat Studi Satwa Primata). To increase the translational value of the study and to recreate the variations in human exposures (hormonal, dietary, and sun exposure), the monkeys studied represented both premenopausal and surgically postmenopausal subjects, monkeys that had consumed a moderately atherogenic diet for a relatively long period (26 months) and a relatively short period (4 months) and were housed either indoors or indoors with access to an outdoor run (indoor-outdoor). All of the monkeys were determined to be adult (*i.e.*, middle aged and beyond), and all consumed the same amount of vitamin D_3 (a woman's equivalent of 1,000 IU/day). Of the total 119 monkeys, 49 were premenopausal and 70 were post-menopausal (ovariectomized). All monkeys were socially housed with 2-5 monkeys per pen. The indoor-outdoor housing group consisted of 50 ovariectomized monkeys. The indoor housing group (n=49 premenopausal, n=20 ovariectomized) was housed in a facility with large tinted windows along both sides of the building to allow light, but to screen out some of the direct ultraviolet sunlight. All monkeys were fed a controlled diet which was prepared in the animal diet laboratory at the Wake Forest University Primate Center and were formulated to be equivalent for cholesterol, macronutrient content (*i.e.*, protein, fat, carbohydrate), and vitamin D_3.

All procedures involving animals in this study were conducted in compliance with state and federal laws, standards of the US Department of Health and Human Services (DHHS), and guidelines established by the Wake Forest University Institutional Animal Care and Use Committee (IACUC) where the animals were housed.

Vitamin D Measurement:

Plasma vitamin D_3 concentrations were measured in all of the 119 female cynomolgus monkeys (premenopausal n=49; ovariectomized, n=70) at a single time point. A subset of the ovariectomized monkeys had vitamin D_3 re-measured after 6 months (n=23). All vitamin D assays were done at The Reading Hospital and Medical Center. Frozen samples (500 μL) were transported to The Reading Hospital and Medical Center and protected from direct sunlight, a technique which has been shown to yield stable results.[36] We utilized the HPLC/tandem mass spectrometry for the 25(OH)D assay, with a determination for D_3. Our approach utilized the Shimadzu liquid chromatography - mass spectrometry/mass spectrometry (LC-MS/2) technology. The liquid chromatography - mass spectrometry prepares the sample to be ionized, through physical separation capabilities of liquid chromatography, for mass analysis by injection into the AB Sciex 3200 Q Trap mass spectrometer.

Plasma Lipids:

Plasma lipid and lipoproteins and body weight were measured at the time of Vitamin D_3 measurement. All lipid measurements were done in the Clinical Chemistry Laboratory at the Wake Forest University Primate Center. The determinations included total plasma cholesterol (TPC), high density lipoprotein cholesterol (HDL-C), non-HDL cholesterol (VLDL-C + LDL-C)

and plasma triglycerides (TGs). Cholesterol and triglyceride analyses were done using enzymatic methods on the COBAS FARA II analyzer, with protocols and reagents supplied by Boehringer Mannheim. The Clinical Chemistry Laboratory was fully standardized with this method and is in the continuing surveillance phase of the CDC Lipid Standardization Program. HDL-C concentrations were determined using the heparin-manganese precipitation procedure[37] and are described in detail in the Manual of Laboratory Operations of the Lipid Research Clinics Program[38]. In addition to the baseline measurements, 23 of the monkeys had follow-up plasma lipid measurements after receiving an additional 6 months of the moderately atherogenic diet.

Statistical Analysis:

Descriptive statistics including medians, means and standard deviations were used to describe the body weight data. Arithmetic means were used to describe measured values including: TG, HDL-C, TPC, and VLDL-C + LDL-C. For calculated values (*e.g.,* TPC/HDL-C ratios), geometric means were calculated. Vitamin D_3 concentrations and plasma lipids were assessed for normality of distribution and analyzed as continuous variables. Vitamin D_3 values were dichotomized into higher and lower concentrations using the median vitamin D_3 concentration of 48 ng/mL.

To determine the pathologic significance of the TPC/HDL-C ratios for monkeys in the upper half of the distribution of plasma concentrations of vitamin D compared with those in the lower half of the distribution (TPC:HDL-C, 6.2 *vs.* 5.4, respectively), we used a separate database from a study reported previously[39]. In that study, data were available regarding the relationship between plasma TPC/HDL-C and the extent of atherosclerosis found in the iliac arteries of 103 subjects. Iliac artery plaque size (intimal area) was expressed as cross-sectional

area in mm^2. Based on the pathologic convention that intimal areas of ≤0.2 mm^2 equate with fatty streaks and intimal areas >0.2 mm^2 are considered plaques, we determined the relationship between plasma TPC/HDL-C ratios and the individual cases that had fatty streaks *vs.* those with plaques (figure 1).

Inferential statistics consisted of Student *t*-tests to compare lipid profiles by vitamin D_3 group (High vs. Low), all lipid and D_3 values by pre- and post-menopausal status, and type of housing. For comparison of lipid profiles and vitamin D_3 concentrations of the entire cohort (n=119), Pearson's correlation coefficient was used to generate r values for continuous variables. Correlations between variables and high and low vitamin D_3 values were analyzed using Spearman's ranked correlation coefficient to generate rho values.

All analyses were conducted using an *a priori* alpha level of 0.05 such that results yielding $p<0.05$ were deemed statistically significant. SPSS v. 17.0 (SPSS Inc., Chicago, IL 2009) was used for all analyses.

Results:

Housing Condition:

Housing condition (indoor vs. indoor /outdoor) had a significant effect on plasma vitamin D_3 concentrations ($p<0.001$). The mean (± SD) for the monkeys housed indoors was 44.68 ± 9.14 ng/mL (range 24 to 64); whereas those with access to outdoor pens was 53.87 ± 14.96 ng/mL (range 26 to 95).

Time Consuming an Atherogenic Diet:

As expected, the length of time the monkeys consumed an atherogenic diet may have influenced their HDL-C concentrations. The monkeys that consumed the diet for 26 months had a mean HDL-C concentration of 46.35 ± 15.33 mg/dL, whereas those that consumed the diet for 4 months had a mean of 61.04 ± 18.89 mg/dL ($p<0.001$).

The Entire Cohort:

Among the entire 119 monkeys fed an adequate and consistent quantity of vitamin D_3, some with and some without exposure to sunlight, the range of vitamin D_3 concentrations was 24.0 to 95.2 ng/mL (mean ± SD = 48.5 ± 12.7 ng/mL) (figure 2). The body weight (BW) distribution ranged from 2.12 to 4.46 kg (mean ± SD, 2.97 ± 0.46 kg).

Analyzing vitamin D_3 as a continuous variable, plasma vitamin D_3 concentration was positively associated with HDL-C ($r=0.28$, $p=0.003$) (figure 3). In contrast, there were no significant associations between the vitamin D_3 concentrations and any of the following: BW, TPC, TG, VLDL+LDL-C, or TPC/HDL-C ratio. Those with vitamin D_3 concentrations ≥48 ng/mL, compared to those with vitamin D_3 concentrations <48 ng/mL, showed no significant differences in BW, TPC, TG, or VLDL+LDL-C. Conversely, monkeys with vitamin D_3 concentrations ≥48 ng/mL had a significantly higher plasma HDL-C than those with vitamin D_3 concentrations <48 ng/mL (57.9 mg/dL *vs.* 47.1 mg/dL, respectively; $p=0.001$) (figure 4). Although the difference was not significant ($p=0.120$), monkeys with vitamin D_3 concentrations ≥48 ng/mL had a lower TPC/HDL-C ratio than those <48 ng/mL, (5.4 and 6.2, respectively) (figure 4).

The difference between the TPC/HDL-C ratio in those with lower *vs.* higher vitamin D_3 concentrations appeared large, but did not meet statistical significance. A *post hoc* power analysis showed that a total sample size of 380, or 190 in each group (divided at the median), would afford 80% power to detect a difference in means of 0.88 (the difference between a Group 1 TPC/HDL-C ratio of 6.2 and a Group 2 TPC/HDL-C ratio of 5.4) assuming that the common standard deviation was 3.0, using a two-group t-test with a 0.05 two-sided significance level.

There were 23 ovariectomized monkeys fed the moderately atherogenic diet for 4 months that were followed prospectively with additional plasma lipid measurements 6 months later. As expected with additional months of diet, all variables, with the exception of triglyceride, moved in the direction of a more atherogenic plasma lipid profile. However, the finding that monkeys with higher plasma vitamin D_3 concentrations (High group) tended to have lower adverse changes in plasma lipids than those with lower values (Low group) was unexpected, although these relationships did not reach statistical significance. TPC increased 35.3% *versus* 45.9% in the high *versus* low vitamin D concentration groups, respectively. Likewise, the LDL-C + VLDL-C concentrations increased 49.3% *versus* 63.9%, the HDL-C decreased 12.1% *versus* 17.1% while the TPC/HDL-C ratio increased by 2.9 *versus* 4.3, all in the high *versus* low vitamin D concentration groups, respectively (table 1).

The TPC/HDL-C ratio for both the Low and High plasma vitamin D cohorts was 4.8 (n=23). After six months of consuming a moderately atherogenic diet, the ratio was 9.1 in the low vitamin D group compared with 7.7 in the high vitamin D group. In our analysis of a separate database, from a study published previously[39], we considered the significance of the TPC/HDL-C ratio. From that database, we determined that the progression of atherosclerosis (defined as an intimal area of >0.2 mm^2) begins at a TPC/HDL-C ratio of approximately 6, and

there was a strong and highly significant correlation between intimal area and TPC/HDL-C ratio ($r=0.489$, $p<0.001$) (Figure 1). Taken together, these findings suggest that vitamin D modulated differences in the TPC/HDL-C ratio may affect atherosclerosis progression and thus have clinical significance.

Discussion:

Despite an abundance of data suggesting an association between low vitamin D_3 and CHD risk[21-30], recent data have challenged this belief[9,10,40]. A report from the Women's Health Initiative (WHI), looking at calcium and vitamin D supplementation and its link to the prevention of coronary artery calcification, showed no significant effect. It should be noted, however, that patients were receiving only 400 IU of vitamin D_3 per day[40]. In addition, plasma vitamin D concentrations were not known, compliance was known to be poor, and patients were allowed to continue their baseline vitamin supplementation. Current data suggest that the daily vitamin D intake should be at least 600-800 IU per day for better health outcomes.[8-10]

In the study presented here, a large variation in plasma vitamin D_3 concentrations (24.0 to 95.2 ng/mL) was observed, in a cynomolgus monkey population in which all monkeys were consuming a diet nearly equivalent in cholesterol, protein, fat, carbohydrates, and vitamin D (1,000 IU human equivalents) at the time of sampling. We question whether these differences may be due, at least in part, to genetic variations in vitamin D metabolism (although the time on each diet and environmental factors likely played a role as well). While variations in absorption could also play a role, it would seem unlikely to have such a high prevalence of poor vitamin D absorption in this cohort of monkeys. The wide variation also raises the question as to the

usefulness of 1,000 IU of vitamin D supplementation in those with low levels. In other words, it is not known whether supplementing low vitamin D concentrations in general, or even with 1,000 IU, makes a clinically meaningful impact, or whether low vitamin D concentrations are a marker for other processes (potentially unrelated to vitamin D supplementation). Therefore, this finding warrants further study related to vitamin D supplementation and its effects. It is interesting to note that despite a paucity of data or understanding of what vitamin D supplementation does, it is becoming a more popular phenomenon to test and aggressively treat decreased plasma vitamin D concentrations.

The current study demonstrates an association between low plasma concentrations of vitamin D and a more atherogenic lipid profile. The major findings, therefore, propose an explanation for why vitamin D-deficient individuals may be at increased risk for CHD. In our sample, cynomolgus monkeys with higher plasma vitamin D concentrations had significantly higher HDL-C levels (figure 3 and 4). These findings suggest that low HDL-C levels, one of the well recognized risk factors for CHD, may be causally linked to a deficiency in vitamin D in the monkey model. The monkeys with higher plasma concentrations of vitamin D also had a lower TPC/HDL-C ratio (figure 4). TPC/HDL-C ratios greater than 6.0, based on our additional analysis, are shown to be highly atherogenic in this model (figure 1). An increasingly higher TPC/HDL-C ratio, as shown in figure 1, is correlated with a greater plaque size, suggesting a worsening risk with increasing TPC/HDL-C ratios. The fact that monkeys in the higher vitamin D group had TPC/HDL-C ratios <6, while those in the lower vitamin D range had ratios >6, although not statistically significant, has clinical implications. These findings, in combination with greater HDL-C values in the higher vitamin D cohort ($p=0.001$), provide evidence for a more favorable lipid profile in those monkeys with higher vitamin D concentrations.

A subset of 23 monkeys, followed prospectively for 6-month changes in their lipid profiles, revealed interesting findings (table 1). Although none of the differences were statistically significant (largely due to the small sample size), it is noteworthy that the changes, evaluated as both actual and percentage differences, were all lower in the monkeys with higher baseline vitamin D concentrations. A *post hoc* power analysis determined the need for at least 38 monkeys to have 80% power to be able to detect a statistically significant difference in these parameters. While the findings are not statistically significant, the consistency of the finding along with the degree of disparity in the percent change may imply clinical relevance. This could imply that higher vitamin D concentrations may help abate the development of an atherogenic lipid profile, despite a high-fat diet.

A study of human subjects which determines if and to what extent HDL-C levels increase and TPC/HDL-C ratios decrease with vitamin D supplementation would be clinically beneficial. Additionally, it would be helpful to analyze the role of further supplementation and more specifically, the response of cholesterol measurements when Vitamin D supplementation is sequentially increased. With adequate and maximal dosing, what is the vitamin D response and does it vary widely among individuals? Further studies should also analyze whether patients who are hypercholesterolemic benefit from vitamin D assessment, both in lipid parameters and measures of atherosclerosis.

The variation in HDL-C concentrations and vitamin D_3 concentrations based on diet and housing, respectively, is not surprising. We expected a worsening lipid profile in those monkeys with a larger exposure to a moderately atherogenic diet. Likewise, we expected higher vitamin D_3 concentrations in those monkeys with outdoor exposure. Differences in the time on diet and housing arrangements helped create a more realistic translational model that gave the necessary

distribution of study variables. The small population size of cynomolgus monkeys could have prohibited our ability to see significant differences due to a low power and hence a beta (Type II) error. Studies to confirm these results in a human cohort would be helpful. However, the cynomolgus monkeys have been shown to be a reliable model to study potential causes of postmenopausal atherosclerosis[41], a model in which standardized dosing, compliance, sun exposure, time of dosing, food intake, and plasma measurements can all be controlled.

Conclusion:

Despite the limitations mentioned above, the clinical relevance of our findings is important. To the best of our knowledge, this is the first report suggesting lower HDL-C concentrations and a worsening lipid profile over time in association with low Vitamin D concentrations. It was also done in a way that controlled the sun exposure, diet, and vitamin D_3 intake. Given that all monkeys consumed a diet that was replete in vitamin D_3, it appears that individual differences (presumably genetic) in vitamin D absorption or metabolism may have determined whether the monkeys had high or low plasma concentrations of vitamin D_3. Lower concentrations of vitamin D_3 were associated with significantly lower concentrations of HDL-C, a higher TPC/HDL-C ratio, and an enhancement of the adverse lipid effects of a moderately atherogenic diet, all established risk factors for progressive coronary artery atherosclerosis of both the monkey model and in women. The results add to existing knowledge by identifying vitamin D deficiency as a potential cause of atherogenic lipid parameters and increased CHD.

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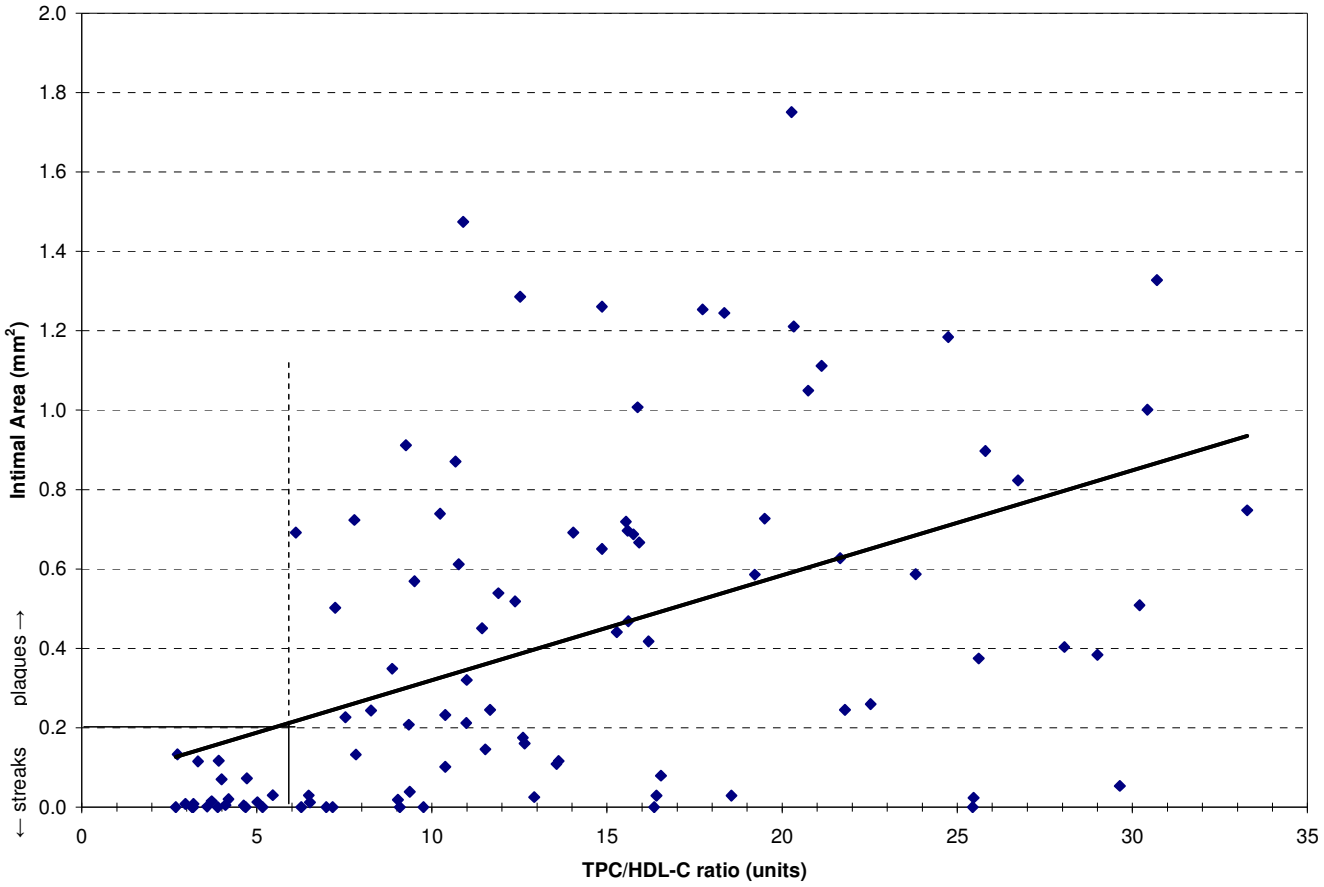
Table 1. Actual and percentage changes in lipid profiles after 6 months on a high-fat diet

	Low Vitamin D*		High Vitamin D*	
lipid parameter	actual chg	% chg	actual chg	% chg
TPC (mg/dL)	117.9	45.9	92.7	35.3
TG (mg/dL)	-5.5	-10.6	-3.3	-7.7
HDL-C (mg/dL)	-9.8	-17.1	-7.3	-12.1
LDL-C+VLDL-C (mg/dL)	127.7	63.9	100	49.3
TPC/HDL-C ratio (units)	4.3	87.9	2.9	61.6

*Low vitamin D was defined as a plasma vitamin D_3 concentration less than the median (48 ng/mL) while High vitamin D was defined as a plasma vitamin D_3 concentration greater than or equal to the median.

TPC: total plasma cholesterol
TG: Plasma Triglyceride
HDL-C: high density lipoprotein cholesterol
LDL-C + VLDL-C: non HDL-C (or low density lipoprotein cholesterol and very low density lipoprotein cholesterol)

Figure 1. This figure shows the progression of atherosclerosis as the TPC/HDL-C ratio increases. Plaques are defined as iliac artery intimal areas above 0.2 mm^2; this figure suggests the TPC/HDL-C ratio at which the progression of atherosclerosis begins is 6.



TPC/HDL-C – Total Plasma Cholesterol: High Density Lipoprotein ratio

Figure 2 Frequency of vitamin D_3 concentrations in cynomolgus monkeys (*Macaca fascicularis*) after controlled diet consumption.

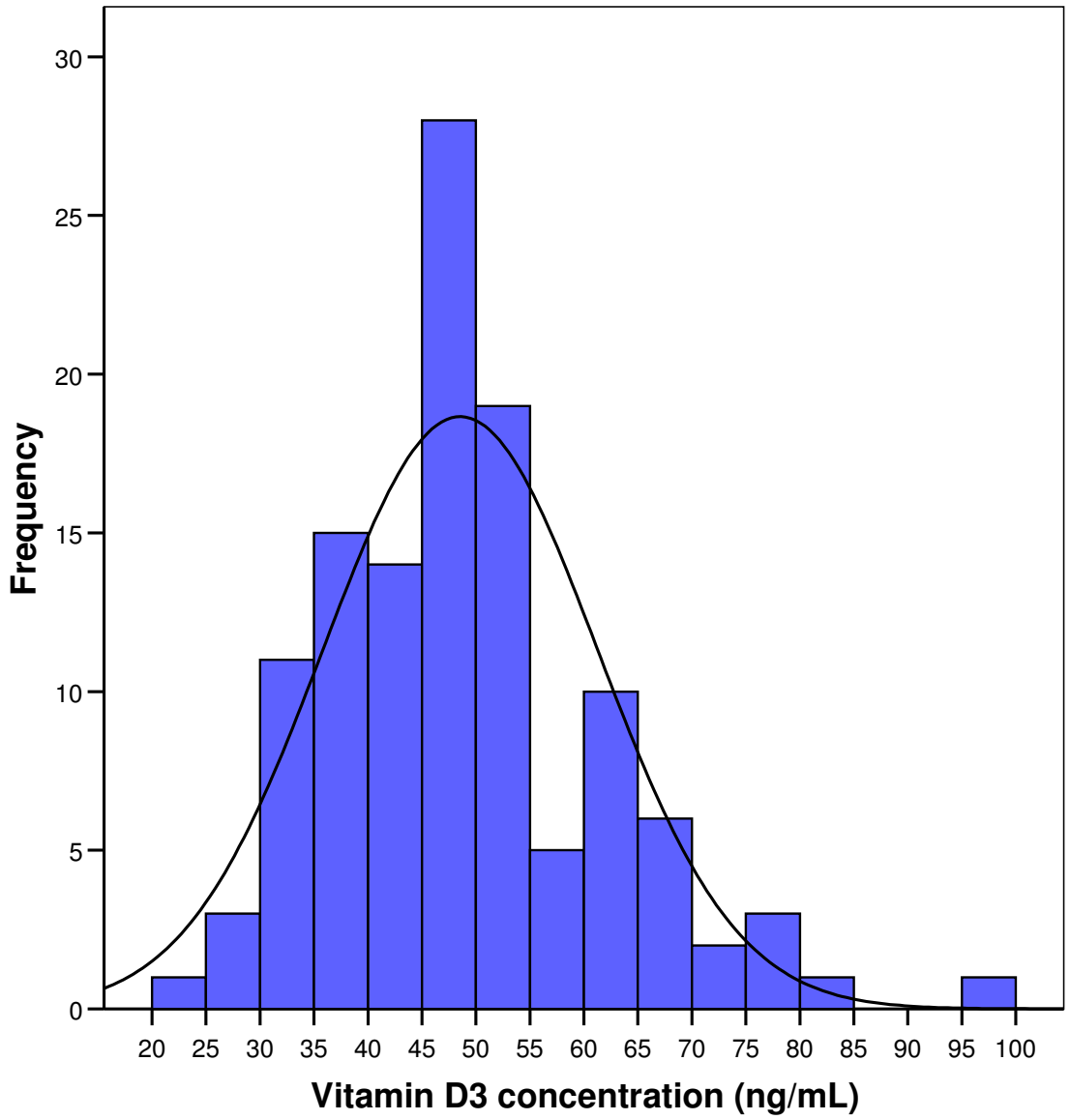


Figure 3. Vitamin D_3 concentration as a function of baseline HDL-C (mg/dL) concentration in cynomolgus monkeys (*Macaca fascicularis*) after controlled diet consumption.

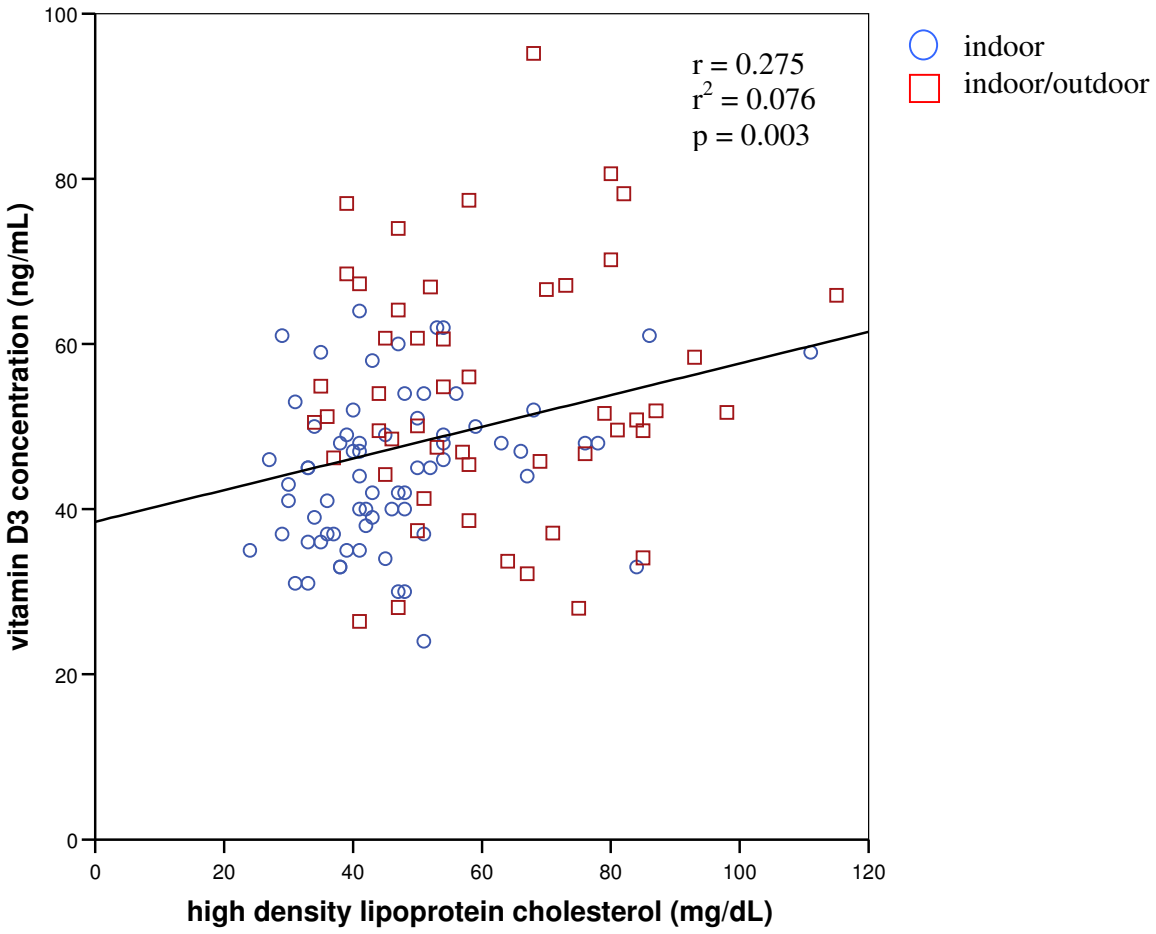
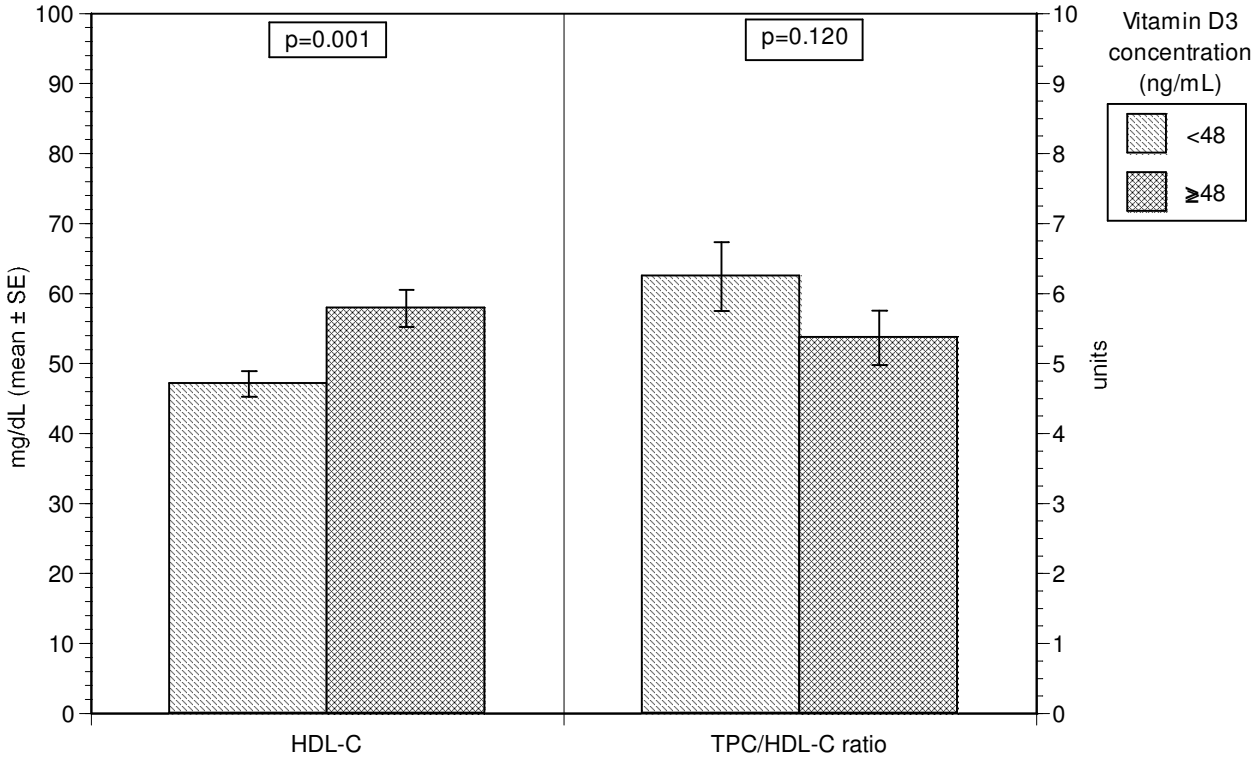


Figure 4. Mean HDL-C (mg/dL) concentration and TPC/HDL-C ratio for cynomolgus monkeys (*Macaca fascicularis*) with vitamin D3 concentrations ≥48 ng/mL and <48 ng/mL



HDL-C = high density lipoprotein cholesterol
TPC/HDL-C = total plasma cholesterol / high density lipoprotein cholesterol