# 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] 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 [5]. 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; [6]), 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 data re-analyzed from [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)

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.

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

Considering the direction of this effect, patients with symptomatic primary hyperparathyroidism have elevated parathyroid hormone and calcium levels and reduced vitamin D. The results of some case-control studies evaluating cholesterol levels are mixed. Two reports show that these patients also have significantly elevated total and/or LDL-cholesterol [7,8], though several others show either non-significant effect or decreases [9–15]. 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.

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 or physical activity.

BONE MASS

There are several strengths of this study. We present data on a large number of mice roughly equally divided between sexes and two diets. We consider the secondary analysis of data from two independent genetically diverse mice populations a major strength of this work. These relationships are robust over a wide variation in genetics and not restricted to findings in inbred mouse populations. Furthermore, this relationship holds over multiple diets, sexes, investigators, sites, and genetic backgrounds further strengthening the rigor of these findings.

## Limitations of the present study

While there were multiple measurements of calcium in this dataset (at week 8 and week 19), cholesterol levels were stable through this period, so it is not possible to evaluate the longitudinal association between cholesterol and calcium without an earlier time point. In addition, this cross-sectional association does ascribe a directionality to this relationship, at this stage we think it reasonably likely that calcium may increase cholesterol as cholesterol might increase calcium. 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.

# References

1. Chick, J.M.; Munger, S.C.; Simecek, P.; Huttlin, E.L.; Choi, K.; Gatti, D.M.; Raghupathy, N.; Svenson, K.L.; Churchill, G.A.; Gygi, S.P. Defining the Consequences of Genetic Variation on a Proteome-Wide Scale. *Nature* **2016**, *534*, 500–505, doi:10.1038/nature18270.

2. Munger, S.C.; Raghupathy, N.; Choi, K.; Simons, A.K.; Gatti, D.M.; Hinerfeld, D.A.; Svenson, K.L.; Keller, M.P.; Attie, A.D.; Hibbs, M.A.; et al. RNA-Seq Alignment to Individualized Genomes Improves Transcript Abundance Estimates in Multiparent Populations. *Genetics* **2014**, *198*, 59–73, doi:10.1534/genetics.114.165886.

3. Andreux, P.A.; Williams, E.G.; Koutnikova, H.; Houtkooper, R.H.H.; Champy, M.-F.F.; Henry, H.; Schoonjans, K.; Williams, R.W.; Auwerx, J. Systems Genetics of Metabolism: The Use of the BXD Murine Reference Panel for Multiscalar Integration of Traits. *Cell* **2012**, *150*, 1287–1299, doi:10.1016/j.cell.2012.08.012.

4. Mulligan, M.K.; Mozhui, K.; Prins, P.; Williams, R.W. GeneNetwork: A Toolbox for Systems Genetics. In *Methods in Molecular Biology*; 2017; Vol. 331, pp. 75–120 ISBN 978-1-4939-6427-7.

5. R Core Team R: A Language and Environment for Statistical Computing 2019.

6. Therneau, Terry; Atkinson, Beth Rpart: Recursive Partitioning and Regression Trees.

7. Procopio, M.; Barale, M.; Bertaina, S.; Sigrist, S.; Mazzetti, R.; Loiacono, M.; Mengozzi, G.; Ghigo, E.; Maccario, M. Cardiovascular Risk and Metabolic Syndrome in Primary Hyperparathyroidism and Their Correlation to Different Clinical Forms. *Endocrine* **2014**, *47*, 581–589, doi:10.1007/s12020-013-0091-z.

8. Luigi, P.; Chiara, F.M.; Laura, Z.; Cristiano, M.; Giuseppina, C.; Luciano, C.; Giuseppe, P.; Sabrina, C.; Susanna, S.; Antonio, C.; et al. Arterial Hypertension, Metabolic Syndrome and Subclinical Cardiovascular Organ Damage in Patients with Asymptomatic Primary Hyperparathyroidism before and after Parathyroidectomy: Preliminary Results. *Int J Endocrinol* **2012**, *2012*, 408295, doi:10.1155/2012/408295.

9. Luboshitzky, R.; Chertok-Schaham, Y.; Lavi, I.; Ishay, A. Cardiovascular Risk Factors in Primary Hyperparathyroidism. *J Endocrinol Invest* **2009**, *32*, 317–321, doi:10.1007/BF03345719.

10. Ring, M.; Farahnak, P.; Gustavsson, T.; Nilsson, I.-L.; Eriksson, M.J.; Caidahl, K. Arterial Structure and Function in Mild Primary Hyperparathyroidism Is Not Directly Related to Parathyroid Hormone, Calcium, or Vitamin D. *PLoS One* **2012**, *7*, e39519, doi:10.1371/journal.pone.0039519.

11. Farahnak, P.; Lärfars, G.; Sten-Linder, M.; Nilsson, I.-L. Mild Primary Hyperparathyroidism: Vitamin D Deficiency and Cardiovascular Risk Markers. *J Clin Endocrinol Metab* **2011**, *96*, 2112–2118, doi:10.1210/jc.2011-0238.

12. Christensson, T.; Einarsson, K. Serum Lipids before and after Parathyroidectomy in Patients with Primary Hyperparathyroidism. *Clinica Chimica Acta* **1977**, *78*, 411–415, doi:10.1016/0009-8981(77)90074-2.

13. Ejlsmark-Svensson, H.; Rolighed, L.; Rejnmark, L. Effect of Parathyroidectomy on Cardiovascular Risk Factors in Primary Hyperparathyroidism: A Randomized Clinical Trial. *The Journal of Clinical Endocrinology & Metabolism* **2019**, *104*, 3223–3232, doi:10.1210/jc.2018-02456.

14. Hagström, E.; Lundgren, E.; Rastad, J.; Hellman, P. Metabolic Abnormalities in Patients with Normocalcemic Hyperparathyroidism Detected at a Population-Based Screening. *European Journal of Endocrinology* **2006**, *155*, 33–39, doi:10.1530/eje.1.02173.

15. Kaji, H.; Hisa, I.; Inoue, Y.; Sugimoto, T. Low Density Lipoprotein-Cholesterol Levels Affect Vertebral Fracture Risk in Female Patients with Primary Hyperparathyroidism. *Exp Clin Endocrinol Diabetes* **2010**, *118*, 371–376, doi:10.1055/s-0029-1224152.

# 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.