

Cross-sectional association between blood cholesterol and calcium levels in genetically diverse strains of mice.

Short Title: Calcium and cholesterol in DO mice

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Abstract

Genetically diverse outbred mice allow for the study of genetic variation in the context of high dietary and environmental control. Using a machine learning approach we investigated clinical and morphometric factors that associate with serum cholesterol levels in 840 genetically unique Diversity Outbred mice of both sexes (n=417 male and 423 female), and on both a control chow (% kcals in diet: Protein 22%, Carbohydrate 62%, Fat 16%, no cholesterol) and high fat high sucrose (% kcals in diet: Protein 15%, Carbohydrate 41%, Fat 45%, 0.05% cholesterol). We find expected elevations of cholesterol in male mice, those with elevated serum triglycerides and/or fed a high fat high sucrose diet. The third strongest predictor was serum calcium which correlated with serum cholesterol across both diets and sexes ($r=0.39-0.48$) in both diversity outbred ($p=3.0 \times 10^{-43}$) and BXD ($p=0.005$) mice. This is in-line with several human cohort studies which show associations between calcium and cholesterol, and calcium as an independent predictor of cardiovascular events.

Keywords: Cholesterol, Calcium, Diversity Outbred, Cross-Sectional

Introduction

Elevated blood cholesterol, particularly in the forms of atherogenic LDL particles are causal of cardiovascular disease, the major cause of death in Western societies [1]. Cholesterol levels in humans vary widely depending on multiple factors including genetics, diet and other lifestyle factors, with genetics and lifestyle each contributing roughly equally to cardiovascular disease risk [2].

Human genetics has led to a sophisticated understanding of how cholesterol synthesis and excretion is regulated, and how this varies between individuals and diets [3]. Understanding complex genetic traits like cholesterol is a challenge in human studies where diet and lifestyle data is subject to substantial instrument error. Here genetically diverse panels of mice, where the diet and environment are tightly controlled, and genetics can be well defined, can provide solutions. Among several resources, the Diversity Outbred [4] and UM-HET3 [5] mice represent genetically diverse outbred populations, while the Collaborative Cross [6] and BXD [7] resources provide data on recombinant inbred mice. Collectively these resources are valuable tools to understand complex traits such as cholesterol.

In this study we performed a secondary data analysis of Diversity Outbred mice to understand clinical and phenotypic correlates of elevated cholesterol levels. Using a machine learning approach, we demonstrate that sex, diet, and serum triglycerides are strong predictors of cholesterol levels in these mice, but also that even with high dietary control serum calcium provides an independent correlate of serum cholesterol levels.

Methods and Materials

Diversity Outbred Data

The phenotype data for diversity outbred mice (RRID:IMSR_JAX:009376) contains data on 840 mice from the diversity outbred collection of both sexes [8]. The dataset includes 162 phenotypes for each mouse, measured once, twice, or weekly in the case of body weights. Animals were first received at wean age (3 weeks old) and then distributed into cages of five same-sex animals per cage. Animals were housed in pressurized, individually ventilated cages (Thoren Caging Systems, Hazelton, PA) with pine bedding (Crobb Box, Ellsworth, ME) and had ad libitum access to food. At time of weaning mice were placed on a high fat high sucrose diet (HFHS; Harlan TD.08811), or kept on a normal chow diet (NCD; LabDiet 5K52). In the final dataset there were 225 female mice on NCD, 224 male mice on NCD, 198 female mice on HFHS, and 193 male mice on HFHS. Blood from mice was obtained from the retro-orbital sinus after administration of tetracaine HCl using a heparin-coated microcapillary tube and collected into a 1.5 ml Eppendorf tube. For collection of blood plasma, approximately 150µl of whole blood was collected into a tube and plasma was separated by centrifugation at 10,000 rpm for 10 min at 4° Celcius and removed into a clean Eppendorf tube. Cholesterol, triglycerides, and calcium were quantified in plasma using the Beckman Synchron DXC600Pro Clinical chemistry analyzer. Body composition was collected by dual x-ray absorbitrometry (DEXA) on Lunar PIXImus densitometer (GE Medical Systems). Additional details about these mice can be found in [9].

BXD Data

Calcium and cholesterol levels from male and female BXD were described in [10]. These data were downloaded from GeneNetwork (<http://www.genenetwork.org/>) [11,12] as datasets BXD_12844, BXD_12914, BXD_12951, and BXD_12881. These datasets included 17 female and 36 male strains (72 mice) and 36 male strains (254 mice; RRID:MGI:2164899). These mice were maintained on a chow diet (SAFE; D04) with blood collected at 14 weeks of age. Data were averaged and analyzed by strain and sex.

Statistics

All statistical analyses were performed using R version 4.2.0 [13]. Cholesterol data were not normally distributed within groups ($p < 0.05$ by sex and diet stratified Shapiro-Wilk tests), so non-parametric pairwise tests were used. Summarized data is reported as mean \pm standard deviation for summary statistics and standard deviation of the error for multivariate models. For all comparisons sex was first tested as a modifier, and then as a covariate. If there was significant evidence of sex modification, pairwise sex-stratified analyses are also reported. Regression trees were generated using the rpart package (version 4.1.19; [14]), and pruned based on the number of branches at the minimum cross-validated standard error rate. Partial effect sizes were estimated using the effectsize package (version , reporting ω^2_p [15,16]) Causal mediation analyses were performed with 1000 bootstraps with both the full and mediator models adjusted for sex using the mediation package (version 4.5.0; [17]), using the method described in [18]. Statistical significance was set at an alpha of 0.05. All data and reproducible code are available for this manuscript at <https://github.com/BridgesLab/PrecisionNutrition>.

Results

Diversity outbred mice exhibit diet and sex dependent variation in cholesterol levels

We first evaluated the cholesterol levels in the diversity outbred mice measured at 8 and 19 weeks (5 and 16 weeks of HFHS or NCD feeding). Cholesterol levels for each group were similar at both time points ($p = 0.474$ by pairwise Wilcoxon test, see Supplementary Figure 1). This indicates that cholesterol levels are stable between both time points. We stratified cholesterol levels by sex and diet. Via multivariate regression, we found the expected cholesterol elevations in mice on a HFHS diet (33.7 ± 2.0 mg/dL, $p = 1.4 \times 10^{-56}$), and male sex (16.9 ± 2.0 mg/dL, $p = 3.0 \times 10^{-17}$; Figure 1A). There was no evidence of a significant interaction between diet and sex ($p = 0.636$).

The HFHS diet is deployed primarily as an obesogenic diet in rodents. We evaluated the role of body weight and fat mass on cholesterol levels in these mice. As expected, after adjusting for sex, increases in both body weight (16.9 ± 1.6 mg/dL per 10g weight; $p = 2.2 \times 10^{-24}$) and percent fat mass (15.2 ± 1.1 mg/dL per 10% increase in percent fat mass; $p = 3.1 \times 10^{-38}$) correlated with cholesterol levels at 19 weeks of age (Figure 1B). We next asked whether diet played a role aside from increasing percent fat mass and found that this is true as well. We performed a causal mediation analysis to interrogate this relationship and estimate that, after

adjusting for sex, differences in percent fat mass explained 25 percent of the HFHS diet effect on cholesterol levels in these mice (95% CI 17-34%; $p_{\text{mediation}} < 1 \times 10^{-15}$). This suggests that there are both obesity-dependent and HFHS-diet-dependent contributions to cholesterol levels in these mice.

Diet, triglycerides and calcium associate with cholesterol levels.

To define other potential associations between cholesterol and measured phenotypes in this dataset we generated a regression tree using the 165 phenotypes in this dataset (Figure 1C). The major classifier of cholesterol levels was the diet, and the second was triglycerides measured at 19 weeks. Serum calcium measured at 19 weeks was the third phenotype that associated with cholesterol levels, and body weight measured at 19 weeks was the fourth (Figure 1C).

Dyslipidemia often includes elevations of both triglyceride and cholesterol levels in both mice and humans, so the association of triglycerides with cholesterol was not unexpected. Via multivariate modelling accounting for the effects of diet and sex, a 100 mg/dL increase in triglycerides was associated with a 17.7 +/- 1.7 mg/dL elevation in cholesterol ($p = 3.8 \times 10^{-24}$, Figure 2A). Within subgroups, the triglyceride-cholesterol relationship varied somewhat, with weaker correlations in HFHS populations (Spearman's rho for males 0.190, females 0.276) than on NCD (males 0.491, females 0.453). Via multivariate regression with interactions, we observed significant sex x diet x triglyceride effect modification with respect to cholesterol levels ($p = 4.5 \times 10^{-4}$, $\omega^2_p = 0.014$).

The strong cross-sectional association of calcium with cholesterol was not predicted by our research team. As shown in Figure 2B and Table 1, after adjusting for diet and sex, a one mg/dL increase in calcium is associated with a 12.7 +/- 0.8 mg/dL increase in cholesterol ($p = 3.0 \times 10^{-43}$). Linear models predicting serum cholesterol including sex and diet, and calcium as covariates had an adjusted R^2 of 0.45 with a partial effect size (ω^2_p) of 0.22 for serum calcium. We performed sub-group analyses and found that each diet-sex combination had broadly similar estimates for Spearman's rho (ranging from 0.39 for HFHS females to 0.48 for HFHS males), each of which had a p-value of less than 2.2×10^{-7} . In this study, calcium levels were not affected by rodent sex ($p = 0.59$) and only modestly by diet (increased by 0.3 mg/dL in HFHS; Supplementary Figure 2A). Due to the strong impacts of diet and sex on cholesterol levels, these suggest that calcium may work largely independently of those factors.

Given the differences in the physiology and cardiovascular disease associations between HDL versus LDL cholesterol concentrations, we repeated this analysis assessing the diet- and sex-adjusted associations between HDL cholesterol and non-HDL cholesterol and calcium (Supplementary Figures 2B-C). Both of these fractions have positive associations with calcium levels, indicating that the positive associations with calcium are found in both apolipoprotein fractions. The relationships between all clinical parameters and endpoint cholesterol both in terms of non-parametric correlations and diet/sex adjusted associations are presented in the Supplementary Table.

To externally test these findings, we evaluated a distinct dataset of genetically diverse mice, the BXD mouse collection. In a secondary data analysis using data in [10], we replicated this finding, showing a cross-sectional association between cholesterol and calcium levels after adjusting for sex differences. Similar to the data from the diversity outbred mice, we estimate a 14.8 +/- 5.1 mg/dL increase in cholesterol was observed for every 1 mg/dL increase in calcium ($p=0.005$). In the smaller BXD dataset there appeared to be a stronger relationship in male mice (Spearman's $\rho=0.393$ $p=0.018$, $n=36$ strains) than female mice ($\rho=0.269$; $p=0.297$, $n=17$ strains), but in multivariate modelling there was no significant modifying effect of sex ($p=0.178$), likely due to the relatively small number of female strains.

In the diversity outbred mice, serum calcium levels are not significantly altered by sex ($p=0.59$), and only modestly increased by HFHS diets (0.30 ± 0.07 mg/dL; $p=5.1 \times 10^{-5}$; Supplementary Figure 2C). Since calcium is normally tightly controlled by homeostatic mechanisms regulating calcium absorption and bone remodeling, we tested whether bone mineral content and density in these mice was associated with cholesterol levels. As shown in Supplementary Figure 2D and E, there was no evidence of an association between bone mass or density measured at 21 weeks and cholesterol levels measured at 19 weeks ($p=0.93$ and 0.90 respectively) in the diversity outbred dataset.

Conclusions

In this study we report an association between calcium and cholesterol in two distinct mouse datasets. This relationship was similar across both sexes and over both normal chow and obesogenic high fat, high sucrose diets. We were not surprised that HFHS feeding raised cholesterol, as this has been widely observed in mice, rats, and humans. This is likely due to a combination of increased dietary cholesterol, triglycerides, and body fat in these mice. The calcium relationship with cholesterol that was identified here is a unique observation in mice. As the magnitude of elevations of cholesterol and calcium from HFHS diet were similar, and because diet did not substantially alter calcium levels, it is possible that calcium and diet are independent predictors of cholesterol homeostasis. The finding that the calcium-cholesterol association is largely independent of sex, diets or triglyceride levels suggests that serum calcium may represent a novel biomarker of dysfunctional cholesterol homeostasis and may point to novel mechanisms by which cholesterol could be controlled.

To our knowledge this is the first demonstration of an association between serum calcium and cholesterol in rodents. That being said, several large cross-sectional studies have consistently demonstrated a correlation between serum calcium and cholesterol in multiple populations [19–32]. In addition, calcium is also a longitudinal predictor of cardiovascular events in humans independent of BMI or blood pressure [21,32–37]. A meta-analysis of these associations show that an increase of one standard deviation of serum calcium is associated with an eight percent increased risk of subsequent cardiovascular events [38]. These data are consistent with the

hypothesis that the calcium-cholesterol relationship we report here in mice is concordant with increased cardiovascular risk.

The present study does not speak to the directionality of this association, but there are some hints in the literature. A meta-analysis by demonstrates a 31% increased risk of myocardial infarction in patients with calcium supplementing with calcium compared to placebo [39]. Patients with primary hyperparathyroidism have elevated parathyroid hormone and calcium levels and are an interesting population to examine. The results of case-control studies evaluating cholesterol levels are mixed and there is limited evidence that PTH causes elevated cholesterol levels. While reports show that these patients also have significantly elevated total and/or LDL-cholesterol [40,41], though most others show either non-significant effect or even decreases [42–48].

In terms of whether cholesterol could be driving hypercalcemia, there is some positive evidence. Two interventional studies using statins have showed a reductions in calcium levels [49,50], whereas another single-arm study showed declines that did not reach significance [51]. A Mendelian Randomization approach using LDL-C as the instrument was also associated with elevated calcium levels [52], further supporting a potential causal relationship with cholesterol driving calcium levels. As this is a cross-sectional associative relationship we are not able at present to define directionality, much less the underlying biological mechanism(s). Whether calcium can modify serum cholesterol, or cholesterol can modify calcium are both important nutritional and pathophysiological questions, and future controlled mouse studies should shed light on the directions and mechanisms explaining this association.

There are several other strengths of this study. We present data on a large number of mice roughly equally divided between sexes and two diets and find consistent results across all groups. We have exceptional control of confounders such as diets, environment, activity levels, and other exposures that could affect the interpretation of the human studies. Our supervised machine learning approach used a large number of measured phenotypes to predict calcium levels, and set cutoffs in a data-driven manner. We consider the support of data from two independent genetically diverse mice populations another strength of this work. Calcium-cholesterol associations appear to be robust over a wide variation in genetics and not restricted to findings in inbred mouse populations. As such, this relationship holds over multiple diets, sexes, investigators, sites, and genetic backgrounds.

Limitations of the present study

While there were multiple measurements of calcium and cholesterol in this dataset (at week 8 and week 19, after 5 and 16 weeks of HFHS/NCD respectively), cholesterol levels were stable through at these points. Therefore, it was possible to effectively evaluate the longitudinal association between cholesterol and calcium, nor the effects of advanced age in modifying this relationship. In addition, this cross-sectional association does not ascribe a directionality to this relationship, at this stage we think it plausible that calcium may increase cholesterol, that cholesterol might increase calcium, or that a third, unmeasured factor drives both factors. As this is a secondary data analysis, we are unable to evaluate differences in calcium-regulatory

hormones, which we predict would vary more than the relatively homeostatic blood calcium levels. Another limitation is that cholesterol homeostasis is substantially different in mice and humans, especially in the fraction of cholesterol present in the HDL versus LDL fractions, due to the absence of CETP in mice. These data therefore largely reflect associations between calcium and the HDL pool. Finally, as cardiovascular disease is extremely rare in mice of this age we did not assess cardiovascular disease, or atherogenic lesions as an endpoint in this study.

In conclusion, in this work we use a machine learning approach to describe that diet, sex, triglyceride levels and calcium all contribute independently to the serum cholesterol levels in Diversity Outbred mice, and that the relationship between calcium and cholesterol holds true in BXD mice. These data support that the observed human relationships between serum and cholesterol levels are true in mice, and present an opportunity for further physiological and genetic dissection of this relationship.

Funding Information

We would like to thank the members of the Bridges Laboratory for helpful discussions regarding this work. We would also like to acknowledge funding from the National Institutes of Diabetes and Digestive Kidney Diseases (NIDDK; R01DK107535), the National Institutes of General Medical Sciences (NIGMS; R01GM07068), the Undergraduate Research Opportunity Program (UROP to KL), and a Pilot and Feasibility Grant from the Jackson Laboratory. We would also like to thank the developers and funders of the Diversity Outbred Database, Diversity Informatics Platform and GeneNetwork for their commitment to open science and for providing the data used in these analyses.

Author Contributions

DB and CMC conceptualized this research study, decided and validated the methodologies, performed the investigations, wrote the original draft, the final draft, and prepared visualizations. Formal analyses were done by CMC, DB and KL. Data was provided by GAC. This work was administered and supervised by DB who along with GAC performed the data validation. Funding for this work was acquired by DB and GAC. All authors have read and agreed to the final published work.

Conflict of Interest

The authors have no conflicts to declare

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Figure Legends

Figure 1: Description of cholesterol levels in diversity outbred mice. A) Violin plot of cholesterol levels of diversity outbred at 19 weeks, mice stratified by diet and sex. B) Scatter plot of cholesterol levels in relation to percent fat mass at 19 weeks of age, stratified by diet and sex. C) Pruned regression predicting cholesterol at 19 weeks. Above the box is the algorithmically generated cutoff for triglycerides (abbreviated TG in mg/dL), calcium (Ca in mg/dL), and body weight (BW in g). Each predictor was a phenotype measured at 19 weeks. Within each box, the value represents the average cholesterol level in that group (in mg/dL) and the number of mice in that group (n=840 for panel A and 818 for panel 2 as 22 mice were omitted due to missing data).

Figure 2: Cross-sectional associations of cholesterol with triglycerides and calcium. Sex and diet stratified scatter plots of A) triglyceride and B) calcium relationships with cholesterol levels at 19 weeks in diversity outbred mice (n=840 mice and strains). C) Cholesterol and calcium associations in male and female BXD strains (n=326 mice from 17 female and 36 male strains; error bars represent within-strain standard error of the mean). P-values indicate the level of significance for the diet and sex adjusted relationship between cholesterol and triglycerides or calcium from a multivariate linear model. The lines represent independent estimates for each group.

Supplementary Figure 1: Cholesterol levels are stable across time in diversity outbred mice. Average cholesterol levels, and levels measured at 8 and 19 weeks, stratified by sex and diet.

Supplementary Figure 2: Calcium is not strongly associated with diet, sex or bone mass/density in diversity outbred mice. A) Violin plot of calcium levels at 19 weeks across diets and sex. Sex and diet stratified scatter plots of HDL cholesterol (B) and non-HDL cholesterol (C as a proxy for LDL-C) and their relationships with serum calcium at 19 weeks. Scatter plots of the relationships between bone mineral content (D) and bone density (E) via DEXA scan and their relationships with cholesterol levels at 19 weeks. For A, the p values represent the significance of diet and sex from a multivariate linear model. For panels B-D, p values indicate the significance for the diet and sex adjusted relationship between cholesterol and the outcome from a multivariate linear model.