nature portfolio

Peer Review File

FNIP1 variants associated with favorable metabolism in 1 million humans

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Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
In this paper, Hindy and colleagues use exome sequencing to identify rare variants that are associated with changes in the triglyceride (TG) to HDL ratio. The authors use this information to validate the use of the TG/HDL ratio as a biomarker for various metabolic phenotypes and cardiovascular disease. The genetic studies and associated clinical data are impressive and will provide an important resource for subsequent studies. However, our review focuses specifically on the data from the mouse models used, and in particular the studies aimed at investigating the role of interfering with the FLCN/FNIP pathway on metabolic health.

1. A range of mutations in FNIP1 are identified and predicted to be LoF. Some of these mutations are located towards the C-terminus of the protein. Is there any direct evidence that all the mutations are LoF (particularly the mutations in the C-terminal region)?

2. The approach used to investigate the functional significance of candidate genes identified from the exome sequencing requires some justification. Presumably, the mutations in humans are present in all cell types expressing the gene, and the mutations are heterozygous? In order to mimic the human situation a direct approach would be to make heterozygous (whole body) knockouts of candidate genes. Of course, this is a time-consuming approach, and presumably because of this the authors chose to use AAV8-targetted expression of gRNAs to delete candidate genes. This is very likely quite different from the situation in humans carrying mutations, so immediately raises concerns as to how to interpret the data. Moreover, the use of AAV8-targetted deletion raises its own issues if the aim is to delete genes specifically in hepatocytes. AAV8 targets other cell types in addition to hepatocytes, and so it is not possible to attribute directly any phenotypic effects to the liver (hepatocytes). In this regard, conditional deletion of candidate genes (e.g. using Alb-cre of floxed alleles) would be a better approach. As it stands, it is difficult to make firm conclusions from the mouse models (see further comments below).

3. The choice of which candidate genes to explore is not made obvious. As the authors mention, previous studies have reported that liver-specific deletion of FLCN in mice protects against hepatic steatosis and fibrosis, but in humans LoF mutation in FLCN lead to autosomal dominant BHD syndrome, leading to increased risk of cancer, particularly renal cancer. LoF mutations in FNIP1 lead to autosomal recessive abnormalities, including immunodeficiency and cardiomyopathy (overlapping with WPW syndrome). The decision to focus on the inhibition of the FLCN/FNIP pathway as a potential therapeutic area is a little surprising (but clearly this is a subjective decision). That aside, the authors use the AAV8 approach to down-regulate FLCN, FNIP1, FNIP2 or FNIP1/FNIP2 gene expression in Cas9-Ready mice. Confusingly, in Ex Data Fig. 6, FNIP2 expression in the liver increases in FNIP1/FNIP2 gRNA mice. The authors point out that in addition to hepatocytes, the liver contains other cell types but make no further attempt to explain how this may contribute to their results. It is not obvious why FNIP2 expression increases in the FNIP1/FNIP2 gRNA mice. At the very least, the authors should determine gene expression levels in hepatocytes isolated from the mouse livers, but a deeper understanding of expression levels in different cell types (e.g. using scRNA approaches) would be very informative here. Circling back to the clinical finding in humans, does down-regulation of FNIP1 or FNIP1/FNIP2 lead to a decrease in circulating TG levels?

4. Related to the above point, it would be informative to have some data looking at the protein levels of FLCN/FNIP1/FNIP2

in liver (and isolated hepatocytes) from the different mouse lines. Depending on the outcome of these additional studies, the conclusions drawn from these mouse models may need to be reconsidered in their totality.

5. Do the authors have any mechanistic explanation for the protection against diet-induced obesity following deletion of FLCN or FNIP1/FNIP2 in the liver (Fig. 4c)? Is the effect observed at thermoneutrality?

6. The authors also fail to discuss the potential deleterious effects of FLCN loss in the liver. Specifically, supplementary Fig.S4 panel D shows higher odds of disease outcome for liver cirrhosis in predicted loss of function FLCN mutations in humans. In addition, a recent study (Custode et al PMID: 40189703) reported liver cell damage occurring following liver specific deletion of FLCN (using Alb-cre). In that study, male mice showed signs of liver damage as early as 4 weeks of age. In the current study, the effect of FLCN deletion on ALT levels in male mice 30 weeks after AAV8 treatment fed HFHFD were measured. However, ALT levels in mice fed a chow diet, as well as the effect of FNIP1/FNIP2 deletion should also be measured.

7. Were female mice used in any of the studies? If not, the reason why they were not included should be discussed.

Referee #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #3

(Remarks to the Author)
The authors conducted the largest-to-date rare variant burden analysis of the TG/HDL ratio and identified 60 genes. The analytical framework is robust and substantially increases the number of genes implicated in the TG/HDL ratio, a proxy for human energy metabolism. In particular, the identification of FNIP1, a previously poorly characterized gene in lipid metabolism, is highly novel and promising for future drug development for metabolic health. The authors further validate their findings in a rodent model and observe a robust causal effect of gene knockdown on the phenotype. The manuscript is concise and very well written. I have several comments that I believe will further enhance the readability and robustness of the paper.

Major Comments

pLoF FNIP1 in Humans
The LOEUF for FNIP1 is reported as 0.39 in gnomAD project, consistent with borderline constraint. The pattern differs substantially from that observed for PCSK9 (LOEUF = 1.25), which is the common example of the existing of human knockout as a proxy of knockdown in the humans. Although the authors report an extensive PheWAS of FNIP1 protein-truncating variants, the stance of the authors is unclear. Do the authors claims FNIP1 knockout is safe for humans? or need cautions with any undescribed complications.

Related to this, the statement in line 187 - that no individuals carried pLoF variants in both FNIP1 and FNIP2 - is not supported by a statistical analysis. Please clarify whether this absence reflects an expected frequency under independence or indicates an exceptionally rare event consistent with digenic impact within this pathway. An estimate derived from cumulative allele frequencies would allow assessment of whether the observed co-occurrence (or lack thereof) deviates from chance expectations.

Experimental Validation in Human Metabolism
The authors conducted extensive metabolic analysis using mice model. However, as the author described, the lipid metabolism is far different from humans and mice. To bridge findings, in-vitro experiments could be considered in human cell types. Especially, it is important that only FNIP1 knock out is sufficient to induce favorable metabolic pattern in humans not by dual knock down of FNIP1 and FNIP2 observed in the mice.

Natural History of FNIP1/FNIP2 Knockout in Mice
The protective effect under high-fat/high-fructose diet in the intervention group (FNIP1 and FNIP2 knockdown) is highly remarkable, however no data is shown in the regular diet. This also address the safety concerns raised by human genetic evidence above.

TG/HDL Ratio in the Diverse Population
The multi-ancestry aspect of this project is not sufficiently addressed in the manuscript. As shown in Table 1, the means of HDL-C, TG, and TG/HDL differ significantly across cohorts. It is well known, often called the “triglyceride paradox” that individuals of African ancestry tend to have relatively low TG and high HDL-C (clear if we focus on BioMe, PMBB, DBS), while worse clinical outcomes. Leveraging this research network to examine this phenomenon using the TG/HDL ratio is both intriguing and important. Population-stratified epidemiologic and genetic analyses would further increase the value of this study, clinically and scientifically.

Ratio as an Outcome
One fundamental question is whether using a ratio as the outcome is free from bias. Several previous works note that ratios

computed from two continuous variables can introduce collider bias. That said, the TG/HDL ratio is widely used and well established in this field. Within this research network, demonstrating that non-ratio measures (e.g., HDL-C residualized on TG, TG conditioned by HDL-C) yield consistent results would substantially increase the paper's impact and robustness.

Functional Clusters
Extended Data Figure 3 provides an excellent visualization of effect direction of each component as well as overall TG/HDL ratio. For example, ABCA1 lowers HDL-C more than it lowers TG, resulting in an increased TG/HDL ratio. In contrast, A1CF increases TG but no effect on HDL-C, leading to a increased TG/HDL ratio as well. Do these distinct mechanism imply different clinical associations? Moreover, could these differences be mapped to distinct biological pathways?

eQTL Integration Analysis
The idea of integrating common genetic signals to enhance rare variant association is excellent. I think the authors could further deepen the discussion by considering the effect of direction. From beta-eQTL and beta-TG/HDL, we can infer the impact of decreased gene expression on outcomes (TG/HDL) by flipping the effect. This could be aligned with the pLoF/missense burden beta for TG/HDL. For example, ANGPTL3 pLoF/missense variants decrease TG/HDL. Additionally, the sentinel variant 1:62604866:AGTTAATGTG:A in this region decreases expression of ANGPTL3 and lowers TG/HDL, which is a fascinating example of convergence in gene function and expression. I briefly confirmed that 9 out of 9 genes with EWS and significant colocalization align beautifully with this observation. In this right, the line 238 could be started with "Consistently,". The next question is whether a more liberal threshold can maintain this consistency. If alignment persists even under lenient thresholds, the RVAS likely captures true associations with this level of association.

Minor Comments

Replication
The study design is robust and sufficiently controlled by exome wide significance. However, still replication analysis can add additional value. With whole genome sequence data now available in All of Us Research program, there is no justification for withholding these resources. Although a small number of samples may overlap with existing cohorts, including this cohort in the analysis will strengthen the paper's robustness.

Conditioning
Conditioning on common genetic variants is an excellent way to reduce linkage between common and rare variants. I agree to report the conditioned analysis, but I am curious whether these adjustments change the statistics for rare variants, e.g., some genes might show decreased association, and vice versa. Also, the authors use REGENIE as an association framework, which regresses out global effect from common-variant in step 1. Doesn't this additional conditioning overlap with that process and risk overcorrection?

Effect of LD in Rare Variant Analysis
Another question is that ld sharing in rare variants. In Fig. 3 we see clusters on chromosome 19 1) APOC3, ZNF285 (and APOE), 2) LDLR and KEAP1. ZNF285 and KEAP1 are both not well characterized genes as I quickly searched. Is this association potentially driven by shared haplotypes between these genes? The rare haplotype is sometimes longer than common haplotypes. Previously this is not testable as small number of samples, but in this unprecedented scale of study, we may answer this question.

Novel Gene
In line 109-111, the authors described that they identified 15 genes found in the previous study. If this discussion is based on Supple Table S13 (Hindy et al, AJHG 2022, PMID: 34932938), they reported 17 genes including 2 genes (APOE and TMEN136) not identified in this study while larger sample size. Is there any reasonable account for this non-identification? Also, how many of 60 genes identified were in the known (or identified in this study) by common variant association analysis.

Data Representation
The presentation of dates in Fig. 2 might be somewhat unfamiliar for readers. Considering the putative causal relationships between the TG/HDL ratio and clinical outcomes (CAD, T2D), harmonizing the x-axis across panels to the TG–HDL ratio—as demonstrated in Fig. 2a, would enhance interpretability. Moreover, pairwise correlations are not well suited to reflecting differential effect sizes across outcomes. Estimating and reporting coefficients per 1 SD increase in the TG–HDL ratio for each outcome, accompanied by error bars (e.g., 95% confidence intervals), would more effectively quantify heterogeneous effects and their uncertainty.

Phenome Wide Analysis
Because the authors combine metabolic traits to demonstrate benefit (line 150), presenting the unfavorable outcomes in the same combined manner (line 161, Supplementary Fig. S3) would allow fairer comparisons.

Phenotype Definition
In lines 26–38 (Methods), the authors state that median values were used for measured traits. For the TG/HDL ratio, were TG and HDL measured at the same time point, or were values from different time points used? If different, please describe the matching strategy.

Statistical Finemapping
In lines 248-256 (Methods), he authors conducted fine-mapping with SuSiE on the meta-analysis summary statistics and

stated that LD was computed using the individuals included in the association analysis. How was LD computed across participants from different biobanks? Was a combined genetic dataset available to compute an LD matrix across all participants? Also, 30.3% of participants were non-European. Did this ancestry mixture affect LD structure and the fine-mapping results? LD mismatch can introduce bias in fine-mapping. In addition, the number of most credible sets (the L parameter in SuSiE) is not reported. How many loci reached saturation (number of causal signals == L)? This is a quick sanity check for LD mismatch.

Supplementary Table S3
Please confirm that the reported Beta and SE are unconditioned (marginal) effect sizes and standard errors from the primary association model. I note that many variants are not significant (|Z| < 1.96). It would be helpful to include SuSiE fine-mapping metrics to aid interpretation - specifically, the posterior inclusion probability, locus-membership, credible-set membership, and the maximum LD (R2) to the lead variant. These additions would clarify overall procedure in this process.

Methods in Common-Variant Analysis
The description of common-variant analyses is insufficient. Please report basic summary of results to characterize the genetic architecture of this phenotype. For example (1) the number of non-overlapping associated loci described in lines 248-256 (Methods), (2) the genomic inflation factor, and, if inflation is observed, the LD Score regression intercept to assess confounding, (3) standard visualizations (Manhattan and QQ plots). These additions would help demonstrate that the study design, particularly phenotype definition and population stratification, has been appropriately controlled.

Multiple Testing
The manuscript adopts a exome-wide significance threshold of 1.04×10-7. While the authors constructed 24 variant masks, these appear to have been combined via the Cauchy combination test to yield a single P value per transcript. If so, the effective multiplicity should correspond to the number of transcripts assessed, rather than to the number of masks times number of transcripts. In addition, the manuscript does not clearly state the total number of transcripts (genes) tested.

Extended Data Figs. 6-10
The coloring of bar plot is a bit difficult to distinguish. Please consider different color scheme.

Line 108-109
Supplementary Note S1 is somewhat disjointed from context.

Referee #4

(Remarks to the Author)
OVERALL ASSESSMENT

The authors present a large multi-ancestry exome-wide association study of TG/HDL ratio. Rare variant burden tests identify 60 associated genes, whiel common variant association analyses yield 1,617 loci. The study then focuses on biological characterization of selected genes in the FNIP/FLCN gene family and HPN in hepatocytes, finding experimental evidence of their functional roles. The manuscript is novel, well-structured, and has a strong narrative from genetic discovery through fine-mapping, functional follow-up, biological characterization, and experimental validation. However, several methodological and interpretational issues require clarification and sensitivity before the paper can be recommended for publication.

MAJOR COMMENTS

1. The meta-analyses include individuals from 5 distinct genetic ancestries, yet the manuscript does not address how ancestry influences the associations. Results appear to be based on pooled analyses across all ancestries. Please describe whether signals are driven primarily by European ancestry (the largest subgroups) or whether they generalize across other ancestries. Also clarify how ancestry was accounted for in cohort-specific analyses.

2. The TG/HDL ratio is mathematically dependent on two correlated traits, which may introduce collider bias and obscure signals specific to TG or HDL. To increase confidence in the findings, please examine the behavior of the identified variatns and genes with respective to TG and HDL individually, clarify whether associations are driven by TG, HDL, or both, and discuss the potential role of covariates that may differentially influence TG and HDL.

3. Please clarify whether TG and HDL were measured in fasting or non-fasting state across cohorts. Given that TG levels are more variable than HDL, it is important to discuss whether TG/HDL ratio can be considered a stable trait across these states.

4. TG/HDL ratio is strongly related to fat distribution, which often differs by sex. Stratified analyses by sex would be relevant to understand whether associations are consistent across men and women.

5. To complement the biological interpretation, consider linking exome-wide signals to cis-pQTLs to clarify whether the genetic burden may act through protein expression changes relevant to lipid metabolism.

6. The burden-test results suggest enrichment in liver, whereas previous UK Biobank TG/HDL GWAS based on common variants reported predominant enrichment in adipose tissue. To clarify these differences, consider performing tissue-enrichment analyses on the 1,617 independent common variants as well.

MINOR COMMENTS

1. Consider reporting cell type-specific expression levels for FNIP/FLCN family and HPN in human and mouse liver.

2. Lines 105-107: Clarify the basis for the exome-wide statistical significance threshold applied.

3. Lines 189-190: The claim that “genes associated with favorable metabolic state are enriched in liver” seems unsubstantiated. Please provide supporting evidence or revise.

4. Methods: Clarify whether fine-mapping was multi-ancestry framework or stratified by ancestry, and specify the LD reference panels used.

5. Methods: Specify the co-localization method applied and the LD reference panels used.

6. Extended Figure 3: Clarify the burden test methodology and the variants included.

7. Supplementary Table 3: Add P values, sample sizes, and posterior inclusion probabilities.

8. Supplementary Table 6: Add effect sizes and P values.

Referee #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
In their revised manuscript the authors have made substantial changes and include new experimental data which significantly improves the paper and greatly strengthens the conclusions of the study. The revised manuscript adequately addresses all of the major concerns raised in our original review and so we are happy to recommend publication of the paper. Below we provide some brief comments to the authors revisions responding to our concerns raised for the previous submission:

R1C1:
Evidence supporting the predicted loss-of-function mutations of FNIP1 is satisfactorily provided through both proteomic analysis and relevant support from previous studies. Inclusion of the FNIP1 variants in Fig. S20 is very helpful.

R1C2:
The rationale for liver-specific inhibition of FNIP1 is sound and heterozygous FNIP1 mutations are shown not to be associated with adverse cardiometabolic phenotypes.

R1C3:
Specific Flcn/Fnip knockdown in hepatocytes leading to the relevant metabolic phenotype is thoroughly confirmed. Using GalNAc-siRNA for hepatocyte-specific knockdown is particularly relevant due to its translational potential.

R1C4:
Measurement of FLCN and FNIP2 using a MS-based approach, together with immunoblot analysis of FNIP1 in human hepatocytes addresses our previous concerns.

R1C5:
The limitations of a mouse preclinical model are noted. Exploring circulating TG levels in the mouse models was an important phenotype to explore and this is now addressed despite the variability of the model.

R1C6:
Changes in lipid catabolism, oxidative phosphorylation and energy expenditure are now included providing a mechanistic explanation for the observed phenotype. Additionally, the reduction of the hepatokine Inhbe as a potential driver for the adipose phenotype is a promising new mechanism for FLCN/FNIP liver biology.

R1C7:
The new data showing that the protective effects on the metabolic phenotype are maintained when mice are housed at thermoneutrality strengthens the translational impact of the findings.

R1C8:
The authors address the Custode et al. paper and the possible deleterious effects in the text and importantly through mouse experiments. Monitoring of ALT levels as requested and performing a chronic (30 weeks) DIO model provides additional evidence against adverse effects of inhibiting FLCN/FNIP signalling in the liver.

R1C9:
The authors provide a satisfactory explanation for not including female mice in their study.
Confirming generalizability of the human genetic findings for both men and women strengthens the information in the manuscript despite the lack of data for female mice.

Referee #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #3

(Remarks to the Author)
I appreciate the authors' clear and thoughtful responses to my questions and concerns. I was particularly impressed by the careful balance they struck between the human genetic evidence and the therapeutic potential of FNIP1 inhibition. I sincerely hope that this discovery will one day benefit patients with metabolic disorders.

Referee #4

(Remarks to the Author)
The authors have thoroughly addressed my comments, and I have no further remarks.

Referee #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Dear Dr. and Nature Editors,

We are grateful to you and the Reviewers for the thorough evaluation of our work and for the constructive comments which have helped refine and improve our manuscript. We are submitting a revised manuscript which addresses the points raised during review.

In the revised manuscript, we have performed a vast set of new genetic analyses and experiments, which have substantially strengthened our results and conclusions, including:

New Genomic Analyses

- Replication of TG-HDL associations in 114,942 All of Us participants.
- Ancestry-stratified epidemiological and genomic analyses.
- Sex-stratified analyses revealing a novel sex-interaction for *PDE3B.*
- Proteomics associations for TG-HDL genes in ~60,000 participants.
- Pathway analyses showing enrichment for energy metabolism pathways.
- Multiple robustness and sensitivity analyses— all confirming the reported associations.

New Experimental Data

- New *in vitro FNIP1* knockdown experiments in primary human hepatocytes demonstrating that single-gene inhibition of *FNIP1* is sufficient to activate energy metabolism gene-expression programs – this was a critical point raised by Editors and Reviewers and the new experiments directly address it.
- New mouse experiments showing that GalNAc-siRNA-mediated *Flcn* silencing recapitulates the protective metabolic phenotype of AAV8-gRNAs, confirming the phenotype is due to hepatocyte silencing.
- New mouse AAV8-gRNA experiments of *Flcn* and *Fnip1/Fnip2* silencing at thermoneutrality, confirming protective effects are independent of thermogenic demand.
- New mouse AAV8-gRNA experiments of *Flcn* and *Fnip1/Fnip2* silencing in a chow diet model showing favorable metabolic profile.
- New mouse data on AAV8-gRNA mediated *Flcn* silencing confirming beneficial metabolic effects over 30 weeks (long-duration follow-up).
- Independent set of new experimental replicates for the AAV8-gRNA mouse experiments, providing an even more robust characterization of the metabolic effects of liver silencing of *Flcn* and *Fnip1/Fnip2.*
- Development of mass-spectrometry protein assays confirming FNIP2 and FLCN protein knockdown in mouse experiments. Immunoassay for FNIP1 protein demonstrating protein depletion in human hepatocyte model.
- Discovery of *Inhbe*-mediated liver-to-adipose crosstalk as a mediating mechanism for the protective adipose phenotype, providing greater mechanistic understanding for our associations.

We attach a point-by-point reply to each of the comments raised during review.

We look forward to hearing back about your evaluation of this revised manuscript and thank you again for your consideration of our work.

Very best wishes,

Luca A. Lotta, MD, PhD (on behalf of co-authors)
Vice President and Chief Translational Genetics Officer, Regeneron Genetics Center
Regeneron Pharmaceuticals Inc. Tarrytown, New York (USA)

Reviewer #1 – co-reviewed with Reviewer #2

Reviewer 1 Opening Statement (R1OS): "In this paper, Hindy and colleagues use exome sequencing to identify rare variants that are associated with changes in the triglyceride (TG) to HDL ratio. The authors use this information to validate the use of the TG/HDL ratio as a biomarker for various metabolic phenotypes and cardiovascular disease. The genetic studies and associated clinical data are impressive and will provide an important resource for subsequent studies. However, our review focuses specifically on the data from the mouse models used, and in particular the studies aimed at investigating the role of interfering with the FLCN/FNIP pathway on metabolic health."

R1OS – Author's reply: We thank the Reviewer for their thorough evaluation of our work, for the supportive remarks regarding the genetic studies and clinical data, and for the valuable suggestions which have greatly helped improve our manuscript.

Below, we provide a point-by-point response to each comment.

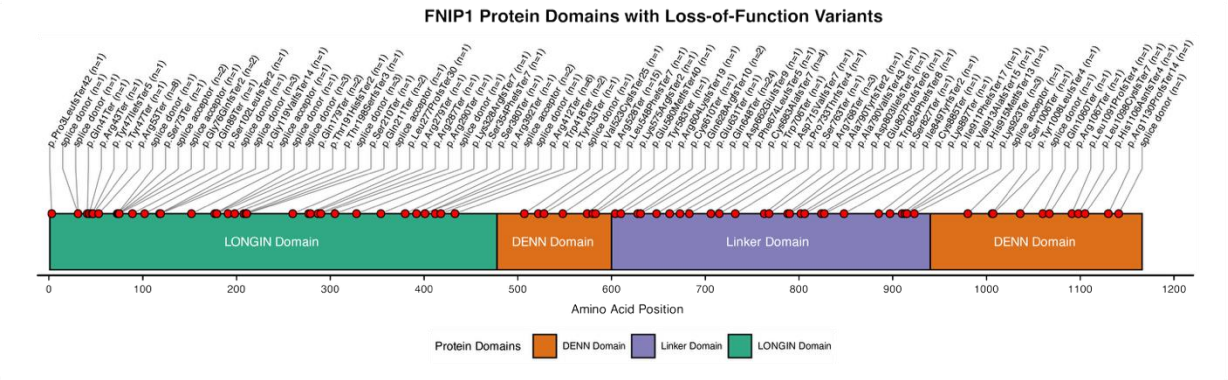
Reviewer 1 Comment 1 (R1C1): "A range of mutations in FNIP1 are identified and predicted to be LoF. Some of these mutations are located towards the C-terminus of the protein. Is there any direct evidence that all the mutations are LoF (particularly the mutations in the C-terminal region)?"

R1C1 – Author's reply: This is an excellent point and there are several lines of evidence supporting a loss-of-function mechanism for this association.

Our *FNIP1* association was driven by 86 distinct and ultra-rare protein-truncating mutations, including stop-gain or frameshift mutations occurring throughout the gene and predicted to truncate the protein in different domains (**Inset Fig. 1**). It is highly unlikely that such a large collection of protein-truncating mutations would result in neutral or gain-of-function effect, leaving loss-of-function as the most likely explanation for the association signal.

Indeed, a stop-gain mutation identified in our analysis (p.Arg290Ter) has been also reported in homozygous state in a patient with immunodeficiency-93 and hypertrophic cardiomyopathy (i.e. homozygous FNIP1 deficiency), who had no detectable FNIP1 protein on immunoblotting (*Blood. 2021;137:493-499*), consistent with a complete loss-of-function effect.

Inset Fig. 1. Distribution of predicted loss-of-function *FNIP1* mutations across protein domains.

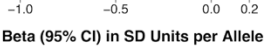


In the revised manuscript, we performed a new analysis showing that the association of *FNIP1* mutations with lower TG-HDL ratio is robust and consistent when considering variants predicted to result in nonsense mediated decay (NMD; p-value for heterogeneity in effect estimates between NMD vs non-NMD variants, $p_{heterogeneity}$=0.81; **Inset Fig. 2**). NMD variants accounted for 81% (n=126) of the *FNIP1* pLOF carriers in our data. Also, N-terminal protein-truncating mutations showed comparable effect size as C-terminal protein-truncating mutations ($p_{heterogeneity}$=0.32; **Inset Fig. 2**).

Inset Fig. 2. Association with TG-HDL ratio for *FNIP1* predicted loss-of-function mutations with different protein location and nonsense mediated decay annotation.

FNIP1 pLoF, AAF<0.01%

Gene Variant	Genotype Count RR / RA / AA	AAF	Beta (95% CI) per Allele in Clinical Units	P–Value	Heterogeneity P-Value
All Variants	1,031,961 / 155 / 0	0.008%	−1.66 (−2.17, −1.16) ratio	1.1e−10	
NMD classification					
NMD Variants	1,031,511 / 126 / 0	0.006%	−1.62 (−2.18, −1.06) ratio	1.5e−8	0.81
Non−NMD/splice site Variants	931,010 / 29 / 0	0.002%	−1.88 (−3.05, −0.71) ratio	0.002	
Location of pLOF mutation					
N−Terminus Variants	1,026,423 / 68 / 0	0.003%	−1.96 (−2.72, −1.20) ratio	4.4e−7	0.32
C−Terminus Variants	1,031,549 / 87 / 0	0.004%	−1.43 (−2.11, −0.76) ratio	3.1e−5	



Importantly, both N- and C-terminal domains of FNIP1 are required for its heterodimerization with folliculin (*Nat Commun. 2016:7:12037; Proc Natl Acad Sci U S A. 2006;103:15552-7*), and this interaction protects FNIP1 from proteasomal degradation (*PLoS Genet. 2020;16:e1009187*). Truncating mutations in either region would therefore be expected to disrupt this interaction and lead to FNIP1 protein loss.

This is consistent with our observation of comparable genetic effect sizes for N- and C-terminal mutations and with the absence of FNIP1 protein in patients with various homozygous *FNIP1* mutations in the C-terminus (*Blood. 2021;137:493-499; J Clin Immunol. 2023;43:1751-1753; Eur J Immunol. 2020;50:1078-1080*).

We also provide broader validation of our pLOF prediction methodology through proteomics analyses in our response to **R4MC5**.

Taken together, the distribution and nature of the truncating mutations, the direct functional evidence from patients with homozygous FNIP1 deficiency, the NMD analyses, and the functional evidence that C-terminal domain is needed to prevent FNIP1 degradation all converge to strongly support a loss-of-function mechanism underlying the association of *FNIP1* mutations with TG-HDL ratio.

R1C1 – Manuscript changes: The 86 pLOF mutations in *FNIP1* and the associations for different mutation types (e.g. N- vs C-terminal) are described in the **Results** (Lines 172-178) and in **Supplementary Fig. S20** and **Table S20**. The proteomics validation of pLOF predictions is in **Supplementary Fig. S18** (see also **R4MC5**).

Reviewer 1 Comment 2 (R1C2): "The approach used to investigate the functional significance of candidate genes identified from the exome sequencing requires some justification. Presumably, the mutations in humans are present in all cell types expressing the gene, and the mutations are heterozygous? In order to mimic the human situation a direct approach would be to make heterozygous (whole body) knockouts of candidate genes. Of course, this is a time-consuming approach, and presumably because of this the authors chose to use AAV8-targetted expression of gRNAs to delete candidate genes. This is very likely quite different from the situation in humans carrying mutations, so immediately raises concerns as to how to interpret the data. […] The choice of which candidate genes to explore is not made obvious. As the authors mention, previous studies have reported that liver-specific deletion of FLCN in mice protects against hepatic steatosis and fibrosis, but in humans LoF mutation in FLCN lead to autosomal dominant BHD syndrome, leading to increased risk of cancer, particularly renal cancer. LoF mutations in FNIP1 lead to autosomal recessive abnormalities, including immunodeficiency and cardiomyopathy (overlapping with WPW syndrome). The decision to focus on the inhibition of the FLCN/FNIP pathway as a potential therapeutic area is a little surprising (but clearly this is a subjective decision)."

R1C2 – Author's reply: We agree with the Reviewer and in the revised manuscript we clearly outline the rationale for our focus on *FNIP1* and for choosing liver silencing as experimental model.

Our focus on *FNIP1* stems from the novel association of pLOF mutations in this gene with favorable metabolism and protection against cardiometabolic disease. Protective associations for naturally-occurring human pLOF mutations have led to the development of pharmacologic inhibitors for a broad range of therapeutic targets, including FDA-approved drugs targeting PCSK9 (human genetics: *NEJM 2006;354:1264-72*; clinical trials: *NEJM 2018;379:2097-2107, NEJM 2017;376:1713-1722*), ANGPTL3 (human genetics: *NEJM. 2010;363:2220-7, NEJM. 2017;377:211-221*; clinical trials: *NEJM 2020;383:711-720*) and APOC3 (human genetics:

Science 2008;322:1702-5, NEJM 2014;371:32-41, NEJM 2014;371:22-31; clinical trials: *NEJM 2025;392:127-137*).

Our focus on liver specific inhibition relates to the therapeutic tractability of this approach. Specifically, *FNIP1* and *FLCN* are expressed in hepatocytes and previous *in vivo* studies in mice had suggested a role for the folliculin pathway in liver metabolism (e.g. *Science. 2022;376:eabf8271*). Importantly, there are 7 FDA-approved N-acetylgalactosamine (GalNAc)-conjugated siRNA therapeutics that effectively and durably silence a variety of genes in hepatocytes in humans (e.g. *NEJM. 2020;382:2289–2301; NEJM 2020;382:1507–1519; NEJM. 2021;384:1216–1226; NEJM 2025;392:127–137*). This precedent makes hepatocyte modulation of this pathway a tractable therapeutic approach.

As it relates to the Mendelian syndromes, in the revised manuscript, we specifically investigated associations of heterozygous *FNIP1* or *FLCN* mutations with the corresponding Mendelian phenotypes (see **Inset Fig. 3**, which is also discussed in **R3MiC6**). While *FLCN* mutations were clearly associated with the Mendelian features of Birt-Hogg-Dubé syndrome, we found that *FNIP1* mutations are associated with protection from cardiometabolic disease without a statistically-significant increase in the composite phenotype for immunodeficiency 93 and hypertrophic cardiomyopathy (**Inset Fig. 3**).

Inset Fig. 3. Association of *FNIP1* and *FLCN* mutations with cardiometabolic disease risk and with a composite phenotype for their respective Mendelian syndromes.

Outcome Gene Variant	Count (Cases) RR / RA / AA	Count (Controls) RR / RA / AA	AAF	Odds Ratio (95% CI) per Allele	P–Value
FNIP1					
Cardiometabolic Disease					
FNIP1 pLoF, AAF<0.01%	227,613 / 23 / 0	265,063 / 51 / 0	0.008%	0.39 (0.22, 0.69)	0.001
FNIP1 Mendelian Composite					
FNIP1 pLoF, AAF<0.01%	282,152 / 53 / 0	270,267 / 34 / 0	0.008%	1.39 (0.87, 2.21)	0.163
FLCN					
Cardiometabolic Disease					
FLCN pLoF, AAF<1%	220,594 / 69 / 0	255,715 / 103 / 0	0.018%	0.78 (0.55, 1.10)	0.152
FLCN plus deleterious missense (5/5 or ESM1vp), AAF<1%	226,822 / 813 / 1	264,078 / 1,036 / 0	0.19%	0.95 (0.85, 1.05)	0.303
FLCN Mendelian Composite					
FLCN pLoF, AAF<1%	181,136 / 123 / 0	398,080 / 101 / 0	0.019%	2.98 (2.25, 3.97)	5.3e−14
FLCN plus deleterious missense (5/5 or ESM1vp), AAF<1%	180,488 / 770 / 1	396,722 / 1,459 / 0	0.19%	1.20 (1.09, 1.32)	2.2e−4

These findings suggest that adverse phenotypes of FNIP1 deficiency are recessive and driven by loss-of-function in cardiomyocytes and immune cells. A liver-directed therapeutic approach using GalNAc-conjugated siRNA would be expected to mitigate safety concerns associated with systemic inhibition as these agents specifically silence their targets in hepatocytes and not in other cell types, and typically achieve partial rather than complete target knockdown. In the revised manuscript, we also investigate hepatic safety in depth as noted in **R1C8** below.

In summary, our focus on FNIP1 and liver knockdown is motivated by a series of strong precedents for protective mutations and for liver siRNA development. The goal for the validation experiments was not to perfectly phenocopy human genetics with a mouse model, but rather to

assess the hypothesis of whether hepatocyte-specific inhibition via siRNA could be beneficial to metabolism. We now clearly state this in the revised manuscript.

R1C2 – Manuscript changes: We outline the rationale for our focus on *FNIP1* and liver-specific inhibition as validation model in the **Results** (Lines 169-171; 211-224). We describe the expression of *FNIP1* and *FLCN* in human and murine hepatocytes in **Supplementary Fig. S26**. The Mendelian phenotype analysis for *FNIP1* and *FLCN* mutations is in **Supplementary Note S3, Figs. S21–S22 and S24**.

Reviewer 1 Comment 3 (R1C3): "The use of AAV8-targetted deletion raises its own issues if the aim is to delete genes specifically in hepatocytes. AAV8 targets other cell types in addition to hepatocytes, and so it is not possible to attribute directly any phenotypic effects to the liver (hepatocytes). In this regard, conditional deletion of candidate genes (e.g. using Alb-cre of floxed alleles) would be a better approach. As it stands, it is difficult to make firm conclusions from the mouse models (see further comments below). […] The authors use the AAV8 approach to down-regulate FLCN, FNIP1, FNIP2 or FNIP1/FNIP2 gene expression in Cas9-Ready mice. The authors point out that in addition to hepatocytes, the liver contains other cell types but make no further attempt to explain how this may contribute to their results. At the very least, the authors should determine gene expression levels in hepatocytes isolated from the mouse livers, but a deeper understanding of expression levels in different cell types (e.g. using scRNA approaches) would be very informative here."

R1C3 – Author's reply and manuscript changes: We agree with the importance of demonstrating a hepatocyte specific effect, which we have addressed with a series of new experiments in this revision.

First, to validate that our AAV8-gRNAs did not target extra-hepatic tissues, we have measured *Flcn*/*Fnip* expression in several tissues relevant to energy metabolism. These new data, now shown in **Supplementary Fig. S28**, demonstrate that *Flcn/Fnip* gene expression was unchanged in gastrocnemius muscle, heart, kidney, adipose tissue or spleen upon AAV8-gRNA administration.

Next, to assess expression across liver cell types, we generated single cell RNA-sequencing plots for human and mouse liver (**Supplementary Fig. S26**). These data confirmed that *Flcn/Fnip* genes are expressed in both hepatocytes and non-parenchymal cells, so we sought to demonstrate that the metabolic phenotype is specifically attributable to hepatocytes.

To do so, we used a GalNAc-conjugated siRNA to silence *Flcn* expression specifically in hepatocytes (**Inset Fig. 4**, and **Extended Data Fig. 9**), but not non-parenchymal cells.

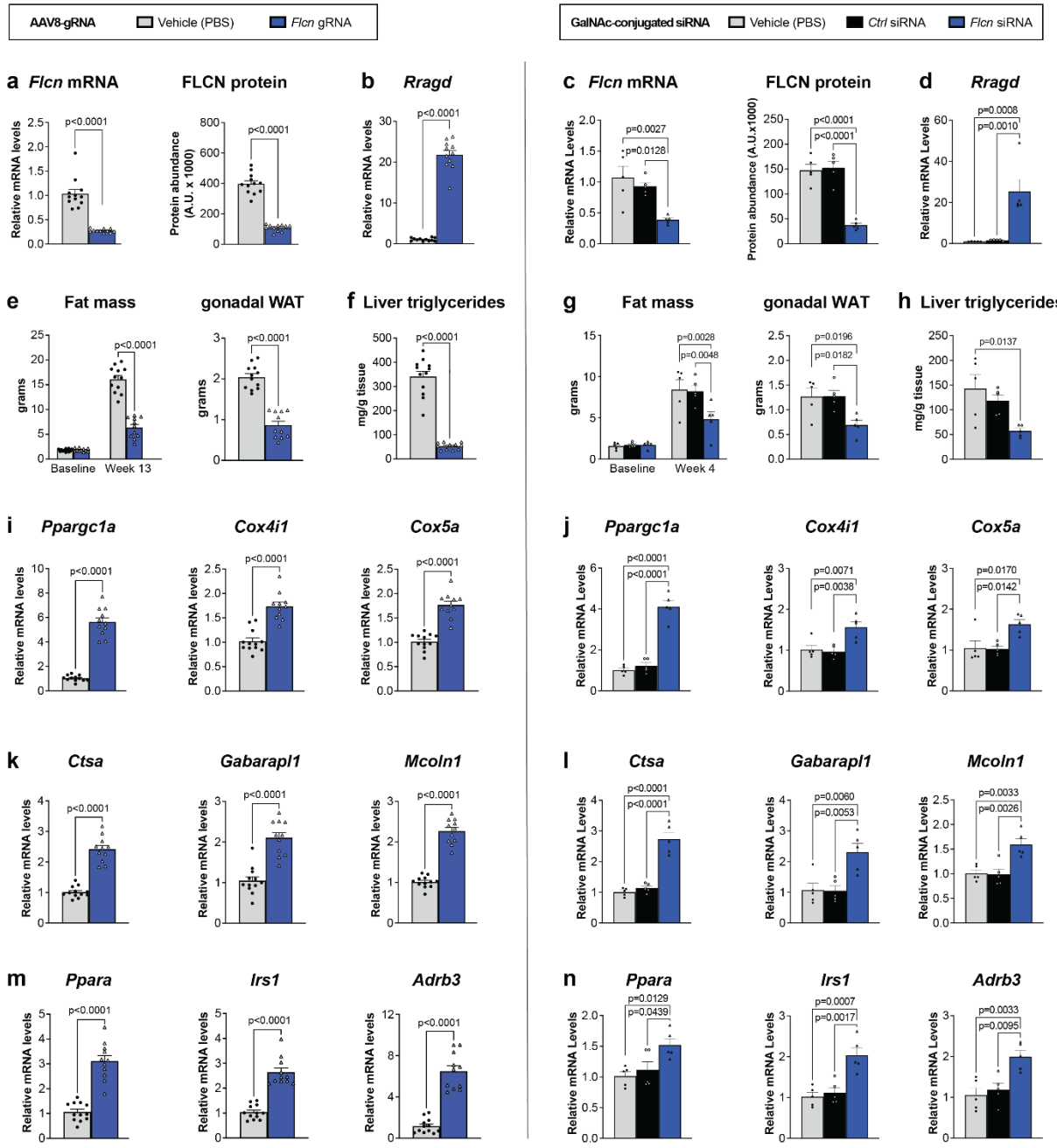
Hepatocyte-specific *Flcn* knockdown with GalNAc-siRNA led to an analogous phenotype as the one observed in the AAV8-gRNA studies, both as it relates to the favorable metabolic

phenotype and the gene expression signatures. **Inset Fig. 4** provides a head-to-head comparison of these experiments showing remarkable similarity.

Consistent with this mouse data, in the revised manuscript we also show that siRNA knockdown of *FNIP1* in primary human hepatocytes induced a lysosomal and lipid catabolism gene signature as expected for loss-of-function of this pathway (see response to **R3MC3**, as well as **Extended Data Fig. 5** and **Supplementary Fig. S27** in the revised manuscript).

The extra-hepatic expression data, hepatocyte-specific GalNAc-siRNA experiments, and human hepatocyte siRNA knockdown studies collectively demonstrate that the metabolic phenotype observed in the experiments can be attributed to hepatocyte knockdown.

Inset Fig. 4. *Flcn* knockdown using GalNAc-conjugated siRNAs to target mouse hepatocytes yields an analogous phenotype as AAV8-gRNA mediated *Flcn* knockdown.



Reviewer 1 Comment 4 (R1C4): "Confusingly, in Ex Data Fig. 6, FNIP2 expression in the liver increases in FNIP1/FNIP2 gRNA mice. […] It is not obvious why FNIP2 expression increases in the FNIP1/FNIP2 gRNA group. […] Related to the above point, it would be informative to have some data looking at the protein levels of FLCN/FNIP1/FNIP2 in liver (and isolated hepatocytes) from the different mouse lines. Depending on the outcome of these additional studies, the conclusions drawn from these mouse models may need to be reconsidered in their totality."

R1C4 – Author's reply: We thank the Reviewer for the suggestion to evaluate protein levels, which helped clarify the apparent discrepancy for *Fnip2*. In the revision, we developed a mass-spectrometry assay for FNIP2 protein, which demonstrated >85% protein knockdown upon *Fnip2* or *Fnip1/Fnip2* gRNA administration (**Inset Fig. 5**).

The overexpression of *Fnip2* mRNA in the *Fnip1/Fnip2* gRNA group likely reflects the well-described feedback loop resulting in upregulation of *Fnip1*, *Fnip2* and *Flcn* upon FNIP/FLCN pathway inhibition (**Extended Data Fig. 4a**, see also *Science* 2023;380:eabj5559; *Elife 2021;10:e61630*). As demonstrated by the protein assay, the *Fnip2* mRNA is not translated into a functional protein in those experiments.

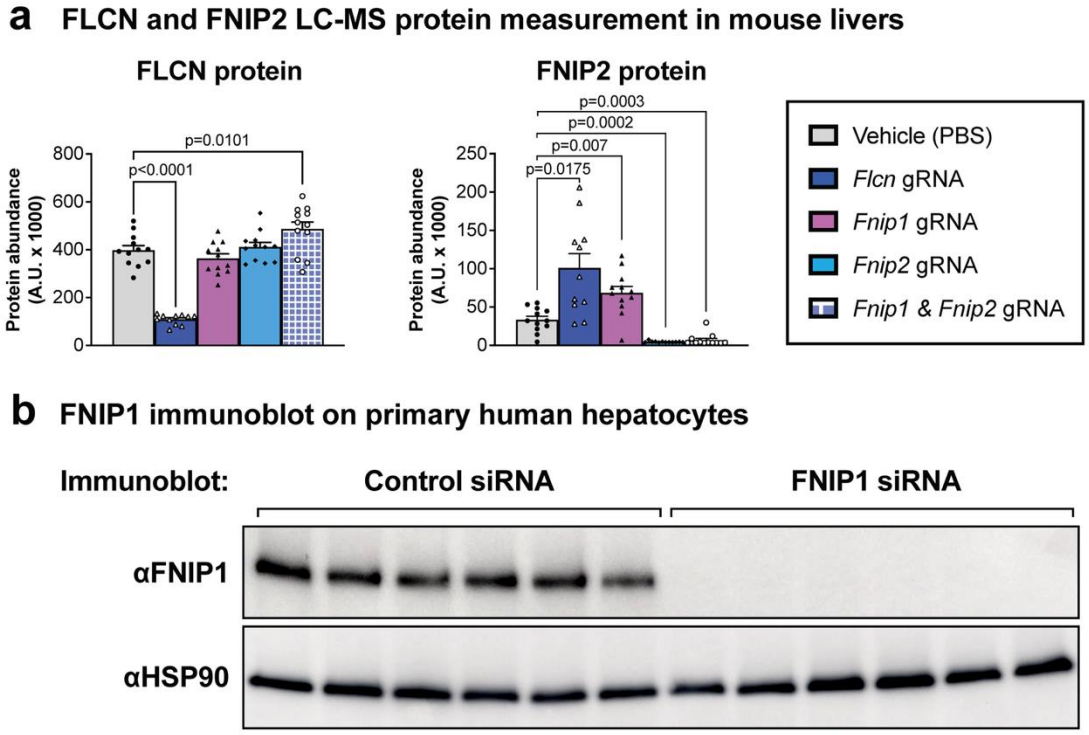
These data confirm that FNIP2 protein is effectively depleted in both the *Fnip2* and *Fnip1/Fnip2* gRNA groups, supporting the conclusions drawn from these models.

We also developed a FLCN protein assay (**Inset Fig. 5**), showing robust knockdown in both the AAV8-gRNA and siRNA experiments, consistent with the mRNA data.

Despite extensive efforts, we have not been able to develop a protein assay for mouse FNIP1. However, we were able to source and validate human FNIP1 antibodies and develop an immunoblot assay. Using this assay in our new experiment with human primary hepatocytes, we clearly demonstrate that the FNIP1 siRNA results in near complete reduction of human FNIP1 protein, consistent with the mRNA results (**Inset Fig. 5;** see also response to **R3MC3**, as well as **Extended Data Fig. 5** and **Supplementary Fig. S27** in the revised manuscript).

Thus, the new protein measurement data generated for the revised manuscript supports the loss-of-function interpretation of the experiments.

Inset Fig. 5. Protein abundance of FLCN and FNIP2 in mouse liver upon AAV8-gRNA administration, and protein abundance of FNIP1 in primary human hepatocytes upon siRNA delivery.



R1C4 – Manuscript changes: The FNIP/FLCN negative feedback loop and the *Fnip2* and *Flcn* mouse protein data are presented in **Extended Data Fig. 4.** We present FNIP1 human protein data in **Extended Data Fig. 5** and **Supplementary Fig. S27.**

Reviewer 1 Comment 5 (R1C5): "Circling back to the clinical finding in humans, does down-regulation of FNIP1 or FNIP1/FNIP2 lead to a decrease in circulating TG levels in mice?"

R1C5 – Author's reply: In the revised manuscript, we have measured circulating triglycerides (TG) across models and found that down-regulation of *Flcn* or *Fnip1/Fnip2* was generally associated with lower circulating TG levels in mice, consistent with the human genetic findings.

Across experiments, TG levels were highly variable, including a well described reduction in circulating TG in the setting of prolonged exposure to high fat diets in mice (*J Lipid Res* 2007;48:278-287; *J Lipid Res* 2024;66:100706).

To overcome this variability, we pooled the overall data across experiments using random-effects models accounting for between-experiment heterogeneity and the nested data structure of

the experimental measurements. This analysis showed a statistically-significant reduction in circulating TG for silencing of *Flcn* (β for *Flcn* silencing [n=40 mice] vs vehicle control [n=40 mice], −22 mg/dL; p=0.002). Silencing of *Fnip1/Fnip2* showed a directionally-consistent but non-statistically-significant difference (β for *Fnip1/Fnip2* silencing [n=27] vs vehicle control [n=40], −11 mg/dL; p=0.29), likely reflecting reduced statistical power due to the smaller sample size.

So, overall and in spite of the inherent limitations/variability of the preclinical models, the experimental results for TGs were consistent with the human genetics results.

R1C5 – Manuscript changes: Triglyceride levels for each experiment are now shown **in Extended Data Fig. 9-10** and **Supplementary Fig. S29-S30**.

Reviewer 1 Comment 6 (R1C6): "Do the authors have any mechanistic explanation for the protection against diet-induced obesity following deletion of FLCN or FNIP1/FNIP2 in the liver (Fig. 4c)?"

R1C6 – Author's reply: Our experimental results, greatly expanded in the revised manuscript, show that liver inhibition of the FLCN/FNIP pathway strongly induces lipid breakdown, oxidative phosphorylation and energy expenditure programs in both liver (directly) and adipose (indirectly), which provides a mechanistic explanation for the favorable metabolic phenotype.

Notably, the expression changes in adipose tissue occurred *without* adipose knockdown of *Flcn* or *Fnip1/Fnip2* and, in the revised manuscript, we provide new evidence for a role of the *Inhbe* pathway in these adipose transcriptomics changes.

Inhbe (activin E) is a hepatokine that is upregulated in liver steatosis (*Nat Commun. 2022;13:4844, doi: 10.1038/s41467-022-32398-7.*) and inhibits adipose tissue lipolysis via the activation of its adipose-expressed receptor Alk7 (*PNAS 2023; 120(32):e2309967120*). *Inhbe* loss-of-function causes adipose-expression changes similar to those observed in our liver *Flcn* or *Fnip1*/*Fnip2* knockdown experiments (*PNAS 2023; 120(32):e2309967120*). Consistent with this, we found that hepatic *Inhbe* mRNA levels were reduced by ~52-55% upon *Flcn* or *Fnip1*/*Fnip2* inhibition. A similar reduction of hepatic *Inhbe* expression was previously reported to be sufficient to elicit body weight reduction, fat loss and increased lipid oxidation in obese mice (*PLoS One 2018, 13(3):e0194798*).

Therefore, reduced liver *Inhbe* expression likely contributes to the protective adipose phenotypes observed upon liver *Flcn* and *Fnip1*/*Fnip2* inhibition.

R1C6 – manuscript changes: Hepatic and adipose gene-expression data across experimental models are presented in **Extended Data Fig. 6 and Extended Data Fig. 8**. The new findings on

Inhbe are presented in the **Results** section (Lines 255-261). Our explanation for the mechanism of the protective effect of *FNIP1* mutations is presented in the **Discussion** (Lines 327-331) section.

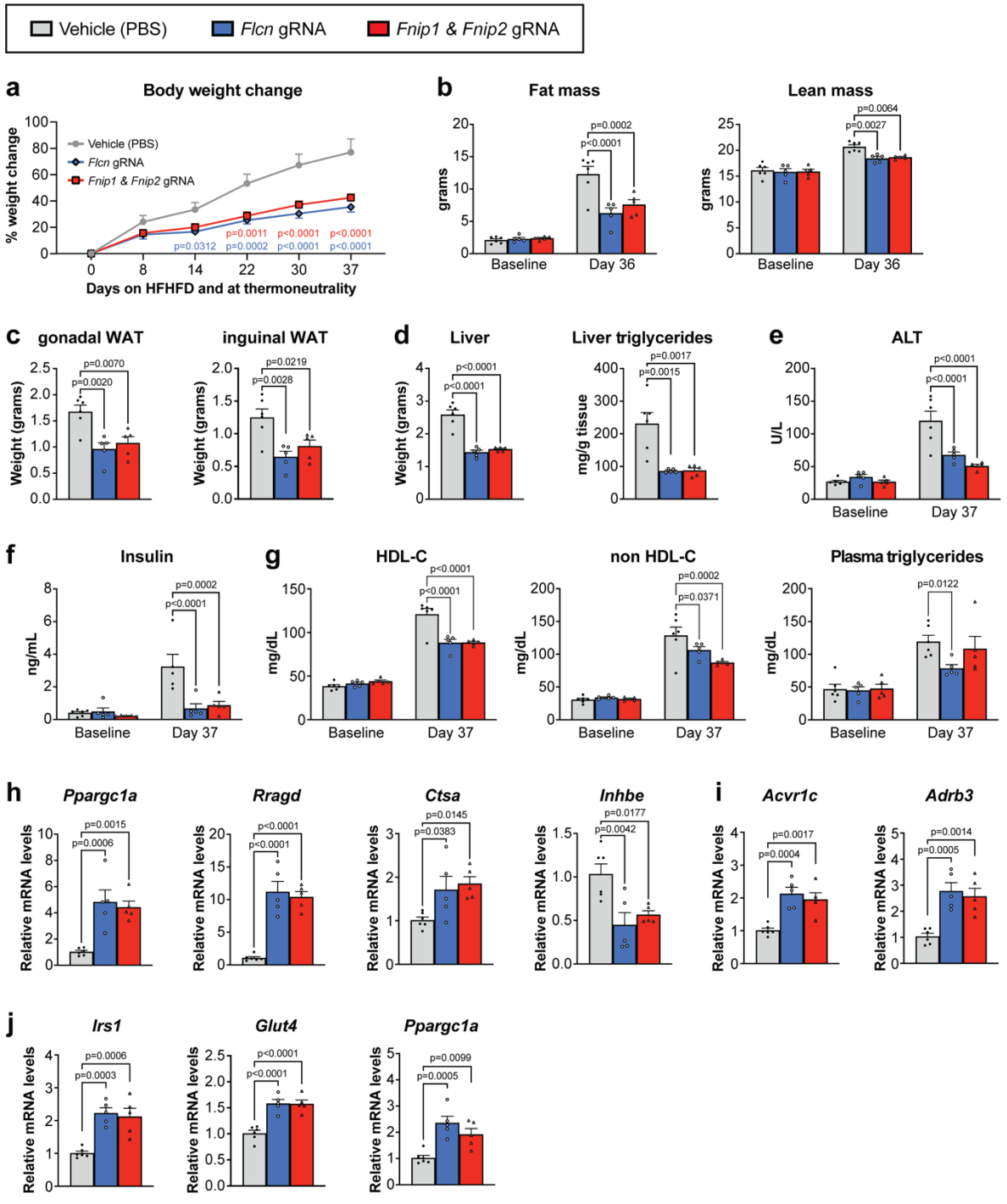
Reviewer 1 Comment 7 (R1C7): "Is the effect [protection against diet-induced obesity following deletion of FLCN or FNIP1/FNIP2 in the liver] observed at thermoneutrality?"

R1C7 – Author's reply: In revised manuscript, we performed a new set of AAV8-gRNA experiments for *Flcn* and *Fnip1*/*Fnip2* inhibition at thermoneutrality.

In these experiments, FLCN/FNIP pathway inhibition led to similarly protective metabolic phenotypes as the ones observed at ambient temperature (**Inset Fig. 6**).

These results indicate that the protective metabolic phenotype is not dependent on increased thermogenic demand at sub-thermoneutral housing, supporting its translational relevance.

Inset Fig. 6. Hepatic *FLCN or FNIP1 & FNIP2* knockdown protects against diet-induced weight gain and metabolic dysfunction at thermoneutrality.



R1C7 – manuscript changes: The thermoneutrality study is now presented in **Supplementary Fig. S29** and discussed in the **Results** section (Lines 267-272).

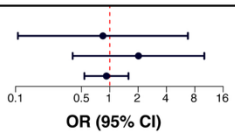
Reviewer 1 Comment 8 (R1C8): "The authors also fail to discuss the potential deleterious effects of FLCN loss in the liver. Specifically, supplementary Fig.S4 panel D shows higher odds of disease outcome for liver cirrhosis in predicted loss of function FLCN mutations in humans. In addition, a recent study (Custode et al PMID: Cell Death Differ. 2025 Aug;32(8):1460-1472) reported liver cell damage occurring following liver specific deletion of FLCN (using Alb-cre). In that study, male mice showed signs of liver damage as early as 4 weeks of age. In the current study, the effect of FLCN deletion on ALT levels in male mice 13 weeks after AAV8 treatment fed HFHFD were measured. However, ALT levels in mice fed a chow diet, as well as the effect of FNIP1/FNIP2 deletion should also be measured."

R1C8 – Author's reply: We thank the Reviewer for highlighting the important study by Custode et al., which we now cite and discuss in the revised manuscript. We also carefully examined the totality of evidence around liver safety, particularly for FLCN loss-of-function, and performed further experiments to address this question.

Overall, human genetic data from this study and the literature is reassuring with regards to liver phenotypes. In our data, *FNIP1* mutations were associated with lower ALT, lower liver fat and lower risk of metabolic liver disease (**Fig. 4 and Extended Data Fig. 3**). In the revision, we also specifically investigated associations with liver cancer and found no association (**Supplementary Fig. S24b and Inset Fig. 7**). A survey of the literature on cases of homozygous FNIP1 deficiency identified no mention of any adverse liver phenotypes (*Eur J Immunol. 2020 Jul;50(7):1078-1080*; *Blood. 2021 Jan 28;137(4):493-499*; *J Clin Immunol. 2023 Nov;43(8):1751-1753*; *J Pediatr Hematol Oncol. 2024 Aug 1;46(6):e472-e475*; *Immunogenetics. 2024 Nov 14;77(1):2*; *Curr Issues Mol Biol. 2025 Apr 18;47(4):290*).

Inset Fig. 7. Heterozygous mutations in *FNIP1* and *FLCN* are not associated with liver cancer.

Outcome Gene Variant	Count (Cases) RR / RA / AA	Count (Controls) RR / RA / AA	AAF	Odds Ratio (95% CI) per Allele	P–Value
Gastrointestinal: Hepatocellular or Cholangio Carcinomas					
FNIP1 pLoF, AAF<1%	3,974 / 1 / 0	559,160 / 76 / 0	0.007%	0.8 (0.1, 6.7)	0.874
FLCN pLoF, AAF<1%	3,974 / 1 / 0	559,075 / 161 / 0	0.014%	2.0 (0.4, 10.1)	0.393
FLCN plus deleterious missense (5/5 or ESM1vp), AAF<1%	3,962 / 13 / 0	557,156 / 2,079 / 1	0.19%	0.9 (0.5, 1.6)	0.766



While the Reviewer is correct that the pLOF *FLCN* mutations showed a numerical increase in odds of liver cirrhosis, this did not meet nominal statistical significance (p=0.059). For the same outcome, the pLOF plus deleterious missense variant gene-burden did not show an association (OR=1.2; p=0.286). The same set of pLOF plus deleterious missense mutations had an association with *lower* ALT (−0.84 U/L; $p=8\times10^{-4}$). Also for *FLCN*, we found no statistically-significant associations with liver cancer (**Supplementary Fig. S24b and Inset Fig. 7**).

As pointed out by the Reviewer and clearly discussed in the revised manuscript, results are more nuanced for mouse models of *Flcn* hepatic inhibition where some studies showed benefit in

models of metabolic liver disease (*Science. 2022;376:eabf8271*; *Sci Rep. 2021;11:21268*), whereas Custode et al. showed increased propensity for liver injury and carcinogenesis for hepatic *Flcn* knockout in hepatocytes and cholangiocytes using an Alb-Cre approach (*Cell Death Differ. 2025;32:1460-1472*).

In the revision, we performed various additional experiments. In the new chow diet experiments (**Supplementary Fig. S30**), we did not see statistically-significant differences in ALT levels. However, we detected associations with lower body fat mass and lower liver triglyceride content in the *Flcn* or *Fnip1/2* liver inhibition groups (**Supplementary Fig. S30**), consistent with an overall favorable metabolic/liver phenotype, which was, as expected, less pronounced than in HFHFD challenge setting. In the HFHFD model, now expanded with more experimental replicates, ALT and liver fat were clearly reduced with *Flcn* or *Fnip1/2* liver inhibition at 13 weeks (**Fig. 4f and Extended Data Fig. 7**), consistent with a favorable liver phenotype. We further performed a long-duration experiment (30 weeks) in HFHFD, where *Flcn* silencing was associated with profound and sustained reductions in ALT and liver fat (**Extended Data Fig. 10**). In this experiment, the beneficial liver impact was further supported by gene expression profiling demonstrating that long-term *Flcn* inhibition prevented the induction of both (a) fibrosis genes and (b) cholangiocyte/hepatic progenitor genes that was observed in control animals on HFHFD (**Extended Data Fig. 10**).

In summary, the human genetic data and experimental data in the revised manuscript is overall reassuring about the favorable impact on liver metabolism with loss-of-function of this pathway, particularly in the HFHFD setting. However, we fully acknowledge the concerns raised by the Custode et al. paper and we discuss that in the context of the totality of evidence in the revised manuscript.

R1C8 – manuscript changes: The additional genetic analyses for liver cancer are in **Supplementary Fig. S24b**. The new chow diet experiment results are shown in **Supplementary Fig. S30** and discussed in **Results** (Lines 272-275). The 13-week AAV8-gRNA experiments are in **Fig. 4 and Extended Data Fig. 7**. The 30-week AAV8-gRNA *Flcn* experiment is in **Extended Data Fig. 10** and discussed in **Results** section (Lines 276-281). In the **Discussion** section, we present a balanced summary on the evidence on liver safety for FLCN-pathway loss of function (Lines 340-352) and directions of future work in this area (Lines 358-359).

Reviewer 1 Comment 9 (R1C9): "Were female mice used in any of the studies? If not, the reason why they were not included should be discussed."

R1C9 – Author's reply and manuscript changes: We thank the Reviewer for raising this point. Our validation experiments were designed to maximize the ability to detect a metabolic phenotype using an established high-fat diet model where effect sizes are best characterized in males. Therefore, the studies involved male mice only, which historically have shown more robust

metabolic disease phenotypes than females (*PLoS One 2012;7:e46057*; *Life Sci. 2022;301:120636*; *Physiol Behav. 2020; 221:112894*; *J Biol Rhythms 2024;39:413–422*). We have provided this explanation in the reporting checklist, and now also include a statement in the **Methods** section (Lines 434-436 at page 11 of the Methods section which is page 125 of the combined PDF file), also acknowledging the potential generalizability limitations of this experimental design choice.

It is important to note that the *FNIP1* human genetic association is robust and consistent in both men and women (p-value for heterogeneity/interaction in association estimates between men and women, p=0.22; see **Supplementary Table S11** of the revised manuscript), confirming the generalizability of our human genetics findings across men and women.

Reviewer #3

Reviewer 3 Opening Statement (R3OS): "The authors conducted the largest-to-date rare variant burden analysis of the TG/HDL ratio and identified 60 genes. The analytical framework is robust and substantially increases the number of genes implicated in the TG/HDL ratio, a proxy for human energy metabolism. In particular, the identification of FNIP1, a previously poorly characterized gene in lipid metabolism, is highly novel and promising for future drug development for metabolic health. The authors further validate their findings in a rodent model and observe a robust causal effect of gene knockdown on the phenotype. The manuscript is concise and very well written. I have several comments that I believe will further enhance the readability and robustness of the paper."

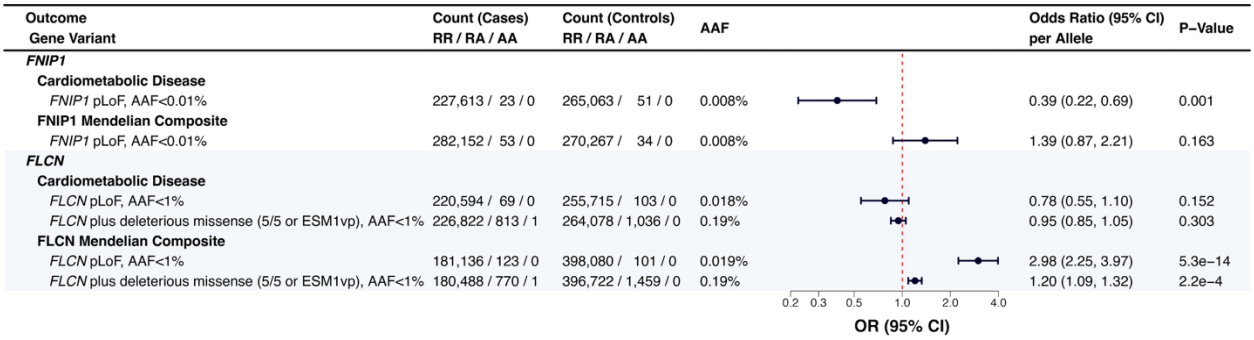
R3OS – Author's reply: We thank the Reviewer for the comprehensive assessment of our work, for the supportive remarks regarding the analytical framework and the novelty of the *FNIP1* findings, and for the valuable suggestions which have truly helped us improve the manuscript. We provide a point-by-point reply to each comment below.

Reviewer 3 Major Comment 1 (R3MC1): "The LOEUF for FNIP1 is reported as 0.39 in gnomAD project, consistent with borderline constraint. The pattern differs substantially from that observed for PCSK9 (LOEUF = 1.25), which is the common example of the existing of human knockout as a proxy of knockdown in the humans. Although the authors report an extensive PheWAS of FNIP1 protein-truncating variants, the stance of the authors is unclear. Do the authors claims FNIP1 knockout is safe for humans? or need cautions with any undescribed complications."

R3MC1 – Author's reply: We thank the Reviewer for raising this interesting point. While there is no definitive explanation for the borderline level of constraint of *FNIP1* mutations, it is possible that the development of immunodeficiency-93 and hypertrophic cardiomyopathy in homozygous mutation carriers might have led to selection. Another very intriguing possibility is that what we observe today as a favorable metabolic phenotype, in the calorie-rich "obesogenic" environment of modern age, might have been deleterious to humans during thousands of years of their evolution in a calorie-deprived environment.

With regards to the safety of inhibiting the FNIP1 pathway therapeutically, Mendelian genetics shows that lifelong complete systemic "human knockout" of *FNIP1* results in recessive immunodeficiency-93 and hypertrophic cardiomyopathy. However, our new results show that heterozygous *FNIP1* mutations are associated with profound protection from cardiometabolic disease without a statistically-significant association with the Mendelian phenotype composite of immunodeficiency 93 and hypertrophic cardiomyopathy (see **Inset Fig. 8**, where Mendelian features of FNIP1 deficiency have been combined as requested by the Reviewer in **R3MiC6**).

Inset Fig. 8*. Association of *FNIP1* and *FLCN* mutations with cardiometabolic disease risk and with a composite phenotype for their respective Mendelian syndromes. *This figure is identical to Inset Fig. 3 included in the R1C2 reply above, it is reproduced here for ease of review.



Another mitigating factor is the possibility of inhibiting the FNIP1 pathway in a tissue specific way (e.g. in liver hepatocytes), sparing other organs and therefore minimizing the potential side effects in other areas of the body (e.g. the immune system or cardiomyocytes; see also **R1C2**). Liver safety is also discussed in detail in the revised manuscript and **R1C8**.

Importantly, as of today there are 7 FDA-approved GalNAc-conjugated siRNA therapeutics that effectively and durably silence a variety of genes in hepatocytes in humans (e.g. *NEJM. 2020;382:2289–2301; NEJM 2020;382:1507–1519; NEJM. 2021;384:1216–1226; NEJM 2025;392:127–137*), indicating that therapeutic modulation of this pathway in the liver would be a feasible approach.

R3MC1 – Manuscript changes: In the revised manuscript, we added the Mendelian phenotype composite analysis for *FNIP1* and *FLCN* in **Supplementary Note S3, Figs. S21–S22, S24**. We discuss safety and tissue-specific inhibition rationale in the **Results** (Lines 217-224) and **Discussion** (Lines 340-342) sections. See also **R1C2, R1C8** and **R3MiC6**.

Reviewer 3 Major Comment 2 (R3MC2): "Related to this, the statement in line 187 - that no individuals carried pLoF variants in both FNIP1 and FNIP2 - is not supported by a statistical analysis. Please clarify whether this absence reflects an expected frequency under independence or indicates an exceptionally rare event consistent with digenic impact within this pathway. An estimate derived from cumulative allele frequencies would allow assessment of whether the observed co-occurrence (or lack thereof) deviates from chance expectations."

R3MC2 – Author's reply and manuscript changes: The absence of dual carriers in our data is entirely expected based on the allele frequency of the respective genotypes. The genotypes are completely independent in our data (correlation r= −0.00014; p=0.88). Given their ultra-rare frequency (alternative allele frequency of 0.0075% for *FNIP1* and 0.0073% for *FNIP2*), we expected 0.02 double heterozygote carriers and observed 0 in our >1 million people dataset. We have included the statistics in the revised manuscript (**Supplementary Table S21**).

Reviewer 3 Major Comment 3 (R3MC3): "The authors conducted extensive metabolic analysis using mice model. However, as the author described, the lipid metabolism is far different from humans and mice. To bridge findings, in-vitro experiments could be considered in human cell types. Especially, it is important that only FNIP1 knock out is sufficient to induce favorable metabolic pattern in humans not by dual knock down of FNIP1 and FNIP2 observed in the mice."

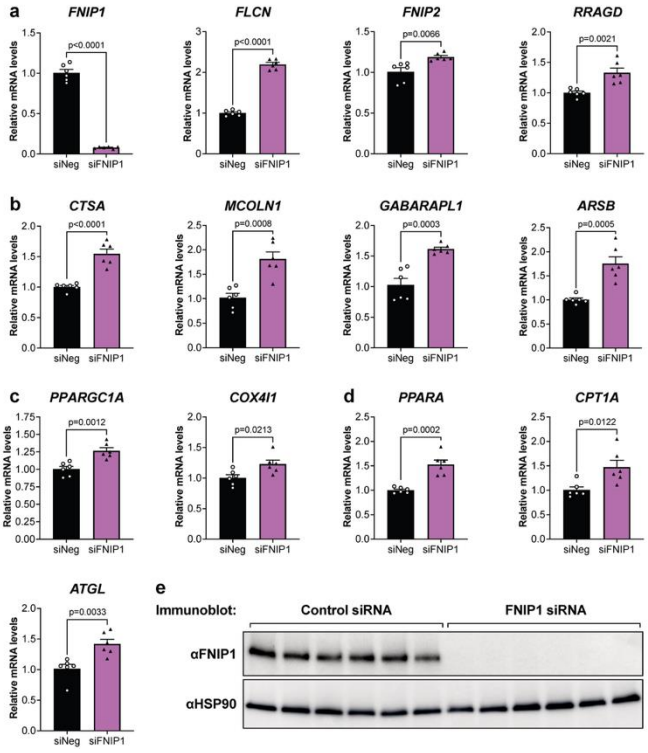
R3MC3 – Author's reply and manuscript changes: We agree with the Reviewer that bridging the mouse and human findings is very important.

For the revised manuscript, we performed knockdown experiments in primary human hepatocytes, which clearly show that *FNIP1* silencing with siRNA induces upregulation of lysosomal and lipid catabolism genes (e.g. *ATGL*, *CTSA*, *ARSB*; **Inset Fig. 9**).

This signature is consistent with that observed in mice in our *Flcn* or *Fnip1* & *Fnip2* silencing experiments as well as with the reported biology of this pathway (*Science 2023;380:eabj5559*; *Science 2022;376:eabf8271*; *JCI Insight 2019;*5:e126939). Importantly, these effects were observed with selective silencing of *FNIP1* alone, without concomitant knockdown of *FNIP2* (**Inset Fig. 9**), directly addressing the question about whether single-gene inhibition is sufficient in human cells.

These results, now added in **Fig 4** and **Extended Data Fig. 5**, provide evidence that FNIP1 inhibition alone is sufficient to activate energy metabolism gene programs in human hepatocytes, helping to bridge the mouse and human data in the revised manuscript.

Inset Fig. 9. Gene-expression signature and FNIP1 protein levels upon *FNIP1* knockdown in primary human hepatocytes.



Reviewer 3 Major Comment 4 (R3MC4): "The protective effect under high-fat/high-fructose diet in the intervention group (FNIP1 and FNIP2 knockdown) is highly remarkable, however no data is shown in the regular diet. This also address the safety concerns raised by human genetic evidence above."

R3MC4 – Author's reply and manuscript changes: This is an excellent point and we have now performed experiments in regular chow diet, which showed a more modest but statistically-significant protective effect also in the chow diet setting (**Supplementary Fig. S30**).

In particular, we observed statistically-significant reductions in fat mass and liver triglyceride content for the *Flcn* and *Fnip1* & *Fnip2* silencing groups vs vehicle control (**Supplementary Fig. S30**). As expected the phenotype was less pronounced than what observed in the HFHFD challenge setting.

The full results of the new chow diet experiments are presented in **Supplementary Fig. S30** of the revised manuscript.

Reviewer 3 Major Comment 5 (R3MC5): "The multi-ancestry aspect of this project is not sufficiently addressed in the manuscript. As shown in Table 1, the means of HDL-C, TG, and TG/HDL differ significantly across cohorts. It is well known, often called the 'triglyceride paradox' that individuals of African ancestry tend to have relatively low TG and high HDL-C (clear if we focus on BioMe, PMBB, DBS), while worse clinical outcomes. Leveraging this research network to examine this phenomenon using the TG/HDL ratio is both intriguing and important. Population-stratified epidemiologic and genetic analyses would further increase the value of this study, clinically and scientifically."

R3MC5 – Author's reply and manuscript changes: We completely agree with the Reviewer on the importance of a deeper exploration of the multi-ancestry nature of our analysis and in the revised manuscript we have performed a series of in depth ancestry-specific epidemiologic association analyses, common variant fine-mapping, and rare variant exome-wide analyses.

For the epidemiology analyses, we have generated and included in the new manuscript association plots and statistics for each ancestry (**Supplementary Figures S2-S6**). TG-HDL ratio was robustly associated with higher cardiometabolic disease risk and with its related anthropometric and biomarker traits across ancestries, including African ancestry.

In the revised manuscript, we also report ancestry-specific genetic association results for both common and rare variants.

For the common variant GWAS, we now report ancestry-specific fine-mapping signals (**Supplementary Table S4**). For the exome-wide analysis of rare coding mutations, we now report ancestry-specific association estimates (**Supplementary Table S9**) showing that associations are consistent across ancestries. Specifically, 58 of 59 genes (98%) had the same direction of association in European vs pooled non-European ancestry estimates (**Supplementary Table S9**). The only exception was the *A1CF* gene, where there was a strong association in European analyses ($p=5.5\times10^{-27}$), but no association in the non-European subsets (p=0.97), which we demonstrate is driven by a gain-of-function variant (p.Gly390Ser; experimentally validated in *Nat Genet. 2017 Dec;49(12):1758-1766*) that is specifically enriched in the European populations (**Supplementary Note S2**).

We also demonstrate the value of the multi-ancestry analysis in detecting certain rare variant associations. In particular, the higher allele frequency of TG-HDL associated mutations in non-European ancestries was instrumental to detect several exome-wide significant associations (e.g. *LIPE*, *PCSK9* and *GALNT2*). This is discussed in the **Results** section of the revised manuscript (Lines 116-119) and **Supplementary Note S2**.

Reviewer 3 Major Comment 6 (R3MC6): "One fundamental question is whether using a ratio as the outcome is free from bias. Several previous works note that ratios computed from two

continuous variables can introduce collider bias. That said, the TG/HDL ratio is widely used and well established in this field. Within this research network, demonstrating that non-ratio measures (e.g., HDL-C residualized on TG, TG conditioned by HDL-C) yield consistent results would substantially increase the paper's impact and robustness."

R3MC6 – Author's reply and manuscript changes: We thank the Reviewer for raising this point and we have performed the requested adjusted analyses (now included in **Supplementary Table S17**).

It is important to note that *both* the ratio and the adjustment (e.g. TG adjusted for HDL-C) approaches can theoretically introduce a collider effect (see *Am J Hum Genet. 2015;96:329-39*; *Am J Hum Genet. 2016;98:392-3*, which provides a robust example of adjustment-induced collider).

However, the two approaches will identify dramatically different sets of genes, as ratio leverages the negative covariance of the traits, whereas the adjustment removes it.

The TG-HDL ratio approach maximizes discovery of genes that affect **both** TG and HDL, particularly in opposite directions. This is the intended goal of the study as that pattern is robustly associated with cardiometabolic disease risk and energy-metabolism biology, as evidenced by the abundant literature on the TG-HDL ratio (*Nat Genet. 2024;56:212-221*; *Nat Commun. 2024;15:8068*).

In contrast, the adjusted approach will remove the co-variance of the two traits and identify genes that are most strongly (or exclusively) associated with the trait that is not being adjusted for (i.e. "TG-dominant" or "HDL-dominant" genes). This would attenuate the signal for genes genuinely associated with the ratio where effect sizes on the two traits are similar in size (i.e. "TG-HDL ratio balanced" genes).

With this in mind, we expected that for TG-HDL, in the absence of major bias, we should observe most genes having a "balanced" association with TG and HDL in opposite direction and that fewer genes should be "trait-dominant".

We defined "effect size balance" as: absolute(beta-TG) / ((absolute(beta-TG) + absolute(beta-HDL)), with all betas expressed in standardized units. Then, we classified genes where balance was ≥ 0.75 as "TG-dominant", genes where balance was ≤ 0.25 as "HDL-dominant" and genes where balance was < 0.75 and > 0.25 as "TG-HDL balanced" genes.

As expected, most genes (39 genes, 66%) were TG-HDL balanced, while 7 (12%) genes were HDL-dominant and 13 (22%) genes were TG-dominant. Even within TG- and HDL-dominant genes, most genes had opposite direction of effect for TG and HDL (**Supplementary Table S17**).

Overall, across the 59 genes, only 3 genes showed statistically-significant associations with both traits but in the same direction (*ANGPTL3*, *LIPG*, *ABCA1;* **Supplementary Table S17**). These 3 genes are well described regulators of lipid metabolism, whereby *ANGPTL3* and *LIPG* are known to biologically interact in both VLDL- and HDL-lipoprotein particle metabolism (*Arterioscler Thromb Vasc Biol. 2007;27:366-72; J Lipid Res. 2015;56:1308-17*; *J Lipid Res. 2020;61:1271-1286*), while *ABCA1* mediates reverse cholesterol transport in the formation of nascent HDL particles (*J Lipid Res. 2001;42:1717-26*; *J Clin Invest. 1999;104:R25-31*).

Consistent with this framework, the adjusted analyses showed the expected results with stronger associations for the dominant genes and weaker associations for the TG-HDL balanced genes (**Supplementary Table S17**).

All these results support the validity of our discovery approach and that these observed patterns of trait-balance reflect relevant biology and different biological groupings (as suggested by the Reviewer in **R3MC7** below), rather than bias.

Based on the Reviewer suggestion, we were intrigued to investigate whether these clusters of dominant and balanced genes reflected different biological underpinnings, so we performed overall and cluster-specific pathway analyses, as described in the reply to **R3MC7** below.

In summary, both theoretical reasoning and empirical analysis of our gene-level results support the validity of the TG-HDL ratio as the discovery trait, and we leveraged the Reviewer's suggestion to further characterize biological groupings among identified genes.

Reviewer 3 Major Comment 7 (R3MC7): "Extended Data Figure 3 provides an excellent visualization of effect direction of each component as well as overall TG/HDL ratio. For example, ABCA1 lowers HDL-C more than it lowers TG, resulting in an increased TG/HDL ratio. In contrast, A1CF increases TG but no effect on HDL-C, leading to an increased TG/HDL ratio as well. Do these distinct mechanism imply different clinical associations? Moreover, could these differences be mapped to distinct biological pathways?"

R3MC7 – Author's reply: We thank the Reviewer for this fantastic suggestion, leading to some intriguing results.

We have now performed pathway enrichment analyses using DEPICT (*Nat Commun. 2015:6:5890*) on the overall set of 59 genes, as well as effect-size balance subgroups (i.e. TG-dominant, HDL-dominant, and TG-HDL balanced gene sets).

Pathway enrichment analysis for the overall and TG-HDL balanced gene-sets revealed 761 and 500 enriched pathways after Bonferroni correction, respectively. There was an overwhelming

representation of energy metabolism pathways including lipid and glucose homeostasis, oxygen and energy consumption, lipoprotein metabolism, insulin resistance, liver fat accumulation, lipolysis regulation, fat storage and more (see **Supplementary Tables S16-S19** for details). For these pathways, an average of 16 of our 59 genes contributed to the pathway enrichment signal, with each gene showing a p-value<0.05 for a given pathway, consistent with an extremely high concentration of true biological signal.

TG-dominant genes showed preferential enrichment for pathways relating to TG-rich very-low density lipoprotein (VLDL) cholesterol and chylomicron particles (**Supplementary Table S18**), HDL-cholesterol level regulation (reflecting the underlying association with TG-HDL ratio even for TG-dominant genes) and susceptibility to atherosclerosis, among the total 365 enriched pathways after Bonferroni correction.

HDL-dominant genes showed preferential enrichment for HDL lipoprotein metabolism (**Supplementary Table S19**), abnormal lipid levels and liver physiology, among the total 145 enriched pathways after Bonferroni correction.

To directly address whether these distinct mechanisms imply different clinical associations, we tested whether effect-size balance predicted the pattern of genetic associations with clinical outcomes, but we found it was not a statistically significant predictor (**Inset Table 1**). This might reflect the fact that the identified genes impact downstream cardiometabolic risk pathways regardless of their primary lipid fraction of association.

Inset Table 1. Gene-burden analysis for effect size balance across the 59 TG-HDL genes and cardiometabolic outcomes. The association is expressed as ln-OR per unit increase in effect-size balance. Associations were estimated using inverse-variance weighed Mendelian randomization (*Genet Epidemiol. 2013;37:658-65*). Abbreviations: TG, triglycerides; HDL, high-density lipoprotein cholesterol; ln-OR, natural logarithm of the odds ratio; MASLD, metabolic dysfunction associated liver disease; T2D, type 2 diabetes; CAD, coronary artery disease.

Predictor variable	Number of genes	Outcome variable	Association between TG-HDL effect-size balance and outcome variable effect estimates, ln-OR (standard error)	P-value
TG-HDL effect size-balance	59	MASLD	0.027 (0.031)	0.39
		Liver cirrhosis	0.033 (0.044)	0.46
		T2D	0.021 (0.020)	0.30
		CAD	0.007 (0.045)	0.88

Reviewer 3 Major Comment 8 (R3MC8): "The idea of integrating common genetic signals to enhance rare variant association is excellent. I think the authors could further deepen the discussion

by considering the effect of direction. From beta-eQTL and beta-TG/HDL, we can infer the impact of decreased gene expression on outcomes (TG/HDL) by flipping the effect. This could be aligned with the pLoF/missense burden beta for TG/HDL. For example, ANGPTL3 pLoF/missense variants decrease TG/HDL. Additionally, the sentinel variant 1:62604866:AGTTAATGTG:A in this region decreases expression of ANGPTL3 and lowers TG/HDL, which is a fascinating example of convergence in gene function and expression. I briefly confirmed that 9 out of 9 genes with EWS and significant colocalization align beautifully with this observation. In this right, the line 238 could be started with 'Consistently,'. The next question is whether a more liberal threshold can maintain this consistency. If alignment persists even under lenient thresholds, the RVAS likely captures true associations with this level of association."

R3MC8 – Author's reply: We thank the Reviewer for their supportive remarks on this analysis and the brilliant suggestion to perform a concordance analysis.

Directional concordance between common eQTL and rare coding variant evidence is observed for 15 genes out of 18 (83%; expected percentage under null hypothesis, 50%; binomial p= 0.0075). Both sets of genes (exome-wide- and experiment-level-significant genes) had a higher percentage of concordance compared to what is expected by chance, 100% (9/9) genes and 67% (6/9) genes for the two groups respectively. This is consistent with true signal and relevant biology for these sets of genes.

R3MC8 – Manuscript changes: We have included this analysis in the **Results** section (Lines 294-298), editing relevant text as suggested. We also report the concordance in **Supplementary Table S23.**

Reviewer #3, Minor Comments

Reviewer 3 Minor Comment 1 (R3MiC1): R3MiC1: "The study design is robust and sufficiently controlled by exome wide significance. However, still replication analysis can add additional value. With whole genome sequence data now available in All of Us Research program, there is no justification for withholding these resources. Although a small number of samples may overlap with existing cohorts, including this cohort in the analysis will strengthen the paper's robustness."

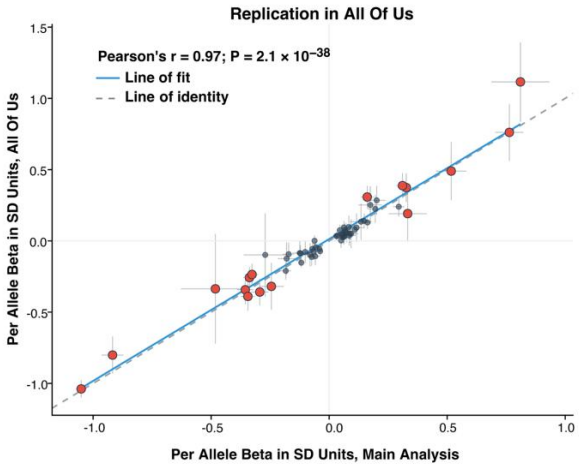
R3MiC1 – Author's reply: This is an excellent suggestion and in the revised manuscript we present a replication analysis in 114,942 participants with TG-HDL ratio measurements and genomic data from the All of Us Research program (AoU version 8).

For the 59 exome-wide significant gene-burden signals, association estimates from the All of Us cohort showed near perfect correlation with estimates from our main analysis (see **Inset Fig. 10** below).

In addition, we successfully replicated all 23 genes for which we estimated we had 90% power to detect an association with alpha < 0.05 in All of Us, given sample size of AoU, and the effect size and frequency of the genetic exposure.

R3MiC1 – Manuscript changes: We added the All of Us replication results to **Supplementary Table S12 and Supplementary Figure S17** of the revised manuscript.

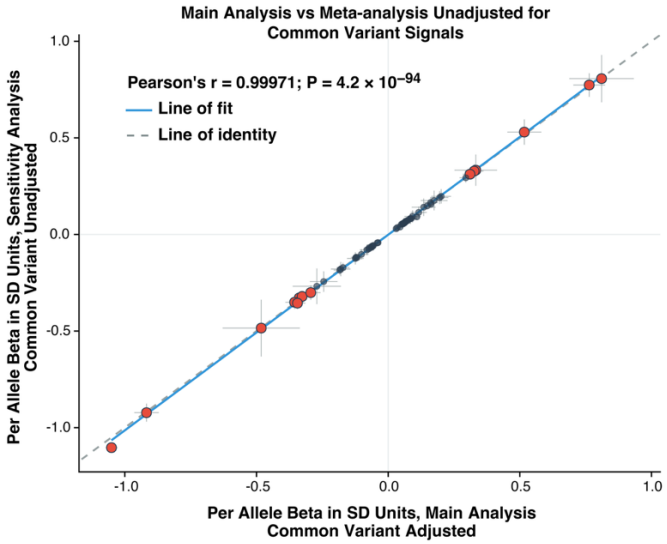
Inset Fig. 10. Correlation of association estimates in the main analysis compared to a replication analysis in 114,942 participants of the All of Us cohort.



Reviewer 3 Minor Comment 2 (R3MiC2): "Conditioning on common genetic variants is an excellent way to reduce linkage between common and rare variants. I agree to report the conditioned analysis, but I am curious whether these adjustments change the statistics for rare variants, e.g., some genes might show decreased association, and vice versa. Also, the authors use REGENIE as an association framework, which regresses out global effect from common-variant in step 1. Doesn't this additional conditioning overlap with that process and risk overcorrection?"

R3MiC2 – Author's reply and manuscript changes: This is an excellent methodological point. As illustrated in **Inset Fig. 11**, association results for the 59 genes were near identical in the main analysis (i.e. adjusted for common variants) vs a sensitivity analysis where we did not perform adjustment for common variants. These results are now included in **Supplementary Fig. S15b and Table S8** of the revised manuscript.

Inset Fig. 11. Correlation of association estimates in the main analysis (i.e. common variant adjusted) compared to a sensitivity analysis without adjustment for common variants.



It is correct that REGENIE regresses out the global effect of common variants by generating polygenic scores (PRSs) in Step 1 that are then used as covariate in Step 2, correcting for relatedness and population stratification. However, it does so by generating leave-one-chromosome-out PRSs, whereby the chromosome of the variant (or gene-burden) being tested is left out in the generation of the PRS (*Nat Genet. 2021;53:1097-1103*. URL: https://rgcgithub.github.io/regenie/). So, linkage disequilibrium *within* a chromosome is not corrected for by the standard REGENIE approach.

In our common variant adjustment analysis, we perform a further correction for fine-mapped common variants that reside in the *same* chromosome as the gene-burden being tested. Therefore, this approach corrects for local common variant signals and local linkage disequilibrium, but does not result in overcorrection. We have modified the **Methods** section of the manuscript to clearly explain this approach (Lines 257-261 at Page 7 of Methods which is Page 121 of the combined PDF file).

Reviewer 3 Minor Comment 3 (R3MiC3): "Another question is that ld sharing in rare variants. In Fig. 3 we see clusters on chromosome 19 1) APOC3, ZNF285 (and APOE), 2) LDLR and KEAP1. ZNF285 and KEAP1 are both not well characterized genes as I quickly searched. Is this association potentially driven by shared haplotypes between these genes? The rare haplotype is sometimes longer than common haplotypes. Previously this is not testable as small number of samples, but in this unprecedented scale of study, we may answer this question."

R3MiC3 – Author's reply and manuscript changes: This is a great point and, in the revised manuscript, we have performed an individual-level data conditional analysis for gene-burden

genotypes, where we adjusted each of the 60 gene-burden signals for other gene-burden signals in the same chromosome.

Of the 60 gene burden signals, 59 remained independently and robustly associated in the conditional analysis, including *KEAP1*, *ZNF285* and the various apolipoprotein encoding genes at the very complex and fascinating chromosome 11 locus containing *APOC3*, *APOA5* and *APOA1*. Only the signal of the *AC126283.2* gene disappeared in the conditional analysis (p=0.99 in the adjusted analysis; **Supplementary Table S5 and Note S2** in the revised manuscript). This reflects the coding sequence overlap with *PLA2G12A*, another exome-wide hit, as described in the revised manuscript in **Results** (Lines 106-109) and **Supplementary Note S2**.

We have now incorporated this exciting analysis in the revised manuscript and we also decided to tweak the presentation of the rest of the manuscript to reflect a total of 59 *independent* rare coding variant signals.

Reviewer 3 Minor Comment 4 (R3MiC4): "In line 109-111, the authors described that they identified 15 genes found in the previous study. If this discussion is based on Supple Table S13 (Hindy et al, AJHG 2022, PMID: 34932938), they reported 17 genes including 2 genes (APOE and TMEN136) not identified in this study while larger sample size. Is there any reasonable account for this non-identification? Also, how many of 60 genes identified were in the known (or identified in this study) by common variant association analysis."

R3MiC4 – Author's reply and manuscript changes: Looking in depth at these two genes in our data, we found that the rare variant *APOE* signal is driven by common variants and attenuated to below exome-wide significance when adjusting for common variants as done in our analysis but not in the AJHG paper.

TMEM136 (*TLCD5*) showed no association in our exome-wide discovery analysis (p=0.69), which likely reflects a false positive association in the original report given our analysis had 3,400 rare mutation carriers compared to the 157 of the AJHG 2022 paper. Genetic results for *APOE* and *TMEM136* are now reported in **Supplementary Table S7** and discussed in **Supplementary Note S2**.

We thank the Reviewer for the valid point about common variant evidence at the rare variant loci. In the revised manuscript, for all 59 rare variant loci we included a report of all common variant signals identified in the loci and putative causative genes from GWAS gene prioritization (i.e. nearest gene, eQTL and pQTL co-localization).

As expected from previous exome-wide analyses across health phenotypes (*Nature. 2021;599:628-634; Nat Genet. 2023;55:1277-1287*) and from the strong polygenic signal

observed for TG-HDL ratio, there was abundant common variant signal across the rare variant loci.

However, in many cases, evidence from common variants did not prioritize the same causative genes as rare variants. This was the case at 19 (32%) of rare variant loci, either because there were no common variant signals (6 loci) or because the common variant GWAS prioritized different genes from the ones identified by the exome-wide analysis (13 loci).

In the remaining 40 rare variant loci, only at 3 loci did the common variant evidence prioritize *only* the exome-wide significant gene. In all other cases, it prioritized multiple genes and often just via the more circumstantial nearest-gene criterion, resulting in ambiguous causal gene identification.

This illustrates the great complementarity and combined value of exome-sequencing alongside traditional GWAS, where rare coding variants provide unambiguous causative gene attribution and directionality of association (e.g. pLOF associated with higher or lower levels of the outcome trait).

The common variant evidence annotation at the rare variant associated loci is now reported in **Supplementary Table S15**.

Reviewer 3 Minor Comment 5 (R3MiC5): "The presentation of dates in Fig. 2 might be somewhat unfamiliar for readers. Considering the putative causal relationships between the TG/HDL ratio and clinical outcomes (CAD, T2D), harmonizing the x-axis across panels to the TG–HDL ratio—as demonstrated in Fig. 2a, would enhance interpretability. Moreover, pairwise correlations are not well suited to reflecting differential effect sizes across outcomes. Estimating and reporting coefficients per 1 SD increase in the TG–HDL ratio for each outcome, accompanied by error bars (e.g., 95% confidence intervals), would more effectively quantify heterogeneous effects and their uncertainty."

R3MiC5 – Author's reply and manuscript changes: thank you for these suggestions, we have modified **Figure 2** accordingly and it indeed looks more impactful.

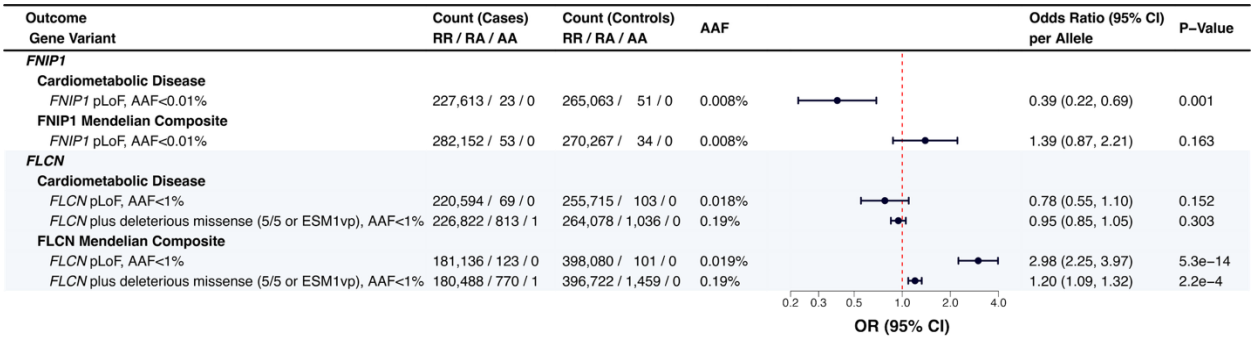
Reviewer 3 Minor Comment 6 (R3MiC6): "Because the authors combine metabolic traits to demonstrate benefit (line 150), presenting the unfavorable outcomes in the same combined manner (line 161, Supplementary Fig. S3) would allow fairer comparisons."

R3MiC6 – Author's reply and manuscript changes: The combined Mendelian phenotype analysis for *FNIP1* and *FLCN* mutations is now reported in **Supplementary Fig. S21 and Inset Fig. 12**.

Heterozygous mutations in *FLCN* were associated with the outcome phenotype combining the clinical features of *dominant* Birt–Hogg–Dubé syndrome (**Inset Fig. 12**), whereas *FNIP1* heterozygous mutations were *not* associated with the outcome phenotype combining the clinical features of *recessive* immunodeficiency-93 and hypertrophic cardiomyopathy (**Inset Fig. 12**). In contrast, *FNIP1* mutations were more strongly associated with cardiometabolic disease protection, compared to *FLCN* mutations (**Inset Fig. 12**).

These results suggest a more favorable balance between cardiometabolic benefit and adverse health manifestations for heterozygous pLOF in *FNIP1* versus *FLCN*. Another mitigation factor could be the possibility of tissue-specific inhibition of the pathway such as hepatocyte specific GalNAc-conjugated siRNA therapeutics, which we extensively explore with experimental models of hepatocyte and liver knockdown in human cells and *in vivo* murine models (see also reply to **R1C2**).

Inset Fig. 12*. Association of *FNIP1* and *FLCN* mutations with cardiometabolic disease risk and with a composite phenotype for their respective Mendelian syndromes. *This figure is identical to Inset Fig. 3 (R1C2 reply) and Inset Fig. 8 (R3MC1 reply), it is reproduced here for ease of review.



Reviewer 3 Minor Comment 7 (R3MiC7): "In lines 26–38 (Methods), the authors state that median values were used for measured traits. For the TG/HDL ratio, were TG and HDL measured at the same time point, or were values from different time points used? If different, please describe the matching strategy."

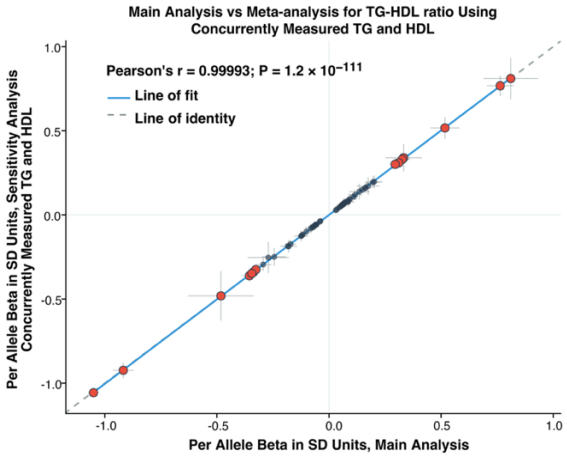
R3MiC7 – Author's reply and manuscript changes: This is a great point. For epidemiological population-based studies, comprising 5 cohorts and 635,115 (62% of total) participants, we used baseline TG and HDL measurements and calculated the TG-HDL ratio at baseline, so at the same time point. For health system cohorts, comprising 6 cohorts and 397,001 (38% of total) participants, we calculated median values of HDL and TG across clinical encounters (considered as the most representative values for each trait) and then calculated the ratio of the median values. In this subset, we did not strictly require that HDL and TG were measured at the same time, as the use of median values across encounters is a standard approach in EMR-based genetic research to define a patient's typical lipid profile (URL: https://www.finngen.fi/en/access_results; *Science*.

2016;354:aaf6814; Genome Med. 2021;13:6). Importantly, this avoids the exclusion of participants with valid but non-concurrent lipid measurements, thereby preserving sample size and discovery power.

We performed a sensitivity analysis where we required concomitant measurements and found that the associations for the top 59 genes were near identical as those from the main analysis (correlation r>0.99, $p=1.2\times10^{-111}$; **Inset Fig. 13 and Supplementary Fig. S15d**).

We now clearly explain the definition of the phenotype in the **Methods** section of the revised manuscript (Lines 37-47 at Page 1 of the Methods section, which is Page 115 of the combined PDF file) and report the results of this sensitivity analysis in **Supplementary Fig. S15d and Table S10**.

Inset Fig. 13. Correlation of association estimates in the main analysis compared to a sensitivity analysis where TG and HDL were required to be measured at the same time.



Reviewer 3 Minor Comment 8 (R3MiC8): "In lines 248-256 (Methods), the authors conducted fine-mapping with SuSiE on the meta-analysis summary statistics and stated that LD was computed using the individuals included in the association analysis. How was LD computed across participants from different biobanks? Was a combined genetic dataset available to compute an LD matrix across all participants? Also, 30.3% of participants were non-European. Did this ancestry mixture affect LD structure and the fine-mapping results? LD mismatch can introduce bias in fine-mapping. In addition, the number of most credible sets (the L parameter in SuSiE) is not reported. How many loci reached saturation (number of causal signals == L)? This is a quick sanity check for LD mismatch."

R3MiC8 – Author's reply and manuscript changes: These are excellent technical questions about the fine-mapping methodology, which we now explain thoroughly in the **Methods** section

("*Common variant association analysis and fine-mapping"* paragraph starting at Line 272 at Page 7 of the Methods section, which is Page 121 of the combined PDF file).

Briefly, we have access to the individual-level data for each cohort, so we used a sample-size-weighted pooled estimate of the covariance matrix as the LD input for fine-mapping. This not only ensures that we used the exact set of individuals included in the meta-analyses, but also that our generated LD matrix captures the exact ancestral composition present in our meta-analyses, thereby avoiding biases typically introduced by using external or mismatched reference panels.

We have also updated the **Methods** section to specify the value used for the L parameter (L = 30). In our common-variant (MAF≥1%) fine-mapping results, the maximum number of observed signals in a region was 24 (**Supplementary Table S3**), consistent with optimal control of LD.

Reviewer 3 Minor Comment 9 (R3MiC9): "Please confirm that the reported Beta and SE are unconditioned (marginal) effect sizes and standard errors from the primary association model. I note that many variants are not significant (|Z| < 1.96). It would be helpful to include SuSiE fine-mapping metrics to aid interpretation - specifically, the posterior inclusion probability, locus-membership, credible-set membership, and the maximum LD (R2) to the lead variant. These additions would clarify overall procedure in this process."

R3MiC9 – Author's reply: It is correct that the betas and standard errors are from the unconditioned analysis and we now report the full set of statistics requested by the Reviewer (i.e. posterior inclusion probability, locus-membership, credible-set membership, and the maximum LD to lead/sentinel variant) in **Supplementary Tables S3-S4**.

Reviewer 3 Minor Comment 10 (R3MiC10): "The description of common-variant analyses is insufficient. Please report basic summary of results to characterize the genetic architecture of this phenotype. For example (1) the number of non-overlapping associated loci described in lines 248-256 (Methods), (2) the genomic inflation factor, and, if inflation is observed, the LD Score regression intercept to assess confounding, (3) standard visualizations (Manhattan and QQ plots). These additions would help demonstrate that the study design, particularly phenotype definition and population stratification, has been appropriately controlled."

R3MiC10 – Author's reply: We agree and we have updated the manuscript accordingly. We now report the number of non-overlapping loci in the **Results** section (Lines 94-96) and **Supplementary Note S1** describing the GWAS results in depth. We also report Manhattan and Q-Q plots in **Supplementary Figures S7-S12**, as well as genomic inflation, LD-score regression intercept and attenuation ratio (**Supplementary Note S1**), which were consistent with appropriate control of genetic sources of confounding.

Reviewer 3 Minor Comment 11 (R3MiC11): "The manuscript adopts a exome-wide significance threshold of 1.04×10-7. While the authors constructed 24 variant masks, these appear to have been combined via the Cauchy combination test to yield a single P value per transcript. If so, the effective multiplicity should correspond to the number of transcripts assessed, rather than to the number of masks times number of transcripts. In addition, the manuscript does not clearly state the total number of transcripts (genes) tested."

R3MiC11 – Author's reply: In the revised manuscript we now clarify our rationale for the selected threshold and the number of transcripts. We used only one transcript per gene, i.e. the canonical transcript, so we tested 19,467 gene-transcripts. We used the Cauchy combination test to combine p-values across the 24 gene-burden exposures, yielding one p-value per gene. The exome-wide significance threshold of 1.04×10^{-7} was calculated as p=0.05 divided by number of genes tested (assumed 20,000) and 24 gene-burden exposures.

This is a strict and robust threshold that optimally controls for type I error, as (1) we are correcting for the 24 gene-burden exposures with both the Bonferroni correction and the Cauchy approach and (2) the actual number of tested genes is 19,467, so just shy of the 20,000 theoretical genes.

The robustness of our approach is strongly validated by the results of the All of Us analysis and also the consistency of associations across multiple sensitivity analyses and the biological enrichment demonstrated in pathway analyses and other evaluations of the identified gene sets.

R3MiC11 – Manuscript changes: We have clarified the multiple testing correction in the **Methods** section (Lines 319-321 at Page 8 of the Methods section, which is Page 122 of the combined PDF file):

Reviewer 3 Minor Comment 12 (R3MiC12): "The coloring of bar plot is a bit difficult to distinguish. Please consider different color scheme."

R3MiC12 – Author's reply: Thank you for this suggestion. We have revised the color scheme.

Reviewer 3 Minor Comment 13 (R3MiC13): "Line 108-109: Supplementary Note S1 is somewhat disjointed from context."

R3MiC13 – Author's reply: We agree and have incorporated the discussion of the *AC126283.2* and *PLA2G12A* sequence overlap in a separate note where we discuss the rare variant conditional analysis and linkage disequilibrium (as discussed in **R3MiC3** above).

Reviewer #4 – co-reviewed with Reviewer #5

Reviewer 4 Opening Statement (R4OS): "The authors present a large multi-ancestry exome-wide association study of TG/HDL ratio. Rare variant burden tests identify 60 associated genes, whiel common variant association analyses yield 1,617 loci. The study then focuses on biological characterization of selected genes in the FNIP/FLCN gene family and HPN in hepatocytes, finding experimental evidence of their functional roles. The manuscript is novel, well-structured, and has a strong narrative from genetic discovery through fine-mapping, functional follow-up, biological characterization, and experimental validation. However, several methodological and interpretational issues require clarification and sensitivity before the paper can be recommended for publication."

R4OS – Author's reply: We thank the Reviewer for their thorough evaluation of our work, for recognizing the strengths of the study narrative and experimental validation, and for the valuable suggestions which have helped us improve the manuscript.

We provide a point-by-point response to each comment below.

Major Comments

Reviewer 4 Major Comment 1 (R4MC1): "The meta-analyses include individuals from 5 distinct genetic ancestries, yet the manuscript does not address how ancestry influences the associations. Results appear to be based on pooled analyses across all ancestries. Please describe whether signals are driven primarily by European ancestry (the largest subgroups) or whether they generalize across other ancestries. Also clarify how ancestry was accounted for in cohort-specific analyses."

R4MC1 – Author's reply: We completely agree with the Reviewer about the importance of this and in the revised manuscript we have performed a series of in-depth analyses across ancestry groups, both for epidemiologic and genetic associations, as also noted in reply to **R3MC5**.

For the epidemiology analyses, we have generated and included in the new manuscript association plots and statistics within each ancestry (**Supplementary Figures S2-S6**). TG-HDL ratio was robustly associated with higher cardiometabolic disease risk and with its related anthropometric and biomarker traits across all ancestries.

In the revised manuscript, we also report ancestry-specific genetic association results for both common and rare variants. For the common variant GWAS, we now report ancestry-specific fine-mapping signals (**Supplementary Table S4**). We further show that our rare variant associations are not affected, whether we adjust for the 1,617 fine-mapped signals of our main analysis or for

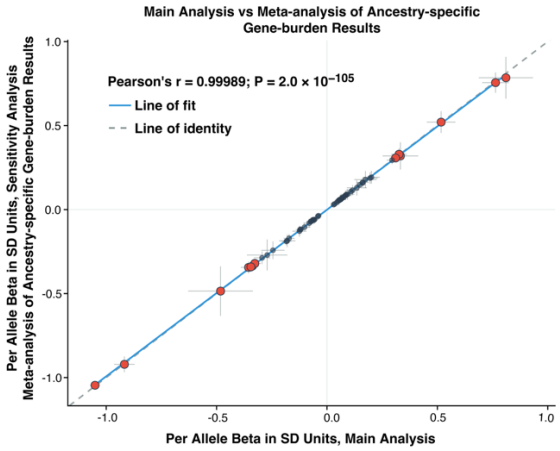
the ones identified in the ancestry-specific analysis (correlation r>0.99, $p=1.3\times10^{-105}$; **Supplementary Figure S15a**).

For the exome-wide analysis of rare coding mutations, we now report ancestry-specific association estimates across each ancestry and pooled across non-European ancestries, showing that associations are directionally consistent across ancestries. Specifically, 58 of 59 genes (98%) had the same direction of association in European vs pooled non-European ancestry estimates (**Supplementary Table S9**). The only exception was the *A1CF* gene, where there was a strong association in European analyses ($p=5.5\times10^{-27}$), but no association in the non-European subsets (p=0.97), which we demonstrate is driven by a gain-of-function variant (p.Gly390Ser; experimentally validated in *Nat Genet. 2017 Dec;49(12):1758-1766*) that is specifically enriched in the European populations (**Supplementary Note S2**).

We also demonstrate the value of the multi-ancestry analysis in detecting certain rare variant associations. In particular, the higher allele frequency of TG-HDL associated mutations in non-European ancestries was instrumental to detect several exome-wide significant associations (e.g. *LIPE*, *PCSK9* and *GALNT2*). This is discussed in the **Results** section of the revised manuscript (Lines 116-119) and **Supplementary Note S2**.

In the **Methods** section of the revised manuscript, we also provide a detailed explanation of how ancestry was accounted for in the combined genetic analyses across ancestries (Lines 251-253 at page 6 of the Methods, which is Page 120 of the combined PDF file). We also show that our results were robust (correlation r>0.99; $p=2.0\times10^{-105}$; see **Inset Fig. 14 and Supplementary Fig. S15c**) to an alternative approach of accounting for ancestry, i.e. performing gene-burden analyses within each cohort and each ancestry and then meta-analyzing results.

Inset Fig. 14. Correlation of association estimates in the main analysis compared to a meta-analysis of ancestry-specific gene-burden results.



R4MC1 – Manuscript changes: We have now added ancestry-specific epidemiologic analyses to **Supplementary Fig. S2-S6** and discussed in the **Results** section (Lines 86-88). We have now added ancestry-specific GWAS Manhattan and QQ plots to **Supplementary Fig. S8-S12** and the ancestry-specific fine-mapped common variants are now reported in **Supplementary Table S4** and discussed in **Results** section (Lines 96-99) and **Supplementary Note S1**. Sensitivity analyses using ancestry-specific fine-mapped loci for adjustment or using ancestry-specific gene burden estimates in a meta-analysis are in **Supplementary Figure S15** and **Supplementary Note S2**.

Reviewer 4 Major Comment 2 (R4MC2): "The TG/HDL ratio is mathematically dependent on two correlated traits, which may introduce collider bias and obscure signals specific to TG or HDL. To increase confidence in the findings, please examine the behavior of the identified variatns and genes with respective to TG and HDL individually, clarify whether associations are driven by TG, HDL, or both, and discuss the potential role of covariates that may differentially influence TG and HDL."

R4MC2 – Author's reply: This is an excellent methodological point and in the revised manuscript we report all associations with TG and HDL individually and also TG-adjusted for HDL and HDL-adjusted for TG as requested by Reviewer 3 in a similar comment (see **R3MC6** and corresponding reply above and also **Supplementary Table S17 and Note S2** of the revised manuscript).

It is important to note that the intended goal of our analysis was deliberately that of identifying genes affecting **both** TG and HDL, particularly in opposite directions, as that pattern is robustly associated with cardiometabolic disease risk and energy-metabolism biology, as evidenced by the abundant literature on the TG-HDL ratio (*Nat Genet. 2024;56:212-221*; *Nat Commun. 2024;15:8068*).

With this in mind, in the absence of a major influence of collider bias, we expected that for TG-HDL ratio we should observe most genes having a "balanced" association with TG and HDL in opposite direction and that fewer genes should be "trait-dominant" (i.e. associated more strongly with one of the two traits). We also expected that even "trait-dominant" genes would be associated with the ratio measure. We also expected that all genes should show some association with either TG or HDL and in most cases this should be in opposite direction.

To assess if these assumptions were correct and to empirically evaluate the presence of bias, we defined "effect-size balance" as: absolute(beta-TG) / ((absolute(beta-TG) + absolute(beta-HDL)), where betas are in the same standard deviation unit scale. Then, we classified genes where balance was ≥ 0.75 as "TG-dominant", genes where balance was ≤ 0.25 as "HDL-dominant" and genes where balance was < 0.75 and > 0.25 as "TG-HDL balanced" genes.

As expected, most genes (39 genes, 66%) were TG-HDL balanced, while 7 (12%) genes were HDL-dominant and 13 (22%) genes were TG-dominant, reflecting the intended performance of the discovery approach. All genes showed an association with either TG or HDL alone, consistent with genuine associations with lipid traits (and not purely introduced by a collider). Even within TG- and HDL-dominant genes, most genes had opposite direction of effect for TG and HDL (**Supplementary Table S17**). Overall, across the 59 genes, only 3 genes showed statistically significant associations with both traits but in the same direction (*ANGPTL3*, *LIPG*, *ABCA1*; **Supplementary Table S17**). These 3 genes are well described regulators of lipid metabolism, whereby *ANGPTL3* and *LIPG* are known to biologically interact in both VLDL- and HDL-lipoprotein particle metabolism (*Arterioscler Thromb Vasc Biol. 2007;27:366-72; J Lipid Res. 2015;56:1308-17*; *J Lipid Res. 2020;61:1271-1286*), while *ABCA1* mediates reverse cholesterol transport in the formation of nascent HDL particles (*J Lipid Res. 2001;42:1717-26*; *J Clin Invest. 1999;104:R25-31*).

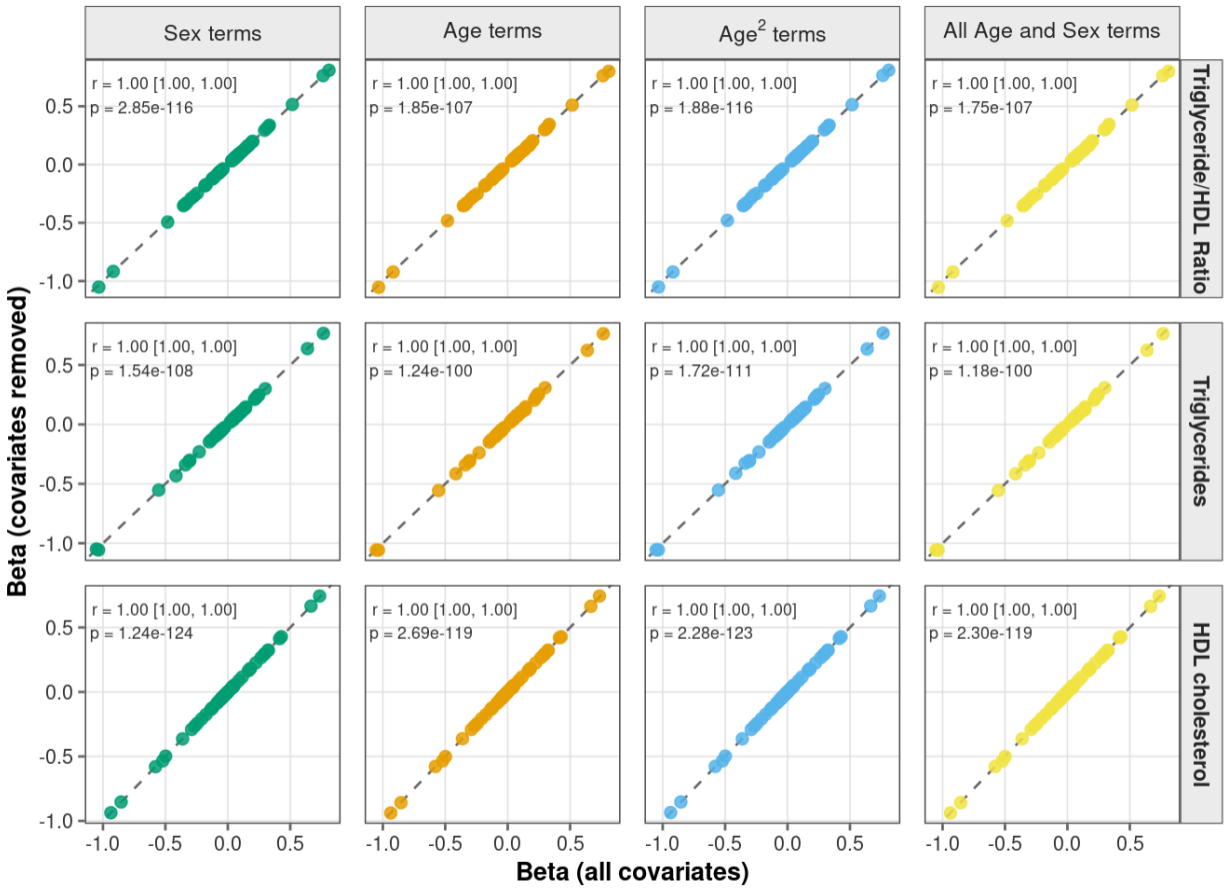
These results support the validity of our discovery approach, and we think that these observed patterns of trait-balance reflect relevant biology and different biological groupings (as also suggested by Reviewer 3 in **R3MC6-R3MC7** above), rather than bias.

In addition, to address the Reviewer's comment on covariates, we performed associations with TG-HDL ratio, HDL and TG before and after removing one covariate at a time and also all non-genetic covariates for the 59 genes and found that the associations were highly correlated across all comparisons (r>0.99 for each analysis; **Inset Fig. 15**).

In summary, both theoretical reasoning and empirical analysis of our gene-level results support the validity of the TG-HDL ratio as discovery trait.

R4MC2 – Manuscript changes: as requested by the Reviewer, we now discuss and report associations with TG and HDL separately for the identified genes (**Supplementary Table S17**). We also discuss covariate adjustment in the **Methods** section (see "*Genetic association analyses*" paragraph on Page 6 of the Methods, which is Page 120 of the combined PDF file).

Inset Fig. 15. Assessing covariate influence on TG, HDL, and TG-HDL ratio associations across the 59 independent genes identified in the exome-wide analysis. Data on the x-axis is based on the full model which adjusts for Sex, Age, Age^2, Age × Sex, Age^2 × Sex. The column strip names represent which covariate terms are removed from the model used for the y-axis beta. Age terms: removes Age, Age², Age×Sex, and Age²×Sex (retains Sex only), Age² terms: removes Age² and Age²×Sex (retains Sex, Age, Age×Sex), Sex terms: removes Sex, Age×Sex, and Age²×Sex (retains Age and Age²), All Age & Sex terms: removes Sex, Age, Age², Age^2×Sex, and Age²×Sex.



Column strip names show covariates removed from the model.

Reviewer 4 Major Comment 3 (R4MC3): "Please clarify whether TG and HDL were measured in fasting or non-fasting state across cohorts. Given that TG levels are more variable than HDL, it is important to discuss whether TG/HDL ratio can be considered a stable trait across these states."

R4MC3 – Author's reply: This is an excellent point. In our study, TG and HDL were measured either in large health-system or epidemiological cohorts. Information on fasting state was collected systematically only in the UK Biobank (UKB) subset (N= 409,602; ~40% of our discovery analysis).

In UKB, 104,276 (25%) samples were collected in fed state (i.e. <2 hours since last meal), 46,550 (11%) samples were collected in fasted state (i.e. >6 hours since last meal) and the remaining samples were collected between 2-6 hours since last meal.

To estimate whether fasting state influenced our findings, we estimated associations in the fasted vs fed state subsets of UKB and tested for interaction. In this analysis, none of the 1,617 common fine-mapped variants or the 59 gene-burden signals met a Bonferroni correction for interaction ($p_{interaction}>3.1\times10^{-5}$ for all common variants and $p_{interaction}>8.5\times10^{-4}$ for all gene burden signals), consistent with the absence of strong interactions with fasting state.

R4MC3 – Manuscript changes: Interaction analyses by fasting state are in **Supplementary Tables S13-S14** of the revised manuscript and are discussed in the **Results** section (Lines 113-116) and **Supplementary Note S2**.

Reviewer 4 Major Comment 4 (R4MC4): "TG/HDL ratio is strongly related to fat distribution, which often differs by sex. Stratified analyses by sex would be relevant to understand whether associations are consistent across men and women."

R4MC4 – Author's reply: This is a fantastic suggestion. In the revised manuscript, we performed a sex-stratified analysis as suggested by the Reviewer.

While results were generally very consistent in men and women (effect size correlation between men and women across the 59 gene-burden associations, r=0.98, $p=2.3\times10^{-41}$; N_{women}=594,942, N_{men}=437,174; **Supplementary Table S11 and Fig. S16**), we identified an interesting interaction for *PDE3B*, where rare mutations showed stronger association with lower TG-HDL ratio in women vs men (I^2=93%; $p_{interaction}=1.8\times10^{-4}$; $beta_{women}$, −0.40 vs $beta_{men}$, −0.28 SD units per allele). *PDE3B* is a known fat distribution gene showing stronger association with waist-to-hip ratio in women vs men (*Nat Commun 2022;13:4844, doi: 10.1038/s41467-022-32398-7*).

R4MC4 – Manuscript changes: We now report the sex-specific analyses in **Supplementary Table S11 and Note S2** and emphasize the *PDE3B* interaction in the **Results** section (Lines 119-122).

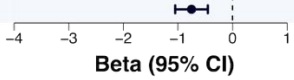
Reviewer 4 Major Comment 5 (R4MC5): "To complement the biological interpretation, consider linking exome-wide signals to cis-pQTLs to clarify whether the genetic burden may act through protein expression changes relevant to lipid metabolism."

R4MC5 – Author's reply and manuscript changes: This is an excellent suggestion, and we performed an analysis using 60,012 participants from the UK Biobank (N=50,437) and the Geisinger Health System cohort (N=9,575) where proteomics was measured using Olink.

First, we studied the impact on circulating protein levels for pLOF mutations in 13 of the 59 independent genes identified in this paper, where we had available circulating protein measurements and the encoded protein was annotated as secreted. For all 13 genes, pLOF variants were strongly associated with lower levels of the encoded protein, consistent with a loss-of-function effect (**Inset Fig. 16 and Supplementary Fig. S18**).

Inset Fig. 16. Association of predicted loss-of-function variants in 13 genes with the respective protein levels. Out of the 59 proteins encoded by top TG-HDL ratio genes, 13 were annotated as "secreted" in Human Protein Atlas and were measured by Olink proteomics in UK Biobank and the Geisinger Health System or by immunoassay (for APOB and APOA1) in the UK Biobank. Genes annotated as only "secreted" are plotted separately from those annotated as both "secreted and "intracellular". AAF, Alternative Allele Frequency; CI, Confidence Interval; pLOF, predicted loss-of-function; RR, Homozygous for the reference allele; RA, Heterozygous; AA, Homozygous for the alternative allele.

Genetic Exposure	Genotype Count RR / RA / AA	AAF	Beta (95% CI) per Allele in SD Units	P–Value
Secreted protein				
GAS6 pLoF, AAF<0.1%	60,002 / 10 / 0	0.008%	−2.37 (−2.99, −1.75)	7.9e−14
ANGPTL3 pLoF, AAF<0.1%	59,860 / 152 / 0	0.13%	−2.19 (−2.35, −2.03)	3.4e−160
APOC1 pLoF, AAF<0.01%	60,001 / 11 / 0	0.009%	−2.15 (−2.74, −1.56)	1.1e−12
APOB pLoF, AAF<0.01%	445,760 / 744 / 0	0.083%	−2.12 (−2.18, −2.06)	2.2e−307
HGFAC pLoF, AAF<1%	59,744 / 265 / 3	0.23%	−2.00 (−2.12, −1.88)	5.4e−240
Secreted and intracellular protein				
APOA1 pLoF, AAF<0.01%	408,987 / 32 / 0	0.004%	−2.65 (−2.97, −2.33)	8.8e−60
LCAT pLoF, AAF<0.01%	59,999 / 13 / 0	0.011%	−2.53 (−3.08, −1.99)	7.3e−20
PCSK9 pLoF, AAF<1%	59,885 / 127 / 0	0.11%	−2.26 (−2.43, −2.08)	8.1e−141
PLTP pLoF, AAF<0.1%	59,981 / 31 / 0	0.026%	−2.09 (−2.45, −1.74)	2.2e−31
ANGPTL4 pLoF, AAF<0.1%	59,911 / 101 / 0	0.084%	−1.88 (−2.08, −1.69)	1.1e−79
ASGR1 pLoF, AAF<0.01%	59,979 / 33 / 0	0.027%	−1.77 (−2.11, −1.43)	2.7e−24
LPL pLoF, AAF<0.1%	50,423 / 14 / 0	0.014%	−1.70 (−2.23, −1.18)	2.0e−10
PKD1 pLoF, AAF<0.01%	59,972 / 39 / 1	0.034%	−0.75 (−1.05, −0.45)	8.5e−7



We also performed a common variant cis-pQTL analysis at the 59 rare variant loci and co-localization analyses with coloc, identifying 25 co-localizing signals between pQTLs and common variant signals for TG-HDL ratio, 14 of which converged on the gene identified in the exome-wide analysis (**Supplementary Table S15**).

These additional analyses complement the interpretation of the association results with TG-HDL ratio as suggested by the Reviewer.

R4MC5 – Manuscript changes: Association of predicted loss-of-function variants in 13 genes with the respective protein levels are now in **Supplementary Fig. S18** and colocalization of

common variants signals at the 59 rare variant loci with cis-pQTLs are in **Supplementary Table S15**.

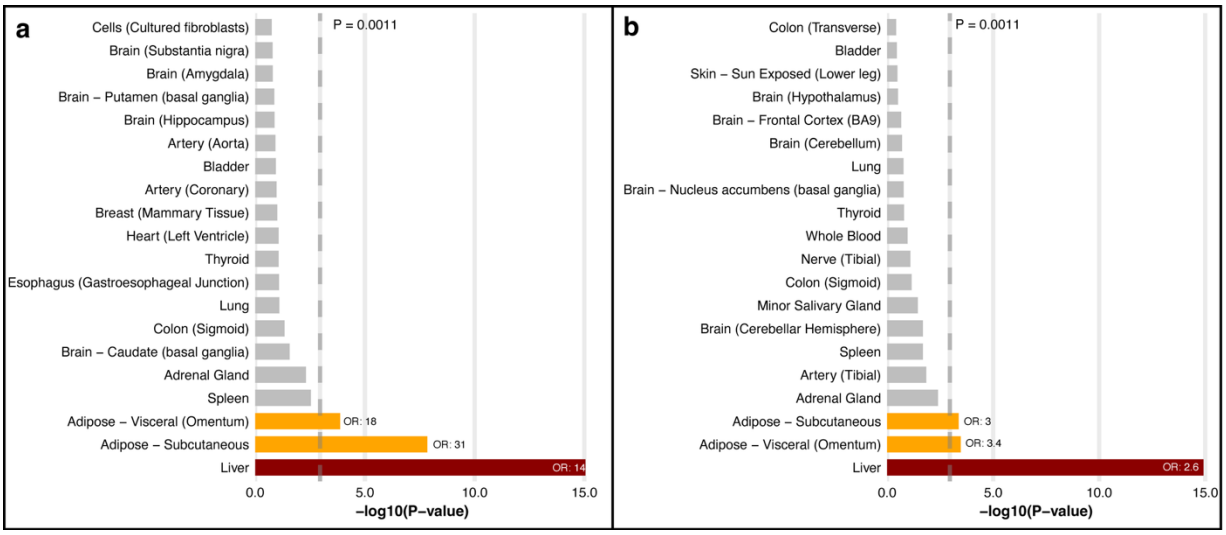
Reviewer 4 Major Comment 6 (R4MC6): "The burden-test results suggest enrichment in liver, whereas previous UK Biobank TG/HDL GWAS based on common variants reported predominant enrichment in adipose tissue. To clarify these differences, consider performing tissue-enrichment analyses on the 1,617 independent common variants as well."

R4MC6 – Author's reply and manuscript changes: this is a valid point. It is important to note that in our exome analysis *there was* enrichment for adipose-expressed genes (odds ratio [OR] for enrichment, 13.6, $p_{enrichment}=9\times10^{-16}$ for liver; OR of 31.2 and 18.1, $p=1\times10^{-8}$ and $p=1\times10^{-4}$ for subcutaneous and visceral adipose tissue, respectively; **Inset Fig. 17**).

In the revised paper, we have now calculated the enrichment for the common variant analysis and identified enrichment for both adipose and liver tissues with a pattern that closely mirrors that of the gene-burden analysis (**Inset Fig. 17**).

R4MC6 – Manuscript changes: We now report these results in **Supplementary Fig. S13** (common variant tissue enrichment) and **Figure 3c** (rare coding variant tissue enrichment). We also reference these analyses in the **Results** section (Lines 99-100; 137-138).

Inset Fig. 17. Tissue enrichment in the rare variant (Panel a) and common variant (Panel b) analyses for TG-HDL ratio.



Minor Comments

Reviewer 4 Minor Comment 1 (R4MiC1): "Consider reporting cell type-specific expression levels for FNIP/FLCN family and HPN in human and mouse liver."

R4MiC1 – Author's reply and manuscript changes: This has been added to the revised manuscript (**Supplementary Fig. S26 and S34**).

Reviewer 4 Minor Comment 2 (R4MiC2): "Lines 105-107: Clarify the basis for the exome-wide statistical significance threshold applied."

R4MiC2 – Author's reply and manuscript changes: This was a Bonferroni correction for 24 gene-burden exposures per gene and 20,000 theoretical genes and is in line with previous literature (*N Engl J Med. 2022;387:332-344; Science. 2021;373:eabf8683*). We now clearly state that in the **Methods** section of the revised manuscript (Lines 319-321 at Page 8 of the Methods section which is Page 122 of the combined PDF file).

Reviewer 4 Minor Comment 3 (R4MiC3): "Lines 189-190: The claim that 'genes associated with favorable metabolic state are enriched in liver' seems unsubstantiated. Please provide supporting evidence or revise."

R4MiC3 – Author's reply and manuscript changes: we agree and have removed the sentence.

Reviewer 4 Minor Comment 4 (R4MiC4): "Methods: Clarify whether fine-mapping was multi-ancestry framework or stratified by ancestry, and specify the LD reference panels used."

R4MiC4 – Author's reply and manuscript changes: As now clearly explained in the **Methods** section of the revised manuscript (see *"Genetic association analyses"* and "*Common variant association analysis and fine-mapping"* paragraphs at Pages 6-7 of the Methods, which are Pages 120-121 of the combined PDF file), we used a multi-ancestry framework as main analysis, but we now also report the results of fine-mapping within each ancestry in the **Results** section (Lines 96-99), **Supplementary Table S4 and Supplementary Note S1**. For both these analyses, we used LD from the exact set of individuals included in each association analysis. Briefly, we have access to the individual-level data for each cohort, so we used a sample-size-weighted pooled estimate of the covariance matrix as the LD input for fine-mapping. This not only ensures that we used the exact set of individuals included in the meta-analyses, but also that our generated LD matrix captures the exact ancestral composition present in our meta-analyses, thereby avoiding biases typically introduced by using external or mismatched reference panels.

Reviewer 4 Minor Comment 5 (R4MiC5): "Methods: Specify the co-localization method applied and the LD reference panels used."

R4MiC5 – Author's reply and manuscript changes: we used coloc (version 5.2.3) in R and this is now noted in the **Methods** section (Lines 391-394 at Page 10 of the Methods, which is Page 124 of the combined PDF file).

Reviewer 4 Minor Comment 6 (R4MiC6): "Extended Figure 3: Clarify the burden test methodology and the variants included."

R4MiC6 – Author's reply and manuscript changes: For each gene we took the best gene-burden exposure for TG-HDL ratio (as listed in Table 1 for each gene) and then estimated associations with each trait for the same exposure. This is now clarified in the legend of **Supplementary Fig. S19** (former **Extended Data Fig. 3**).

Reviewer 4 Minor Comment 7 (R4MiC7): "Supplementary Table 3: Add P values, sample sizes, and posterior inclusion probabilities."

R4MiC7 – Author's reply and manuscript changes: we added the requested statistics in **Supplementary Table S3**.

Reviewer 4 Minor Comment 8 (R4MiC8): "Supplementary Table 6: Add effect sizes and P values."

R4MiC8 – Author's reply and manuscript changes: we added the requested statistics in **Supplementary Table S20** (former **Supplementary Table S6)**.
