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

Short Title: Calcium and cholesterol in DO mice

Authors: Cody M. Cousineau[1], Kaelin Loftus[1], Gary A. Churchill[2] and Dave Bridges[1*]

[1]Department of Nutritional Sciences, University of Michigan School of Public Health
[2]The Jackson Laboratory,
[*]To whom correspondence should be addressed: davebrid@umich.edu

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 x 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 and 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].

74

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 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 +/- 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 x 10^{-56}), and male sex (16.9 +/- 2.0 mg/dL, p=3.0 x 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 x 10^{-24}) and percent fat mass (15.2 +/- 1.1 mg/dL per 10% increase in percent fat mass; p=3.1 x 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

116 adjusting for sex, differences in percent fat mass explained 25 percent of the HFHS diet effect
117 on cholesterol levels in these mice (95% CI 17-34%; $p_{mediation}$<1x10^{-15}).   This suggests that there
118 are both obesity-dependent and HFHS-diet-dependent contributions to cholesterol levels in
119 these mice.
120
121 ### Diet, triglycerides and calcium associate with cholesterol levels.
122 To define other potential associations between cholesterol and measured phenotypes in this
123 dataset we generated a regression tree using the 165 phenotypes in this dataset (Figure 1C).
124 The major classifier of cholesterol levels was the diet, and the second was triglycerides
125 measured at 19 weeks. Serum calcium measured at 19 weeks was the third phenotype that
126 associated with cholesterol levels, and body weight measured at 19 weeks was the fourth
127 (Figure 1C).
128
129 Dyslipidemia often includes elevations of both triglyceride and cholesterol levels in both mice
130 and humans, so the association of triglycerides with cholesterol was not unexpected. Via
131 multivariate modelling accounting for the effects of diet and sex, a 100 mg/dL increase in
132 triglycerides was associated with a 17.7 +/- 1.7 mg/dL elevation in cholesterol (p=3.8 x 10^{-24},
133 Figure 2A). Within subgroups, the triglyceride-cholesterol relationship varied somewhat, with
134 weaker correlations in HFHS populations (Spearman's rho for males 0.190, females 0.276) than
135 on NCD (males 0.491, females 0.453). Via multivariate regression with interactions, we
136 observed significant sex x diet x triglyceride effect modification with respect to cholesterol
137 levels (p=4.5 x 10-4, ω^2_p = 0.014).
138
139 The strong cross-sectional association of calcium with cholesterol was not predicted by our
140 research team. As shown in Figure 2B and Table 1, after adjusting for diet and sex, a one mg/dL
141 increase in calcium is associated with a 12.7 +/- 0.8 mg/dL increase in cholesterol (p=3.0 x 10^{-43}
142). Linear models predicting serum cholesterol including sex and diet, and calcium as covariates
143 had an adjusted R^2 of 0.45 with a partial effect size (ω^2_p) of 0.22 for serum calcium. We
144 performed sub-group analyses and found that each diet-sex combination had broadly similar
145 estimates for Spearman's rho (ranging from 0.39 for HFHS females to 0.48 for HFHS males),
146 each of which had a p-value of less than 2.2 x 10^{-7}. In this study, calcium levels were not
147 affected by rodent sex (p=0.59) and only modestly by diet (increased by 0.3 mg/dL in HFHS;
148 Supplementary Figure 2A). Due to the strong impacts of diet and sex on cholesterol levels,
149 these suggest that calcium may work largely independently of those factors.
150
151 Given the differences in the physiology and cardiovascular disease associations between HDL
152 versus LDL cholesterol concentrations, we repeated this analysis assessing the diet- and sex-
153 adjusted associations between HDL cholesterol and non-HDL cholesterol and calcium
154 (Supplementary Figures 2B-C). Both of these fractions have positive associations with calcium
155 levels, indicating that the positive associations with calcium are found in both apoplipoprotein
156 fractions. The relationships between all clinical parameters and endpoint cholesterol both in
157 terms of non-parametric correlations and diet/sex adjusted associations are presented in the
158 Supplementary Table.

159

160 To externally test these findings, we evaluated a distinct dataset of genetically diverse mice, the

161 BXD mouse collection. In a secondary data analysis using data in [10], we replicated this

162 finding, showing a cross-sectional association between cholesterol and calcium levels after

163 adjusting for sex differences. Similar to the data from the diversity outbred mice, we estimate a

164 14.8 +/- 5.1 mg/dL increase in cholesterol was observed for every 1 mg/dL increase in calcium

165 (p=0.005). In the smaller BXD dataset there appeared to be a stronger relationship in male

166 mice (Spearman's rho=0.393 p=0.018, n=36 strains) than female mice (rho=0.269; p=0.297,

167 n=17 strains), but in multivariate modelling there was no significant modifying effect of sex

168 (p=0.178), likely due to the relatively small number of female strains.

169

170 In the diversity outbred mice, serum calcium levels are not significantly altered by sex (p=0.59),

171 and only modestly increased by HFHS diets (0.30 +/- 0.07 mg/dL; p=5.1 x 10^{-5}; Supplementary

172 Figure 2C). Since calcium is normally tightly controlled by homeostatic mechanisms regulating

173 calcium absorption and bone remodeling, we tested whether bone mineral content and density

174 in these mice was associated with cholesterol levels. As shown in Supplementary Figure 2D and

175 E, there was no evidence of an association between bone mass or density measured at 21

176 weeks and cholesterol levels measured at 19 weeks (p=0.93 and 0.90 respectively) in the

177 diversity outbred dataset.

178

Conclusions

179

180

181 In this study we report an association between calcium and cholesterol in two distinct mouse

182 datasets. This relationship was similar across both sexes and over both normal chow and

183 obesogenic high fat, high sucrose diets. We were not surprised that HFHS feeding raised

184 cholesterol, as this has been widely observed in mice, rats, and humans. This is likely due to a

185 combination of increased dietary cholesterol, triglycerides, and body fat in these mice. The

186 calcium relationship with cholesterol that was identified here is a unique observation in mice.

187 As the magnitude of elevations of cholesterol and calcium from HFHS diet were similar, and

188 because diet did not substantially alter calcium levels, it is possible that calcium and diet are

189 independent predictors of cholesterol homeostasis. The finding that the calcium-cholesterol

190 association is largely independent of sex, diets or triglyceride levels suggests that serum calcium

191 may represent a novel biomarker of dysfunctional cholesterol homeostasis and may point to

192 novel mechanisms by which cholesterol could be controlled.

193

194 To our knowledge this is the first demonstration of an association between serum calcium and

195 cholesterol in rodents. That being said, several large cross-sectional studies have consistently

196 demonstrated a correlation between serum calcium and cholesterol in multiple populations

197 [19–32]. In addition, calcium is also a longitudinal predictor of cardiovascular events in humans

198 independent of BMI or blood pressure [21,32–37]. A meta-analysis of these associations show

199 that an increase of one standard deviation of serum calcium is associated with an eight percent

200 increased risk of subsequent cardiovascular events [38]. These data are consistent with the

201 hypothesis that the calcium-cholesterol relationship we report here in mice is concordant with
202 increased cardiovascular risk.
203
204 The present study does not speak to the directionality of this association, but there are some
205 hints in the literature. A meta-analysis demonstrates a 31% increased risk of myocardial
206 infarction in patients with calcium supplementing with calcium compared to placebo [39].
207 Patients with primary hyperparathyroidism have elevated parathyroid hormone and calcium
208 levels and are an interesting population to examine. The results of case-control studies
209 evaluating cholesterol levels are mixed and there is limited evidence that PTH causes elevated
210 cholesterol levels. While reports show that these patients also have significantly elevated total
211 and/or LDL-cholesterol [40,41], though most others show either non-significant effect or even
212 decreases [42–48].
213
214 In terms of whether cholesterol could be driving hypercalcemia, there is some positive
215 evidence. Two interventional studies using statins have showed a reductions in calcium levels
216 [49,50], whereas another single-arm study showed declines that did not reach significance [51].
217 A Mendelian Randomization approach using LDL-C as the instrument was also associated with
218 elevated calcium levels [52], further supporting a potential causal relationship with cholesterol
219 driving calcium levels.   As this is a cross-sectional associative relationship we are not able at
220 present to define directionality, much less the underlying biological mechanism(s). Whether
221 calcium can modify serum cholesterol, or cholesterol can modify calcium are both important
222 nutritional and pathophysiological questions, and future controlled mouse studies should shed
223 light on the directions and mechanisms explaining this association.
224
225 There are several other strengths of this study. We present data on a large number of mice
226 roughly equally divided between sexes and two diets and find consistent results across all
227 groups. We have exceptional control of confounders such as diets, environment, activity levels,
228 and other exposures that could affect the interpretation of the human studies. Our supervised
229 machine learning approach used a large number of measured phenotypes to predict calcium
230 levels, and set cutoffs in a data-driven manner. We consider the support of data from two
231 independent genetically diverse mice populations another strength of this work. Calcium-
232 cholesterol associations appear to be robust over a wide variation in genetics and not restricted
233 to findings in inbred mouse populations. As such, this relationship holds over multiple diets,
234 sexes, investigators, sites, and genetic backgrounds.
235

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

245 hormones, which we predict would vary more than the relatively homeostatic blood calcium
246 levels. Another limitation is that cholesterol homeostasis is substantially different in mice and
247 humans, especially in the fraction of cholesterol present in the HDL versus LDL fractions, due to
248 the absence of CETP in mice. These data therefore largely reflect associations between calcium
249 and the HDL pool. Finally, as cardiovascular disease is extremely rare in mice of this age we did
250 not assess cardiovascular disease, or atherogenic lesions as an endpoint in this study.
251
252 In conclusion, in this work we use a machine learning approach to describe that diet, sex,
253 triglyceride levels and calcium all contribute independently to the serum cholesterol levels in
254 Diversity Outbred mice, and that the relationship between calcium and cholesterol holds true in
255 BXD mice. These data support that the observed human relationships between serum and
256 cholesterol levels are true in mice, and present an opportunity for further physiological and
257 genetic dissection of this relationship.
258

Author Contributions

270 DB and CMC conceptualized this research study, decided and validated the methodologies,
271 performed the investigations, wrote the original draft, the final draft, and prepared
272 visualizations. Formal analyses were done by CMC, DB and KL. Data was provided by GAC. This
273 work was administered and supervised by DB who along with GAC performed the data
274 validation. Funding for this work was acquired by DB and GAC. All authors have read and
275 agreed to the final published work.
276

Conflict of Interest

278 The authors have no conflicts to declare
279

References
281

282 1.   Grundy, S.M.; Stone, N.J.; Bailey, A.L.; Beam, C.; Birtcher, K.K.; Blumenthal, R.S.; Braun,
283 L.T.; de Ferranti, S.; Faiella-Tommasino, J.; Forman, D.E.; et al. 2018

AHA/ACC/AACVPR/AAPA/ABC/ACPM/ADA/AGS/APhA/ASPC/NLA/PCNA Guideline on the Management of Blood Cholesterol: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Circulation* **2019**, *139*, e1082–e1143, doi:10.1161/CIR.0000000000000625.

2.   Khera, A.V.; Emdin, C.A.; Drake, I.; Natarajan, P.; Bick, A.G.; Cook, N.R.; Chasman, D.I.; Baber, U.; Mehran, R.; Rader, D.J.; et al. Genetic Risk, Adherence to a Healthy Lifestyle, and Coronary Disease. *New England Journal of Medicine* **2016**, *375*, 2349–2358, doi:10.1056/NEJMoa1605086.

3.   Khera, A.V.; Kathiresan, S. Genetics of Coronary Artery Disease: Discovery, Biology and Clinical Translation. *Nature Reviews Genetics* **2017**, *18*, 331–344, doi:10.1038/nrg.2016.160.

4.   Churchill, G.A.; Gatti, D.M.; Munger, S.C.; Svenson, K.L. The Diversity Outbred Mouse Population. *Mammalian Genome* **2012**, *23*, 713–718, doi:10.1007/s00335-012-9414-2.

5.   Jackson, A.U.; Fornés, A.; Galecki, A.; Miller, R.A.; Burke, D.T. Multiple-Trait Quantitative Trait Loci Analysis Using a Large Mouse Sibship. *Genetics* **1999**, *151*, 785–795, doi:10.1093/genetics/151.2.785.

6.   Churchill, G.A.; Airey, D.C.; Allayee, H.; Angel, J.M.; Attie, A.D.; Beatty, J.; Beavis, W.D.; Belknap, J.K.; Bennett, B.; Berrettini, W.; et al. The Collaborative Cross, a Community Resource for the Genetic Analysis of Complex Traits. *Nature genetics* **2004**, *36*, 1133–1137, doi:10.1038/ng1104-1133.

7.   Ashbrook, D.G.; Arends, D.; Prins, P.; Mulligan, M.K.; Roy, S.; Williams, E.G.; Lutz, C.M.; Valenzuela, A.; Bohl, C.J.; Ingels, J.F.; et al. A Platform for Experimental Precision Medicine: The Extended BXD Mouse Family. *Cell Systems* **2021**, *12*, 235-247.e9, doi:10.1016/j.cels.2020.12.002.

8.   Gatti, D.M.; Simecek, P.; Somes, L.; Jeffery, C.T.; Vincent, M.J.; Choi, K.; Chen, X.; Churchill, G.A.; Svenson, K.L. The Effects of Sex and Diet on Physiology and Liver Gene Expression in Diversity Outbred Mice. *bioRxiv* **2017**, 098657, doi:10.1101/098657.

9.   Svenson, K.L.; Gatti, D.M.; Valdar, W.; Welsh, C.E.; Cheng, R.; Chesler, E.J.; Palmer, A.A.; McMillan, L.; Churchill, G.A. High-Resolution Genetic Mapping Using the Mouse Diversity Outbred Population. *Genetics* **2012**, *190*, 437–447, doi:10.1534/genetics.111.132597.

10.   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.

11.   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.

12.   Sloan, Z.; Arends, D.; Broman, K.W.; Centeno, A.; Furlotte, N.; Nijveen, H.; Yan, L.; Zhou, X.; Williams, R.W.; Prins, P. GeneNetwork: Framework for Web-Based Genetics. *Journal of Open Source Software* **2016**, *1*, 25, doi:10.21105/joss.00025.

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

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

15.   Ben-Shachar, M.S.; Lüdecke, D.; Makowski, D. Effectsize: Estimation of Effect Size Indices and Standardized Parameters. *Journal of Open Source Software* **2020**, *5*, 2815, doi:10.21105/joss.02815.

328   16.   Olejnik, S.; Algina, J. Generalized Eta and Omega Squared Statistics: Measures of Effect
329   Size for Some Common Research Designs. *Psychological Methods* **2003**, *8*, 434–447,
330   doi:10.1037/1082-989X.8.4.434.
331   17.   Tingley, D.; Yamamoto, T.; Hirose, K.; Keele, L.; Imai, K. Mediation: R Package for Causal
332   Mediation Analysis. *Journal of Statistical Software* **2014**, *59*, 1–38, doi:10.18637/jss.v059.i05.
333   18.   Preacher, K.J.; Hayes, A.F. Asymptotic and Resampling Strategies for Assessing and
334   Comparing Indirect Effects in Multiple Mediator Models. *Behavior Research Methods* **2008**, *40*,
335   879–891, doi:10.3758/BRM.40.3.879.
336   19.   Lind, L.; Jakobsson, S.; Lithell, H.; Wengle, B.; Ljunghall, S. Relation of Serum Calcium
337   Concentration to Metabolic Risk Factors for Cardiovascular Disease. *BMJ* **1988**, *297*, 960–963.
338   20.   Meng, X.; Han, T.; Jiang, W.; Dong, F.; Sun, H.; Wei, W.; Yan, Y. Temporal Relationship
339   Between Changes in Serum Calcium and Hypercholesteremia and Its Impact on Future Brachial-
340   Ankle Pulse Wave Velocity Levels. *Frontiers in Nutrition* **2021**, *8*.
341   21.   Jorde, R.; Sundsfjord, J.; Fitzgerald, P.; Bønaa, K.H. Serum Calcium and Cardiovascular
342   Risk Factors and Diseases. *Hypertension* **1999**, *34*, 484–490, doi:10.1161/01.HYP.34.3.484.
343   22.   Kennedy, A.; Vasdev, S.; Randell, E.; Xie, Y.-G.; Green, K.; Zhang, H.; Sun, G. Clinical
344   Medicine: Endocrinology and Diabetes: Abnormality of Serum Lipids Are Independently
345   Associated with Increased Serum Calcium Level in the Adult Newfoundland Population. *Clinical
346   medicine. Endocrinology and diabetes* **2009**, *2*, CMED.S2974, doi:10.4137/CMED.S2974.
347   23.   Saltevo, J.; Niskanen, L.; Kautiainen, H.; Teittinen, J.; Oksa, H.; Korpi-Hyövälti, E.;
348   Sundvall, J.; Männistö, S.; Peltonen, M.; Mäntyselkä, P.; et al. Serum Calcium Level Is Associated
349   with Metabolic Syndrome in the General Population: FIN-D2D Study. *European Journal of
350   Endocrinology* **2011**, *165*, 429–434, doi:10.1530/EJE-11-0066.
351   24.   Gallo, L.; Faniello, M.C.; Canino, G.; Tripolino, C.; Gnasso, A.; Cuda, G.; Costanzo, F.S.;
352   Irace, C. Serum Calcium Increase Correlates With Worsening of Lipid Profile. *Medicine
353   (Baltimore)* **2016**, *95*, e2774, doi:10.1097/MD.0000000000002774.
354   25.   De Bacquer, D.; De Henauw, S.; De Backer, G.; Kornitzer, M. Epidemiological Evidence
355   for an Association between Serum Calcium and Serum Lipids. *Atherosclerosis* **1994**, *108*, 193–
356   200, doi:10.1016/0021-9150(94)90114-7.
357   26.   Wilson, P.W.; Garrison, R.J.; Abbott, R.D.; Castelli, W.P. Factors Associated with
358   Lipoprotein Cholesterol Levels. The Framingham Study. *Arteriosclerosis: An Official Journal of
359   the American Heart Association, Inc.* **1983**, *3*, 273–281, doi:10.1161/01.ATV.3.3.273.
360   27.   Chou, C.-W.; Fang, W.-H.; Chen, Y.-Y.; Wang, C.-C.; Kao, T.-W.; Wu, C.-J.; Chen, W.-L.
361   Association between Serum Calcium and Risk of Cardiometabolic Disease among Community-
362   Dwelling Adults in Taiwan. *Sci Rep* **2020**, *10*, 3192, doi:10.1038/s41598-020-60209-w.
363   28.   Sabanayagam, C.; Shankar, A. Serum Calcium Levels and Hypertension Among US Adults.
364   *The Journal of Clinical Hypertension* **2011**, *13*, 716–721, doi:10.1111/j.1751-7176.2011.00503.x.
365   29.   Rohrmann, S.; Garmo, H.; Malmström, H.; Hammar, N.; Jungner, I.; Walldius, G.; Van
366   Hemelrijck, M. Association between Serum Calcium Concentration and Risk of Incident and
367   Fatal Cardiovascular Disease in the Prospective AMORIS Study. *Atherosclerosis* **2016**, *251*, 85–
368   93, doi:10.1016/j.atherosclerosis.2016.06.004.
369   30.   Green, M.A.; Jucha, E. Interrelationships between Blood Pressure, Serum Calcium and
370   Other Biochemical Variables. *International Journal of Epidemiology* **1987**, *16*, 532–536,
371   doi:10.1093/ije/16.4.532.

31.   He, L.; Qian, Y.; Ren, X.; Jin, Y.; Chang, W.; Li, J.; Chen, Y.; Song, X.; Tang, H.; Ding, L.; et al. Total Serum Calcium Level May Have Adverse Effects on Serum Cholesterol and Triglycerides Among Female University Faculty and Staffs. *Biol Trace Elem Res* **2014**, *157*, 191–194, doi:10.1007/s12011-014-9895-9.

32.   Lind, L.; Skarfors, E.; Berglund, L.; Lithell, H.; Ljunghall, S. Serum Calcium: A New, Independent, Prospective Risk Factor for Myocardial Infarction in Middle-Aged Men Followed for 18 Years. *Journal of Clinical Epidemiology* **1997**, *50*, 967–973, doi:10.1016/S0895-4356(97)00104-2.

33.   Foley, R.N.; Collins, A.J.; Ishani, A.; Kalra, P.A. Calcium-Phosphate Levels and Cardiovascular Disease in Community-Dwelling Adults: The Atherosclerosis Risk in Communities (ARIC) Study. *American Heart Journal* **2008**, *156*, 556–563, doi:10.1016/j.ahj.2008.05.016.

34.   Slinin, Y.; Blackwell, T.; Ishani, A.; Cummings, S.R.; Ensrud, K.E.; MORE Investigators Serum Calcium, Phosphorus and Cardiovascular Events in Post-Menopausal Women. *Int J Cardiol* **2011**, *149*, 335–340, doi:10.1016/j.ijcard.2010.02.013.

35.   Jorde, R.; Schirmer, H.; Njølstad, I.; Løchen, M.-L.; Bøgeberg Mathiesen, E.; Kamycheva, E.; Figenschau, Y.; Grimnes, G. Serum Calcium and the Calcium-Sensing Receptor Polymorphism Rs17251221 in Relation to Coronary Heart Disease, Type 2 Diabetes, Cancer and Mortality: The Tromsø Study. *Eur J Epidemiol* **2013**, *28*, 569–578, doi:10.1007/s10654-013-9822-y.

36.   Walsh, J.P.; Divitini, M.L.; Knuiman, M.W. Plasma Calcium as a Predictor of Cardiovascular Disease in a Community-Based Cohort. *Clin Endocrinol (Oxf)* **2013**, *78*, 852–857, doi:10.1111/cen.12081.

37.   Wang, M.; Yan, S.; Peng, Y.; Shi, Y.; Tsauo, J.-Y.; Chen, M. Serum Calcium Levels Correlates with Coronary Artery Disease Outcomes. *Open Medicine* **2020**, *15*, 1128–1136, doi:10.1515/med-2020-0154.

38.   Reid, I.R.; Gamble, G.D.; Bolland, M.J. Circulating Calcium Concentrations, Vascular Disease and Mortality: A Systematic Review. *Journal of Internal Medicine* **2016**, *279*, 524–540, doi:10.1111/joim.12464.

39.   Effect of Calcium Supplements on Risk of Myocardial Infarction and Cardiovascular Events: Meta-Analysis | The BMJ Available online: https://www.bmj.com/content/341/bmj.c3691 (accessed on 4 February 2023).

40.   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.

41.   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.

42.   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.

43.   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.

44.   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.

45.   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.

46.   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.

47.   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.

48.   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.

49.   Soh, J.F.; Bodenstein, K.; Yu, O.H.Y.; Linnaranta, O.; Renaud, S.; Mahdanian, A.; Su, C.-L.; Mucsi, I.; Mulsant, B.; Herrmann, N.; et al. Atorvastatin Lowers Serum Calcium Levels in Lithium-Users: Results from a Randomized Controlled Trial. *BMC Endocr Disord* **2022**, *22*, 238, doi:10.1186/s12902-022-01145-w.

50.   Farhan, H.A.; Khazaal, F.A.; Mahmoud, I.J.; Haji, G.F.; Alrubaie, A.; Abdulraheem, Y.; * A.M.A.; Alkuraishi, M. Efficacy of Atorvastatin in Treatment of Iraqi Obese Patients with Hypercholesterolemia. *AL-Kindy College Medical Journal* **2014**, *10*, 62–69.

51.   Montagnani, A.; Gonnelli, S.; Cepollaro, C.; Pacini, S.; Campagna, M.S.; Franci, M.B.; Lucani, B.; Gennari, C. Effect of Simvastatin Treatment on Bone Mineral Density and Bone Turnover in Hypercholesterolemic Postmenopausal Women: A 1-Year Longitudinal Study. *Bone* **2003**, *32*, 427–433, doi:10.1016/S8756-3282(03)00034-6.

52.   Li, S.; Schooling, C.M. A Phenome-Wide Association Study of Genetically Mimicked Statins. *BMC Med* **2021**, *19*, 1–11, doi:10.1186/s12916-021-02013-5.

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.

A.

B. DO Mice

C.

A. DO Mice

F | M

p<0.001

Diet
— NCD
— HFHS

Cholesterol (mg/dL)

Triglycerides (mg/dL)

B. DO Mice

F | M

p<0.001

Diet
— NCD
— HFHS

Cholesterol (mg/dL)

Calcium (mg/dL)

C. BXD Mice

F | M

p=0.005

Cholesterol (mg/dL)

Calcium (mg/dL)