We thank both reviewers for their well-considered and reasonable critiques to our work. We have revised the manuscript accordingly and detail these changes here as well. Our point-by-point responses are included below in **bold**, and verbatim text added to the manuscript is noted in **red bold** font. We have also included line numbers for the changes for your convenience.

# Point-by-Point Response to Reviewer #1

The manuscript applies AI to investigate which are the factors that are independently associated with plasma cholesterol levels in the diversity outbread mice dataset.  
As expected, gender and diet are the major factors correlating with changes in cholesterol levels. Intriguingly calcium levels appear to the third.  
The manuscript will benefit from the following analysis if feasible:  
-Correlation of plasma cholesterol levels with the extension of the atherosclerotic plaque

**We agree that this is an important pathophysiology that was not measured in this study, so we are unable to perform this analysis. We do look forward to using these data in future studies using mouse models with atherogenic susceptibility, such as *Apoe* or *Ldlr* knockout mice. This is now noted in the limitations section on lines 249-250.**

**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.**

-Correlation of cholesterol content in lipoprotein subsets with calcium levels.

****

**Response Figure 1**: Associations between HDL-C and non-HDL cholesterol with calcium levels at endpoint. These data are presented as Supplementary Figures 2A and 2B in the revised manuscript. Both associations are significant after diet and sex adjustment (p<1x10-7).

**This is an excellent point, given the differing directional associations of LDL vs HDL cholesterol with respect to cardiovascular disease. To answer this question have repeated this analysis for HDL-C which was directly measured and non-HDL cholesterol which was calculated by subtracting HDL-C from the total cholesterol. We are unable to detect other apolipoproteins such as VLDL or chylomicrons or separate those from non-HDL-C, nor are we able to evaluate correlations with subpopulations of lipoprotein particles. The correlations between serum calcium and these values are now presented in Supplementary Figures 2A-B and Response Figure 1. In both cases there was a significant association after adjusting for diet and sex. For HDL-C vs calcium the effect was 10.4 +/- 0.6 mg/dL per mg/dL of calcium (p=3 x 10-47). For non-HDL cholesterol the effect was 2.0 +/- 0.4 (p=4.5 x 10-8). From these data one could reasonably conclude the effect is stronger for the HDL than non-HDL cholesterol association, however we believe these effects are similar in scale since HDL levels are 3.5-7.1x higher than non-HDL cholesterol levels depending on the diet/sex group. Our interpretation is therefore that both subgroups of apolipoprotein fractions are associated with calcium, thus our focus on total cholesterol. It is fascinating that there is only a diet effect (after adjusting for changes in calcium) in the HDL-C but not the non-HDL-C group (comparing the black to the grey lines). While we don’t want to speculate here what that may mean, but we would have expected a stronger diet effect in the non-HDL-C fraction which contains among other things the LDL. This is something we look forward to examining further, and we thank the reviewer for encouraging this analysis. These data are now described in the results section on lines 151-156:**

**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 2A-B). Both of these fractions have positive associations with calcium levels, indicating that the positive associations with calcium are found in both apoplipoprotein fractions.**

-Correlation of cholesterol with the other parameters when a cholesterol rich diet is used.

**We performed the requested analysis of cholesterol associations with all available clinical parameters. These data are presented in two ways in the new Supplementary Table 1 with both spearman correlation coefficients and estimates for association with endpoint cholesterol using sex and diet adjusted linear models. After adjusting for multiple comparisons, out of 80 parameters, we report 35 significant correlations with cholesterol and 28 significant associations after adjusting for sex and diet. 22 parameters, including calcium, were significant in both analyses, including several reported in more detail in the revised manuscript (HDL-C and non-HDL-C at both time points, cholesterol at 8 weeks, and triglycerides at 19 weeks). This table is now described in the manuscript on lines 156-158:**

**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.**

-Evaluation of the translational relevance of the findings by for instance investigating the correlation between plasma chol levels, atherosclerosis and calcium in human datasets.

****

**Response Figure 2: Forrest plot of the associations between cholesterol and calcium in human epidemiological data.** A meta-analysis was performed on studies that evaluated the associations between calcium and cholesterol in a total of 88939 human subjects across 9 studies. In the case of several of these studies data was only presented stratified by subgroups so these are denoted with m, f, f-pm (post-menopausal), or m20-39/m40-69 (males sub-grouped by age). Random effects modelling shows a significant association between calcium and cholesterol with an estimated correlation of r=0.17 (95% CI 0.13-0.20).

**This is an excellent point, and we have revised the discussion for a more thorough consideration of these relationships in humans. As To be more quantitative to this point we performed a meta-analysis of the current papers describing cholesterol-calcium relationships. As shown in Response Figure 2, there is a highly consistent positive relationship between serum total cholesterol and calcium in several different populations of humans (total n=88939 from 10 studies and 17 subgroups; r=0.17, 95% CI 0.13-0.20, p=4.2 x 10-8). These effect estimates are concordant with what we find in mice though the effect sizes of the associations are somewhat smaller (r=0.17 vs r=0.39-0.48 in our more controlled mouse study). Several other studies cited in the revised manuscript have also demonstrated significant positive associations, but were analyzed by quantile regression** [1–4] **or only reported LDL-C not cholesterol [5] so could not be integrated in this meta-analysis. We feel that the work reported in this manuscript in experimental animals is strongly supportive of this human data. Although we could be convinced otherwise, we would prefer to publish these human meta-analytic data separately as the additional figures such as study details, other outcomes (LDL-C, HDL-C), and bias assessments would detract from the more direct mouse data we are reporting in this revised manuscript. That being said we look forward to publishing this meta-analysis of the human calcium cholesterol associations separately.**

**Related to the translational significance to cardiovascular events, rather than just cholesterol we now cite seven human studies showing associations between calcium in the blood and cardiovascular risk, independent of either BMI or blood pressure** [6–12]. **A meta-analysis of serum calcium relative to risk for subsequent cardiovascular disease also showed a positive association between serum calcium and increased risk of MI/CHD (8% increased risk per SD of serum calcium, 95% CI 4-13%, eight studies) and overall mortality (13% [9-18%], nine studies [13]). Finally a large meta-analysis of calcium supplementation studies also showed an association between calcium supplementation and cardiovascular risk both in RCTs (1.31x increased risk of myocardial infarction, p=0.035; [14]). Together, these data are highly supportive of associations between serum calcium and blood cholesterol as well as cardiovascular disease.**

**We have added these insights on lines 194-202 of the revised manuscript:**

**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** [1–7,15–21]**. In addition, calcium is also a longitudinal predictor of cardiovascular events in humans independent of BMI or blood pressure** [6–12]**. 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 [13]. These data are consistent with the hypothesis that the calcium-cholesterol relationship we report here in mice is concordant with increased cardiovascular risk.**

Additional analysis.  
-How do the authors explain the weaker association between plasma chol and plasma TG in figure 2, panel A, male mice on HFHS diet compared to normal diet?

**Response Table 1: Subgroup sensitivity analyses of the cholesterol-triglyceride relationships in DO mice at 19 weeks.** Beta coefficients include estimate +/- standard error in units of mg/mL cholesterol per mg/mL of triglycerides; p-values are from spearman’s rho estimates.

|  |  |  |  |
| --- | --- | --- | --- |
| **Group** | **Beta Coefficient** | **Spearman’s Rho** | **P-value** |
| NCD-F | 0.202 +/- 0.026 | 0.453 | 1.1 x 10-12 |
| NCD-M | 0.194 +/- 0.022 | 0.491 | 1.7 x 10-14 |
| HFHS-F | 0.354 +/- 0.063 | 0.276 | 9.3 x 10-5 |
| HFHS-M | 0.083 +/- 0.036 | 0.190 | 0.01 |

**This is something we had not noticed in our initial analysis of these data, so thank the reviewer for pointing this out. Indeed, a visual analysis of the relationships between circulating cholesterol and triglycerides in Figure 2A shows a positive trend for each group, but a noticeably attenuated relationship in the male mice on NCD. We have performed sub-group sensitivity analyses in Response Table 1 describing these effects in more detail. Indeed, the correlation between serum triglycerides and cholesterol is attenuated in HFHS mice of both sexes, and that the slope of the cholesterol/triglycerides estimate is much lower in the HFHS/Male group.**

**Response Table 2: Modelling of a diet/sex interaction between cholesterol and triglycerides at 19 weeks.** A linear model was constructed with interactions between diet, sex and triglyceride levels in predicting cholesterol. Estimates are in units of mg/dL of cholesterol (diet, sex and diet x sex) or mg/dL cholesterol per mg/dL triglycerides (for estimates involving TG). Partial effect sizes are reported as Ω2p calculated as described in the methods section.

|  |  |  |  |
| --- | --- | --- | --- |
| **Term (reference)** | **Estimate (+/- SE)** | **P-value** | **Partial Effect Size (p)** |
| Intercept | 54.2 +/- 4.5 | NA | NA |
| Diet (HFHS) | 27.1 +/- 6.7 | 5.8 x 10-5 | 0.074 |
| Sex (M) | 13.3 +/- 6.4 | 3.8 x 10-2 | 0.058 |
| TG | 0.202 +/- 0.034 | 4.2 x 10-9 | 0.338 |
| Diet (HFHS) x Sex (M) | 23.9 +/- 9.1 | 9.4 x 10-3 | 0.0075 |
| Diet (M) x TG | 0.152 +/ - 0.062 | 0.015 | 0.0011 |
| Sex (M) x TG | -0.007 +/- 0.044 | 0.87 | 0.00086 |
| Diet (HFHS) x Sex (M) x TG | -0.263 +/- 0.075 | 4.5 x 10-4 | 0.01378 |

**We explored this further via multivariate modeling testing if diet and/or sex significantly modified these relationships. Indeed, a linear model containing terms for a three-way-interactions for triglycerides, diet and sex does support an attenuation of the triglyceride-cholesterol relationship in male mice on a high fat, high sucrose diet. The full model is reported in Response Table 2. This was an overlooked moderation by both sex and diet that we now report in the revised manuscript on lines 133-137:**

**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 x 10-4, p = 0.014).**

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# Point-by-Point Response to Reviewer #2

Comments to the Author  
Comment:  
The authors analyzed in two distinct mouse datasets, Diversity Outbred and BXD, using a machine learning approach. This is an attractive study; however, some concerns are needed to address.  
  
Major concerns:  
1.      How long was the HSFS diet fed to the mice?

**Mice were fed the HFHS diet from week 3 to week 19, so a total of 16 weeks. This is now noted in the methods section on lines 100-101**

**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).**

2.      Are all of these mouse datasets from healthy mice fed NCD or HFHS?

**Yes, they were only on one of those two diets after their weaning at three weeks. Now noted in the methods section on lines 60-64:**

**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).**

Are these phenotypes, including the relationship between cholesterol and calcium, for healthy cases and are different in disease conditions?

**Yes, we report significant calcium-cholesterol associations in both HFHS and NCD fed mice of both sexes. That being said, we don’t think that HFHS necessarily represents a disease condition for all mice. This is now reported on lines 144-146 of the revised manuscript**

**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 x 10-7.**

3.      Aging is also thought to be an important factor in elevated calcium and/or cholesterol levels. Do the authors’ findings apply to both young and old?

**The oldest mice in our study were only 19 weeks old at time of cholesterol and calcium assessment, which relative to the average lifespan of a mouse (48-72 weeks) is quite young, so we did not test whether these effects hold true for older mice in this study. This is a fascinating question that we hope to answer in future studies. Noted in the limitations section on lines 237-241:**

**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.**

4.      Authors described that these data reflect associations between calcium and the HDL pool because of the absence of CETP in mice. Is there possibility that calcium levels affect LDL-C or both?

**Yes, these data are now included in the revised manuscript and we see similar relationships between calcium and both HDL and non-HDL cholesterol (see above response figure 1 and the associated text). The relevance of the absence of CETP is noted in the revised limitations section:**

**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.**

5.      Are patients with hypercalcemia more likely to have dyslipidemia or vice versa? Any reports?

**Yes, as noted above in the response to reviewer #1 the associations between calcium and cholesterol as well as between calcium and cardiovascular disease have been reported previously in humans. This is now described in the revised discussion on lines 197-200:**

**In addition, calcium is also a longitudinal predictor of cardiovascular events in humans independent of BMI or blood pressure** [6–12]**. 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 [13].**   
  
Minor points:  
1.      There is no citation in texts and figure legend in Figure 1C. Please add.

**Thank you for noting this oversight, this is now referenced in lines 125-127 and the figure legend updated.**

**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).**

2.      The lines of HFHS group in Figures 1 and 2 are hard to see due to the light color. Please change it.

**Fixed, making the HFHS points substantially darker in all plots**

# References

**NB: These references are numbered according to this document, and are not the numbers used in the manuscript**

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