

Non-remnant triglyceride-rich lipoproteins due to lipoprotein lipase deficiency increase atherosclerosis in mice

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

I reviewed this manuscript for Nature Metabolism and it was referred to Nature Communications with guidance to the authors to respond to the critiques with a rebuttal and a revised manuscript. I based my review on these responses and the revised manuscript.

This manuscript fundamentally addresses the question of how inducible whole body LPL deficiency influences atherosclerosis on the atherogenic background of functionally deficient LDL receptor mice fed a western diet. The most important conclusion is that LPL deficiency significantly increased atherosclerosis (in two arterial beds in males but only one in females) on this background compared with LDLR deficiency alone. The studies are well-performed and the results very important; they confirm the importance of LPL in protecting against atherosclerosis in a highly atherogenic hypercholesterolemic environment. However, the interpretation of the authors--that this increased atherosclerosis is due to specifically and primarily to hyperchylomicronemia--is not proven based on the data provided.

Specific comments:

1. The conclusion that chylomicrons are the driver of the increased atherosclerosis in this mouse model is not supported by the data presented. The only data presented that include chylomicron measurement are shown in Figure 2 and is described in the Results text as an isolation after a 'brief centrifugation' and the methods section states simply "sequential density ultracentrifugation to separate chylomicrons (CM), VLDL ($d < 1.006$ g/ml)..." More detail on how the chylomicrons were differentiated from other TRLs is needed. In Figure 2C and D the first two bars are labeled 'TRL' but it is unclear how these values were derived separately from the CM, VLDL, and IDL fractions. The other relevant mouse experiments such as those in Figure 1 and Suppl Fig 2 do not report specifically on CM.

The conventional definition of chylomicrons is intestinally-derived apoB-48 containing large ultra-low density lipoproteins. In the response to comment #10, the authors provide data showing that apoB-100 is increased in TRL ($d < 1.006$). It would be helpful to see the characterization of apoB in what the authors are labeling chylomicrons after the 'brief spin.' A substantial increase in apoB-48 and not apoB-100 would support the authors' claim of increased chylomicrons. Also, in Figure 7 the authors show EM images of rabbit TRLs; EM of the mouse CMs would be another useful approach to characterization of these particles.

Even if a clear increase in apoB-48 containing CMs were demonstrated, however, it would be a stretch to attribute the increased atherosclerosis solely or primarily to increased chylomicrons given that it is clear that non-CM apoB-100 TRLs are also increased in this model. Isn't it possible that they contribute substantially to the increased atherosclerosis?

A more conservative interpretation of the data (and title for the paper) would be that LPL deficiency in the setting of LDLR knockdown significantly increases atherosclerosis. This increase could be due to some combination of increased CM, non-CM TRLs, and TG-enriched LDL (in theory even effects of LPL that are unrelated to these lipoprotein effects could play a role).

2. The single cell RNA seq data indicate major differences in gene expression in endothelial cells, macrophages, and VSMCs. It is unclear why the authors chose to highlight endothelial cell inflammation in the manuscript and the title. The experiments shown in Figure 7 using rabbit TRLs with endothelial cells do not address inflammation but rather uptake. More caution is warranted with regard to the interpretation of the mechanism of increased atherosclerosis.

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- The authors responded to the comments of this reviewer who specifically addressed the limited number of animals that were studied resulting in a small number of cells that have been analyzed with single cell seq. The number of animals is low, but the number of cells that have been analyzed are sufficient to draw inferences. No large numbers, but it's not the core datasets and it's aligned with other analyses. Batch correction and harmonization between individual conditions looks properly done (this is crucial, as otherwise is often source of false signals). A pitfall could be the pooling of the samples. It is known that cell types can differ substantially among individual animals and humans. Now it is not known if specific observations in the single cell seq data can be explained by a single animal.

Additional comment:

- Figure 3 numbers do not seem correct. Just add up cross-sectional areas the plaque components (collagen/macrophage/oil red O) and then these numbers are almost 3-fold the total plaque area.

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We thank the reviewer for these comments. We agree that loss of LPL and the resulting increase in circulating non-lipolyzed lipoproteins will lead to a number of changes. Using these LPL-deficient mice, which lack CETP, we do not see changes in LDL and HDL size on FPLC, as would occur in humans. Nonetheless, the LDL does show an increase in TG content.

As noted by the reviewer, VLDL are also increased in the setting of LpL deficiency, and this increase may also contribute to the increased atherosclerosis we have documented in this model. To reflect this, as suggested by the reviewer, we have changed the manuscript title to "Non-remnant triglyceride-rich lipoproteins due to lipoprotein lipase deficiency increase atherosclerosis". This new title is more precise and makes the same point. As noted by the reviewer, loss of LpL should increase non-lipolyzed VLDL as well as chylomicrons.

- A western blot showing an increase of both apoB100 and apoB48 in the 30 min d<1.006 spin (which as noted we had included in our response to this reviewer's comments in our original submission) has now been added to the manuscript as Supplement Figure 2D. On page 6 we now say:

"Immunoblot analysis of the TRL (d<1.006) fraction showed that both apoB48 and apoB100 were substantially augmented, reflecting that LpL deficiency led to increased VLDL and chylomicrons (Supplement Figure 2D, left panel). Ponceau staining highlighting equal loading is shown in right panel."

- In addition, as suggested by the reviewer, electron microscope images of these TRLs are now shown in Figure 7A (right panel). This addition highlights the morphological similarities between rabbit and mouse TRLs, as well as the differences with the particles obtained from cholesterol-fed diabetic rabbits. On page 12 we now say:

"Electron microscopy images of those particles, as well as of TRLs obtained from *iLpL^{-/-}/Ldlr^{kd}* mice, are shown in Figure 7A. Giant TRLs from cholesterol-fed diabetic rabbits were much larger than those from mice or non-diabetic rabbits."

2. The single cell RNA seq data indicate major differences in gene expression in endothelial cells, macrophages, and VSMCs. It is unclear why the authors chose to highlight endothelial cell inflammation in the manuscript and the title. The experiments shown in Figure 7 using rabbit TRLs with endothelial cells do not address inflammation

but rather uptake. More caution is warranted with regard to the interpretation of the mechanism of increased atherosclerosis.

We agree with the reviewer's comments. Our focus on endothelial cells stems in part from our published observations that chylomicron uptake induces endothelial cell inflammation and secretion of extracellular vesicles (EVs) that in turn promote lipid droplet accumulation and inflammatory gene expression in macrophages. We now discuss in more detail the reasons for our focus on endothelial cells as initiating this process. On page 11 we now say: "We have recently shown that chylomicron uptake leads to overexpression of inflammatory genes in ECs, as well as the release of extracellular vesicles with distinct microRNA signatures which in turn induce inflammation in underlying macrophages (PMID: 40093163). Our findings are indicative of a heightened inflammatory response, likely contributing to the progression of atherosclerosis observed under LpL deficiency conditions."

Reviewer #3 (Remarks to the Author):

- The authors responded to the comments of this reviewer who specifically addressed the limited number of animals that were studied resulting in a small number of cells that have been analyzed with single cell seq. The number of animals is low, but the number of cells that have been analyzed are sufficient to draw inferences. No large numbers, but it's not the core datasets and it's aligned with other analyses. Batch correction and harmonization between individual conditions looks properly done (this is crucial, as otherwise is often source of false signals).

A pitfall could be the pooling of the samples. It is known that cell types can differ substantially among individual animals and humans. Now it is not known if specific observations in the single cell seq data can be explained by a single animal.

We thank the reviewer for their thoughtful assessment of our single-cell RNA-seq dataset. We are pleased that the reviewer recognizes the adequacy of the number of cells analyzed, the quality of our batch correction and harmonization procedures, and the alignment of our results with other supporting analyses. We acknowledge the reviewer's comment regarding the potential pitfall of pooling samples. While we agree that inter-animal variability is an important consideration in single-cell studies, pooling was necessary due to the limited number of cells that can be isolated from an individual aortic arch. Pooling allowed us to obtain a sufficient number of viable cells to generate high-quality libraries, permitting robust analysis while minimizing technical variability and maximizing the biological signal at the group level. Importantly, batch correction and harmonization were carefully performed to account for potential technical artifacts, and the consistency of our findings with other core datasets in the study further supports the validity of the observations.

Additional comment:

- Figure 3 numbers do not seem correct. Just add up cross-sectional areas the plaque components (collagen/macrophage/oil red O) and then these numbers are almost 3-fold the total plaque area.

We appreciate the reviewer's observation. The summed areas of macrophages, lipids, and collagen indeed exceed the total plaque area, which reflects the biological reality that these components are not mutually exclusive. Macrophages and lipid deposits often co-localize within foam cells, and collagen can be present throughout both macrophage-rich and lipid-rich regions. The quantification of each marker was performed independently and may include overlapping areas, which is consistent with common histopathological findings in atherosclerotic lesions.

- Reading the other comments (reviewer #1) I feel that the authors could downtone firm statements that can be debated such as in the last sentence of the introduction: "These studies resolve the long-standing controversy on the atherogenicity of large TRLs and debunk the hypothesis that chylomicrons are benign.". Looking at literature it is evident that there is a lot out there that supports the view that chylomicrons are not benign, in contrast. In addition, this an animal study and may suggest but not resolve an issue in human disease. There are GWAS results published clearly showing that SNPs in the LPL gene are associated with complications of human atherosclerotic disease.

Agreed. We have changed this section, and the last sentence of the introduction (page 4) now reads as follows: .

“Despite substantial data implicating TRLs in atherosclerosis, much of the clinical literature has specifically stated that changes in atherosclerosis with TRL reduction are due to a reduction in “remnants”²⁵. These comments imply that TRLs are only toxic after their partial lipolysis by LPL. Our studies do not support this widely accepted hypothesis and are consistent with data implicating both postprandial lipemia and increased non-fasting TG levels as atherogenic states⁴”

This, along with the change in the title, makes clear that the focus is on non-lipolyzed triglyceride-rich lipoproteins to differentiate them from remnants.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The authors have addressed my remaining issues satisfactorily. The new title is an improvement.

Thank you.

Reviewer #3 (Remarks to the Author):

I feel the authors did not address my first two comments.

1- Pooling the mice data. I understand and agree with the response. I understand that the cells were pooled before sequencing which does not allow an assessment of individual mice. I understand that this comment cannot be addressed. I just want to mention that the answers (corrections account for technical artifacts) do not address this question.

We appreciate the reviewer's thoughtful comment and understand the importance of acknowledging the limitation associated with pooling samples for single-cell RNA-sequencing. While we have addressed this point in our response, we now explicitly acknowledge this limitation in the revised manuscript. A sentence has been added to the Discussion section to highlight that pooling precluded assessment of inter-animal variability and that this remains a potential limitation of our single-cell dataset. On page 15 we now say:

"A limitation of our single-cell RNA-seq analysis is the pooling of aortic cells from multiple mice prior to library preparation, which precludes assessment of inter-animal variability. While pooling was necessary to obtain sufficient cell numbers for robust sequencing, we acknowledge that this approach may mask individual differences in cell-type composition or gene expression."

2- The comment about Figure 3. I understand there is overlap in areas that stain for macrophages or fat. However, looking at stains through a microscope will tell that collagen does not overlap with a macrophage or fat. And the answer in fact tells me that the cross-sectional area measurements are not precise. I feel one cannot present cross-sectional areas of stains that are a multifold of the total cross-sectional area of a lesion.

This number does then not reflect the true value. The authors should consider to present these data with another measure.

We thank the reviewer for these comments. We have discussed this with several colleagues, who agreed that the summed areas of macrophages, lipids, and collagen often exceed the total plaque area due to overlap. We have expanded the Methods section to explain our methodology in more detail, and in page 21 we now say:

“Serial sections (6 μm) of roots were obtained by cryosectioning and placed in microscope slides (six sections per slide). CD68 immunostaining was used to determine macrophage content, as described previously⁶⁵⁻⁶⁷. Oil Red O immunostaining was used to determine neutral lipids, and Picrosirius Red staining was used to assess the collagen content under polarized light. Before staining, sections were observed by bright-field microscopy to select those in which all three aortic root leaflets were visible. Six sections per mouse were analyzed for each staining, and the lesion area, as well as area occupied by CD68/Oil Red O/Picrosirius Red in each of those sections were averaged. ImageJ 1.53t software (National Institutes of Health) was used for all the quantifications.”