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

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# Abstract

# Introduction

# Methods

## Diversity Outbred Data

The phenotype data for diversity outbred mice was described in and contains data on 843 mice from the diversity outbred collection of both sexes. These data were downloaded from the Diversity Outbred Database (<https://www.jax.org/research-and-faculty/genetic-diversity-initiative/tools-data/diversity-outbred-database>) as part of the 183\_Svenson\_DO dataset [1,2]. The dataset includes at data for 165 phenotypes, measured once, twice, or weekly in the case of body weights. At 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) [1]. In the final dataset there were 225 female mice on NCD, 223 male mice on NCD, 200 female mice on HFHS, and 196 male mice on HFHS.

## BXD Data

Calcium and cholesterol levels from male and female BXD were described in [3]. These data were downloaded from GeneNetwork (<http://www.genenetwork.org/>) [4,5] as datasets BXD\_12844, BXD\_12914, BXD\_12951, and BXD\_12881. These datasets included 17 female and strains (72 mice) and 36 male strains (254 mice). These mice were maintained on a chow diet (SAFE; D04) and 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 [6]. 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 +/- standard error of the mean. 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; [7]), and pruned based on the number of branches at the minimum cross-validated standard error rate. 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. Cholesterol levels for each group were similar at both time points (p=0.465 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 (33.8 +/- 2.0 mg/dL, p=6.6 x 10-57), and male sex (17.0 +/- 2.0 mg/dL, p=2.09 x 10-17; Figure 1A). There was no evidence of a significant interaction between diet and sex (p=0.667).

## 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 1B). 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 1B).

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 17.9 +/- 1.7 mg/dL elevation in cholesterol (p=5.33 x 10-25, Figure 2A).

The strong cross-sectional association of calcium with cholesterol was not predicted by our research team. As shown in Figure 2B, 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 x 10-43). 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 1.2 x 10-7).

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 [3], 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. 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=0.54), and only modestly increased by HFHS diets (0.31 +/- 0.07 mg/dL; p=2.7 x 10-5; Supplementary Figure 2A). 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 2B and C there was no evidence of an association between bone mass or density measured at 21 weeks and cholesterol levels measured at 19 weeks (p=0.97 and 0.72 respectively) in the diversity outbred dataset.

# Discussion

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. The novel association between calcium and cholesterol study is strengthened by the expected findings that cholesterol is elevated in mice of male sex, with high triglycerides and fed HFHS diets.

Cholesterol-calcium associations are in line with cross-sectional observations in humans linking calcium levels to cholesterol in the blood. OBSERVATIONAL ASSOCIATIONS.

The present study does not speak to the directionality of this association, but there are some hints in the literature. 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. Two reports show that these patients also have significantly elevated total and/or LDL-cholesterol [8,9], though most others show either non-significant effect or even decreases [10–16]. On the other hand, there is no evidence that calcium causes elevations in total or LDL cholesterol <ADD REFS>

In terms of whether cholesterol could be driving hypercalcemia, there is less evidence. 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 of this association.

While the human clinical data is inconsistent, under the conditions of the present study, both the environment and diet are highly controlled reducing the likelihood, possible in human observational studies that cholesterol-calcium associations might be confounded by variation in diets, age, medications, or physical activity.

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. 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. In addition, this cross-sectional association does 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. 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.

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# Author Contributions

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

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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) 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=822 in total).

**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=822 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).

**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 the relationships between bone mineral content (A) and bone density (B) via DEXA scan and their relationships with cholesterol levels at 19 weeks.