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Decreased Circulating Very Small Low-Density Lipoprotein is Likely Causal for Age-Related Macular Degeneration

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Objective: Abnormal changes in metabolite levels in serum or plasma have been highlighted in several studies in age-related macular degeneration (AMD), the leading cause of irreversible vision loss. Specific changes in lipid profiles are associated with an increased risk of AMD. Metabolites could thus be used to investigate AMD disease mechanisms or incorporated into AMD risk prediction models. However, whether particular metabolites causally affect the disease has yet to be established.

Design: A 3-tiered analysis of blood metabolites in the United Kingdom (UK) Biobank cohort to identify metabolites that differ in AMD patients with evidence for a putatively causal role in AMD.

Participants: A total of 72 376 donors from the UK Biobank cohort including participants with AMD (N = 1353) and non-AMD controls (N = 71 023).

Methods: We analyzed 325 directly measured or derived blood metabolites from the UK Biobank for 72 376 donors to identify AMD-associated metabolites. Genome-wide association studies for 325 metabolites in 98 316 European participants from the UK Biobank were performed. The causal effects of these metabolites in AMD were tested using a 2-sample Mendelian randomization approach. The predictive value of these measurements together with sex and age was assessed by developing a machine learning classifier.

Main Outcome Measures: Evaluating metabolic biomarkers associated with AMD susceptibility and investigating their potential causal contribution to the development of the disease.

Results: This study noted age to be the prominent risk factor associated with AMD development. While accounting for age and sex, we identified 84 metabolic markers as significantly (false discovery rate-adjusted *P* value < 0.05) associated with AMD. Lipoprotein subclasses comprised the majority of the AMD-associated metabolites (39%) followed by several lipoprotein to lipid ratios. Nineteen metabolites showed a likely causative role in AMD etiology. Of these, 6 lipoproteins contain very small, very low-density lipoprotein (VLDL), and phospholipids to total lipid ratio in medium VLDL. Based on this we postulate that depletion of circulating very small VLDLs is likely causal for AMD. The risk prediction model constructed from the metabolites, age and sex, identified age as the primary predictive factor with a much smaller contribution by metabolites to AMD risk prediction.

Conclusions: This study underscores the pronounced role of lipids in AMD susceptibility and the likely causal contribution of particular subclasses of lipoproteins to AMD. Our study provides valuable insights into the metabopathological mechanisms of AMD disease development and progression. *Ophthalmology Science 2024;4:100535 Crown Copyright © 2024 Published by Elsevier Inc. on behalf of the American Academy of Ophthalmology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).*

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Age-related macular degeneration (AMD) affects central vision and is the leading cause of irreversible vision loss in individuals >50 years of age in developed countries, accounting for one-half of all legal blindness.[1]

Drusen, the hallmark histopathological feature of AMD, are lipid-rich deposits present in early-stage disease, and accumulate in size and number as the disease progresses. Advanced AMD is characterized by geographic atrophy and macular neovascularization.[1] Drusen deposits predominantly comprise lipids and lipoproteins, prompting researchers to study alterations in these molecules in AMD patients' blood. In aged eyes, lipid deposits also accumulate within Bruch's membrane, which serves as a diffusion barrier between the outer retina and the blood circulation.[2–4] Changes in serum or plasma lipid levels have been identified in several studies, suggesting that dysregulated metabolites may represent important risk factors for AMD.[5,6] In addition, polymorphisms in genes involved in lipid

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homeostasis, including hepatic lipase (LIPC), hepatic cholesterol ester transfer protein (CETP), and apolipoprotein E (ApoE), have been identified by genome-wide association studies (GWAS).[7–9] Interestingly, LIPC, ApoE, and CETP are expressed in the retinal tissue and are involved in lipid transport.[8,10]

Notably, RNA-seq findings indicate distinct expression patterns of ApoE in Müller glia, LIPC in the retinal pigment epithelium layer, and localization of CETP to photoreceptor outer segments and the choriocapillaris.[11–13] This is insightful; however, given the fact that these genes (particularly ApoE) are known to be expressed in multiple tissues, it is unknown whether these lipid-related genes impose a systemic role and/or an intraocular concentrated mechanism of action when considering the pathogenesis of AMD.

Different metabolite profiles of early and intermediate AMD versus late stages of AMD reflect dysregulation of metabolic pathways, particularly those related to lipid metabolism, which provide additional evidence of differences in genetic associations of lipid-related genes and metabolites along the severity spectrum.[7,14,15] This suggests a systemic contribution from metabolites, particularly lipids, to the onset and progression of AMD, and highlights the need to better understand the many potential roles of these metabolites in AMD pathogenesis.[16]

Previous studies showed increased levels of large and extra-large high-density lipoprotein (HDL) subclasses in blood plasma or serum in AMD.[6] Decreased levels of very low-density lipoprotein (VLDL), amino acids, and citrate were also significantly associated with AMD.[6] Lower AMD incidence among subjects on lipid-lowering drugs has been reported as well as some beneficial effects in clinical trials.[17,18] Work in animal models further suggests the importance of metabolic processes in the etiology of AMD.[19] Despite these valuable discoveries, the benefits of elucidating the complex biological role of metabolites in AMD and the translation of these findings in the AMD biological mechanisms area have yet to be realized. Understanding the complex interplay between genetic and environmental factors to discover the mechanisms of action of metabolites in AMD development is crucial to treatment development.[20–22] Circulating metabolites are easily measured biomarkers of disease risk and progression and expand our knowledge to fully investigate the complex metabolic differences in AMD patients.[23,24]

While observational studies cannot avoid unmeasured and residual confounding factors from interventions, Mendelian randomization (MR) is a well-established method that uses genetic variants as instrumental variables to establish causality. This approach has already pinpointed apolipoprotein A1, apolipoprotein B, total cholesterol, and HDL cholesterol to play a role in the etiology of AMD.[25] More recently, studies have tested a larger number of metabolites adding valuable insights into our knowledge of metabolites' role in AMD risk. Particularly lipid- and lipoprotein-related metabolites were shown to be associated with AMD.[14,26] Of note, MR analyses reinforced the findings of previous observational studies in AMD[25–29] thus emphasizing the pivotal role of this statistical approach.

The fast-growing availability of metabolite and genetic information in large databases such as the United Kingdom (UK) Biobank enables tests of metabolite association and disease causation with unprecedented statistical power. Therefore, creating a more comprehensive panel of genetic instruments for metabolites via high-powered GWAS will enhance our statistical power to identify new causal relationships between metabolites and AMD. Additionally, due to the large variety of metabolites and complex relationships between their subcomponents, specific research using integrative approaches is required to elucidate not only risk associations, but also their value as the putative causal role of lipid biomarkers in AMD etiology. In addition, current prediction models for AMD are based on restricted statistical models tested with a limited lipid profile.[30] This restricts us from expanding our knowledge to fully investigate the complex metabolic differences in AMD patients. Using a machine-learning approach on hundreds of metabolites allows us to assess the value of additional metabolites in patients' prognosis.

The UK Biobank cohort recently released nuclear magnetic resonance (NMR)-based metabolite data for 249 circulating metabolites, lipids, and lipoprotein subfractions for approximately 119 000 participants (first release version, March 2021). In addition, Ritchie et al derived 76 ratios of metabolites, making a total of 325 metabolites, or their ratios, available for analysis.[31] This is a valuable dataset for metabolomic clinical studies making it possible to provide in detail insights in biomarker detection in large-scale investigations for a large number of metabolites. Of note, the study of metabolite derivatives and ratios provided invaluable insights into relationships that exist between individual metabolites, enzymatic processes, and clinically useful biomarkers.[32,33] Investigating the clinical lipids measured by NMR platform in this study in addition to investigating derivatives and metabolite ratios may shed light on dynamic changes in metabolite levels in AMD and help target clinically relevant metabolites.

In this study, we addressed 3 specific research questions using metabolites and genetic data from the UK Biobank as follows (depicted in Fig 1):

(1) Which metabolites differ in abundance in AMD patient plasma compared with controls?

(2) Which metabolites may have a causal role in AMD?

(3) Which metabolites have the greatest predictive value for classifying AMD versus control subjects?

In this work, firstly we identify the metabolites that are differentially abundant in AMD patients compared with controls. Secondly, we use the MR approach to identify the causal effects of specific lipid fractions which present avenues for future in vitro validation work. Lastly, we explored the value of these metabolites in AMD risk prediction.

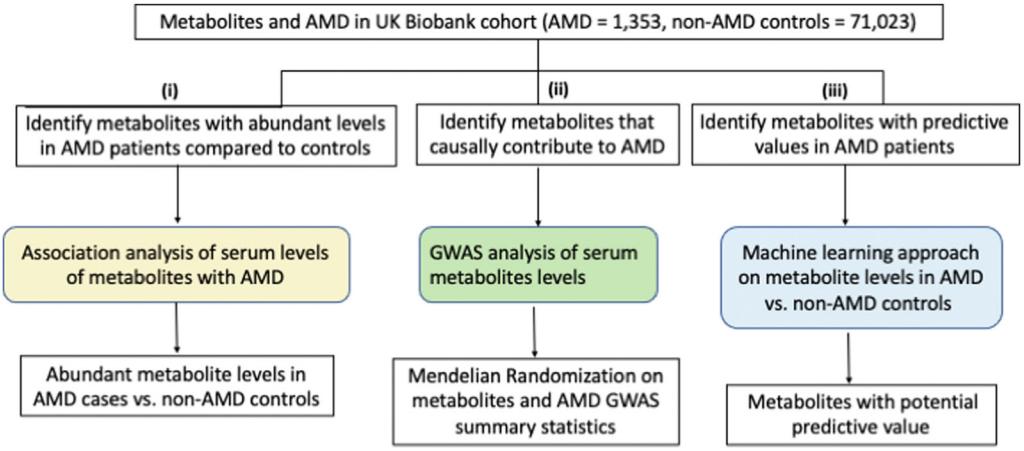


Figure 1. Study design schematic. Colored boxes in yellow, blue, and green present our approach to answering questions (1), (2), and (3) (see text), respectively. AMD = age-related macular degeneration; GWAS = genome-wide association studies; UK = United Kingdom.

Methods

Ethics

This study used data from the UK Biobank cohort under project number 36610. The study was approved by the Walter and Eliza Hall Institute of Medical Research, Human Research Ethics Committee (HREC project number 17/09LR). Ethics approval was obtained by the Northwest Multi-centre Research Ethics Committee and our research adhered to the tenets of the Declaration of Helsinki (UK Biobank research ethics approval). Informed consent was obtained from all study participants and all participants were free to withdraw from the study at any time.

Study Population

The genetic, metabolomic, and demographic data of the UK Biobank cohort were accessed through application number 36610. Details of the UK Biobank (https://www.ukbiobank.ac.uk/) study design and population have been described previously.[34] The UK Biobank study used a high-throughput NMR metabolomics platform[35] to undertake metabolomic profiling in baseline plasma samples from a randomly selected subset of approximately 121 000 participants.[31] The single nucleotide polymorphism (SNP) genotypes of the subset of participants from the UK Biobank cohort with metabolic data were also downloaded. All individuals who had withdrawn from the study as of January 2022 were excluded from the cohort.

Assessment of AMD Status and Non-AMD Controls: Phenotype Definition

For the ~121 000 individuals with metabolomic and genetic data available, AMD status was ascertained through International Classification of Diseases 10th Revision codes for AMD diagnoses (H353) recorded in primary care and hospital admission data.[36] To ensure high-quality clinical diagnostic information for AMD individuals in this study, we excluded individuals with self-reported AMD only (code 1528 in data field 20002, or code 5 in data field 6148), and no clinically recorded disease. For non-AMD controls, we included those participants with neither report of AMD diagnoses (H353) in primary care and hospital admissions data, nor self-report of AMD diagnosis. To limit the potential confounding effect of ancestry differences on metabolite levels, we only included participants with European ancestry. For individuals with >1 visit to a recruitment center, only the first visit (code visit "0") measurements were included in this study (119 020 participants). The samples that were flagged, either due to technical issues or specific sample preparation methods, in the UK Biobank data were excluded. These were samples flagged with high lactate (data field 23652), high pyruvate (data field 23653), low glucose (data field 23654), or low protein (data field 23655). In total, 72 376 individuals including participants with AMD (N = 1353) and non-AMD controls (N = 71 023) remained for further analysis.

Metabolic Biomarker Quantification

Metabolite data acquisition took place between June 2019 and April 2020 (from nonfasting venous blood). This simultaneously quantified 249 metabolic biomarkers (168 directly measured and 81 ratios of these), including lipids, fatty acids, amino acids, ketone bodies, and other low-molecular-weight metabolic biomarkers (e.g. gluconeogenesis-related metabolites), as well as lipoprotein subclass distribution, particle size, and composition.

The UK Biobank metabolic data underwent quality control procedures using the ukbnmr R package (version 1.5) developed by Ritchie et al[31] to remove the effects of technical variation on biomarker concentrations. Metabolite levels were square root transformed to achieve symmetry of the distribution. This transformation was preferred over the log transformation as this was observed to highly skew the distribution of some metabolites. Assuming missingness at random, metabolic values were imputed using the Multiple Imputation Chain Equation by the R-package MICE (version 3.15.0).[37] We imputed 5 different versions of the dataset and took the average of the 5 imputed values for each missing metabolite. Composite metabolites and ratios were then recomputed using ukbnmr (version 1.5). A total of 325 metabolites were included in this study (listed in Table S1). All metabolites were then mean centered and divided by their standard deviation.

Quality Control of Genotype Data

The SNP-genotyping data of the UK Biobank cohort was obtained and underwent quality control and imputation as described previously.[38] Individuals with high heterozygosity or high levels of missingness were excluded prior to imputation. We further excluded individuals who had withdrawn consent (as of January 2022), samples where the self-reported sex did not match the

genetically inferred sex, samples with putative sex chromosome aneuploidy, and samples with an apparent excess of relatives in the cohort (>10 relatives). To decrease the effect of ancestry differences in metabolite levels we only included participants with European ancestry (based upon genetic principal components). The principal components were calculated using the King software version 2.3.0 (released on October 10, 2022).[39] Samples were then restricted to the subset inferred by King to have European ancestry. Variants were filtered to include those with high imputation quality (INFO scores ≥0.7).

Metabolite Associations Analysis

We analyzed metabolite data derived from blood samples of 72 376 participants including AMD (N = 1353) and non-AMD controls (N = 71 023) that had complete metabolite measurements, age at the time of blood sample collection, and sex data (no missing information). To identify differentially abundant metabolites in patients compared with non-AMD controls we conducted linear regression analyses, with age and sex as covariates (model employed: metabolite ~ AMD status + age + sex). The R software (version 4.1.3) was used for the regression analysis. The coefficients resulting from this model represent the estimated change in the metabolite levels for AMD patients versus non-AMD controls while accounting for age and sex.

UK Biobank Metabolomics GWAS

Genotype and metabolite data were available for 119 020 unique samples in the UK Biobank. After imputation and quality control of the genotype data, 98 316 European participants (first visit) were available for association analyses. The GWAS of 325 metabolites were undertaken using the Regenie tool (v.1.0.6.9)[40] and included common variants with minor allele frequency >0.01. Age, sex, 7 principal components, and genotyping batch were included as covariates in the association analyses.

AMD GWAS

We used the GWAS summary statistics data of a recent AMD GWAS via personal communication with associate professor Stuart Macgregor, QIMR, Brisbane, Australia. The GWAS-AMD included 12 711 advanced AMD cases and 5336 intermediate AMD cases with 14 590 controls of European descent from the International AMD Genomics Consortium. Details of this study are described by Han et al.[25]

Genetic Instruments

To identify those metabolites that most likely cause AMD, 2-sample MR analyses were performed to systematically evaluate the causal relationships between 325 metabolites in 98 316 European participants, consisting of the metabolite groups described in Table S1. To determine the genetic instruments to include in MR analysis, we selected genome-wide significant variants ($P < 5 \times 10^{-8}$) associated with 325 metabolites. The independent SNPs taken from the GWAS results for each metabolite ($P < 5 \times 10^{-8}$), and AMD GWAS summary statistics data (no P value filtering) were used as genetic instruments to perform MR analyses (Table S2). To obtain independent SNPs, we set the SNP clumping window to 10 000 kb where SNPs in linkage disequilibrium within a cut-off of $r^2 < 0.001$ were pruned and the SNP with the lowest P value was retained. The SNP clumping step was performed using the "clump_data" function of the "TwoSampleMR" R package, version 0.5.6.[41] We excluded the metabolites that had ≤3 SNPs in common with AMD risk loci (only acetate was excluded). After harmonizing the risk alleles between the exposure and the outcome (using the "harmonise_data" function of the "TwoSampleMR" R package) we included only biallelic SNPs with minor allele frequency ≥0.01 and produced 24-75 genetic instruments (i.e. SNPs) that were selected for MR analysis of 325 plasma biomarkers in European participants of the UK Biobank cohort (Table S2).

MR

Two-sample MR[41] was used to test potential causal relationships between each metabolite (the exposure) and AMD (the outcome). To adequately explore causality, we conducted 3 MR methods with different statistical approaches: (1) MR Egger, which allows ≥1 genetic variants to have pleiotropic effects, as long as the size of these pleiotropic effects is independent of the size of the genetic variants' effects on the outcome;[42] (2) inverse variance weighted, which assumes no pleiotropy;[43] and (3) weighted median which allows for genetic pleiotropy.[42]

We carried out a number of sensitivity analyses to evaluate the validity of our genetic instruments, and to test for violations of the underlying assumptions of MR as described by Hemani et al[41] as follows: (1) the instruments must be associated with the exposure; (2) the instruments must influence the outcome only through the exposure; (3) the instruments must not associate with measured or unmeasured confounders.

To evaluate whether any of the MR estimates were highly influenced by the effect of a particular SNP, leave-one-out analyses were conducted by leaving each genetic variant out of the MR analysis in turn. Further, Cochran's Q statistic was performed to test the heterogeneity by using the function "mr_heterogeneity" of the TwoSampleMR package. In addition, we assessed whether the MR Egger regression analysis resulted in a nonzero intercept estimate; this would suggest either directional pleiotropy or a failure of the instrument strength independent of the direct effect assumption.

Unless otherwise specified throughout the manuscript, the Benjamini-Hochberg method was used to control the false discovery rate (FDR). Statistical significance was defined as FDR-controlled P value < 0.05, throughout this study.

Machine Learning Classification Model

To assess whether circulating NMR biomarkers could help the prediction of AMD risk, we used the extreme gradient boosting (XGBoost, R package version 1.6.0.1) decision tree classification algorithm.[44] The transformed and scaled metabolite dataset was partitioned into 75% for the training stage and 25% for the testing stage for each model, stratified for similar cases and control numbers. We observed that the performance of the XGBoost model was maximized when the case-control ratio was maintained at 1:1, indicating that an equal representation of AMD cases and non-AMD controls led to the most accurate and reliable predictions. Given that the case-control ratio was extremely imbalanced and to ensure the utilization of all available samples and enhance the accuracy and robustness of our predictive models, we adopted an approach to iterate the model on sets of independent non-AMD samples. By breaking down the dataset into 52 subsets, we created a series of models that encompassed a 1:1 ratio of AMD and independent non-AMD samples (each subset includes 1353 AMD and 1353 non-AMD samples). To improve the performance of the developed models by minimizing over-fitting, an iterative 10-fold cross-validation method was used. A grid search across multiple parameter spaces was used to optimize the hyperparameters.

These models were trained including age at blood sample collection and sex (baseline model) and with supplying metabolites as features. The bootstrapping method of the XGBoost tool selects 1 feature among all correlated feature sets. To assess the performance of models we calculated the mean value of the receiver operating characteristic-area under the curve resulting from each model.

Results

Cohort Demographics

Of the original 502 493 UK Biobank participants, NMR metabolite measurements were available for 98 316 European participants (19.6%). Of these, 1353 had AMD based on clinic/hospital records (International Classification of Diseases 10th Revision code: H353). A total of 71 023 individuals who had not reported AMD in primary care records were included in this study as non-AMD controls. The mean age at blood sample collection of participants in this subset of the UK Biobank was 57.8 years (the mean age for cases: 63.8 years and for controls: 57.7 years), and 46.3% (n = 33 494) were males.

Metabolite Associations with AMD

First, we examined the correlation between 325 metabolites in AMD patients (N = 1353) and non-AMD controls (N = 71 023), separately, to explore the strength and direction of relationships among these metabolites (Fig S2). Age showed a highly significant association with metabolite levels, demonstrating that metabolic dynamics and the aging process are intertwined (Fig S3A). As expected, age showed a strong association with AMD (Fig S3B). After adjustment for potential confounding factors (age, sex), 84 of the 325 metabolic biomarkers (including derivatives and metabolite ratios) showed statistically significant associations with AMD at the FDR-adjusted P value < 0.05 level (Table S2). These metabolites are illustrated in Figure 4 and the direction of the association with AMD is shown.

The significant metabolites were identified in 16 metabolic groups (Fig 4). Among these groups, a glycolysis-related metabolite (glucose b = 0.12, adj.P = 0.004) showed the strongest association with AMD, demonstrating a 0.12 increment of glucose in individuals with AMD in comparison to the control group (Fig S5A).

Very small VLDL lipoprotein (cholesteryl esters in very small VLDL, b = −0.11, adj.P = 0.004) showed the second strongest association with AMD with a 0.11 decrease in the level of cholesteryl esters in very small VLDLs in AMD cases compared with controls (Fig S5B). Two other members of lipoprotein subclasses (phospholipids in intermediate-density lipoprotein b = −0.1, adj.P = 0.003; cholesterol in very small VLDL b = −0.1, adj.P = 0.003) showed highly significant associations with AMD.

The lipoprotein subclasses comprised the majority of AMD-associated metabolites (33 of 84). Among these, medium VLDL ratios were the most significant particles. The large low-density lipoproteins and 3 types of VLDLs including very small-, small-, and very small VLDL ratios were the most significant particles with the highest number of lipoprotein to lipid relative concentrations, respectively. Various intermediate-density lipoproteins compositions containing free cholesterol, total lipids, cholesterol, and cholesteryl esters were the other highly significantly associated lipoprotein to lipid relative moieties with AMD. Cholines, phosphatidyl, and sphingomyelins were among other lipids negatively associated with the risk of AMD (Fig 4, Table S2).

The majority of metabolites (N = 76) showed a negative association with AMD, suggesting a lower risk of AMD with the abundance of these metabolites in blood plasma (Fig S6A). Conversely, concentrations of only 8 metabolites were shown to be positively associated with AMD which suggests higher concentrations of these metabolites were associated with higher AMD risk (Fig S6B). The ratios of triglyceride concentrations to total lipids in different lipoprotein subgroups showed a consistent positive association with AMD.

Lipoprotein components of positively associated metabolites include various subgroups of very small, small, medium, and large VLDLs. No amino acids were significantly associated with AMD in this study (Fig 4).

Genome-Wide Study of Circulating Metabolites in the UK Biobank Cohort

To find suitable genetic instruments for MR analyses of 325 metabolites (consisting of metabolite groups described in Table S1) in AMD, a series of GWAS were performed on 98 316 European participants. We report independent SNPs with a genome-wide significant association threshold ($P < 5 \times 10^{-8}$) for 325 metabolites in Table S3. To validate these findings we compared the results of 6 randomly chosen metabolites including members of amino acids, lipoproteins, and lipids (apolipoprotein A1, XL_HDL_TG, XS_VLDL_L, glycine, phenylalanine, and alanine) with the previously published data by Lotta et al and Kettunen et al.[45,46] The comparison was conducted for effect sizes of variants identified in our study ($P < 5 \times 10^{-8}$), and variants identified in Kettunen et al ($P < 5 \times 10^{-8}$), or in Lotta et al (at suggestive significant level: $P < 1 \times 10^{-6}$), and showed high correlations (Fig S7).

Genetic Overlap Between Metabolite Levels and AMD Risk

We investigated the potential overlap between genetic variants associated with 325 metabolites and those implicated in AMD risk, as reported by Fritsche et al.[8] This revealed intersections between the identified metabolite-associated SNPs and established AMD risk variants, particularly those associated with lipid-related AMD risk regions (genes)[10] including rs429358 (APOE), rs17231506 (CETP), rs2043085, rs2070895 (LIPC), and rs2740488 (ABCA1). Notably, these identified SNPs showed enrichment in GWAS results of 2 metabolite groups: lipoprotein subclasses and relative lipoprotein: lipid concentrations. Single nucleotide polymorphisms linked to LIPC were among the most significant signals in metabolite GWAS, while rs2740488 (ABCA1) was observed more frequently among metabolites, albeit at a lower significance level (Fig S8).

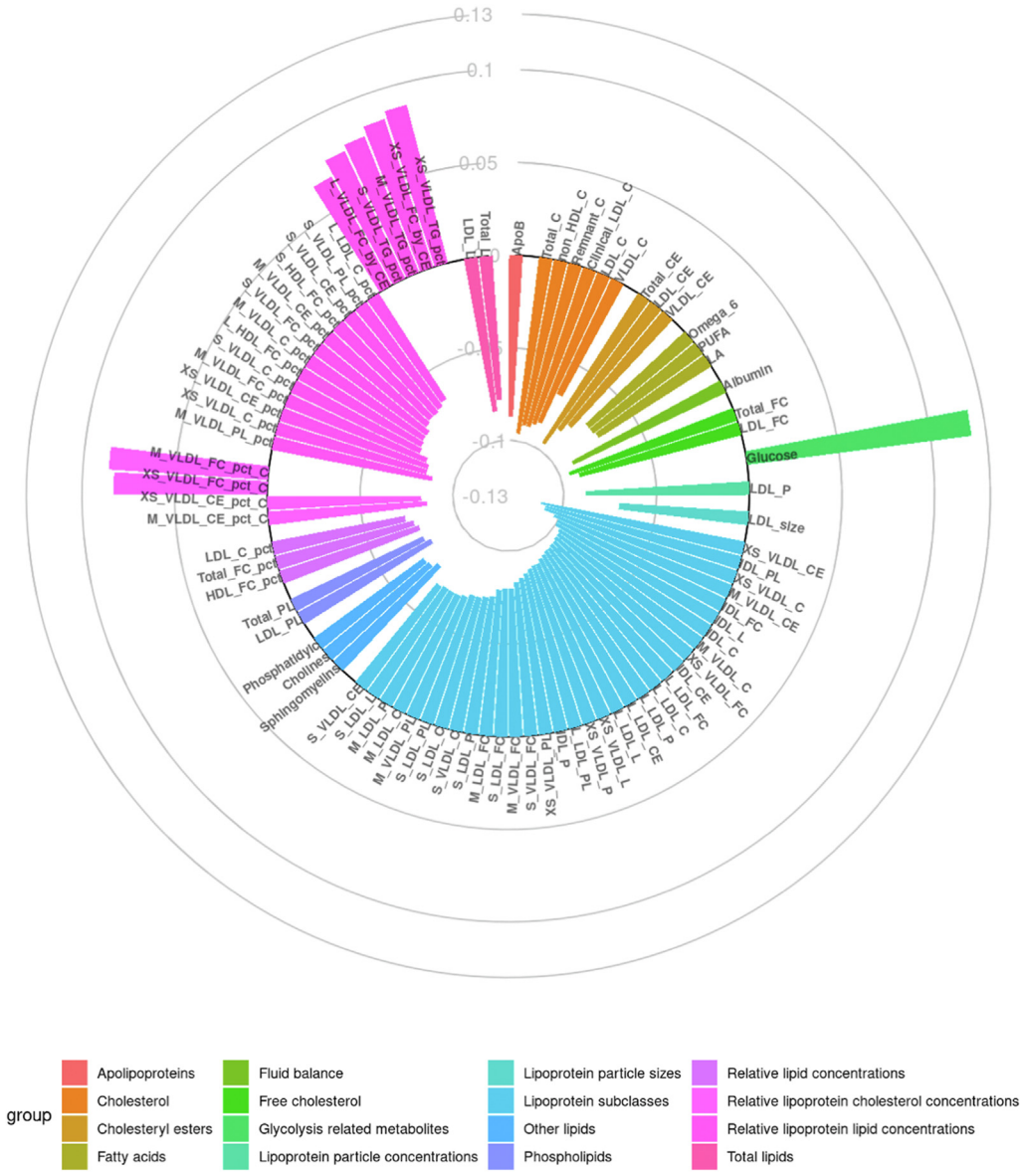


Figure 4. Metabolites that are significantly associated with age-related macular degeneration (AMD) in patients versus non-AMD controls. A circular bar plot depicting 84 metabolites, negatively or positively associated with AMD after accounting for sex and age. Only metabolites meeting a false discovery rate-corrected significance level (adjusted *P* value < 0.05) are displayed. Gray circles denote the circularized y-axis. Bars pointing outward of the black circle denote increased levels in AMD (effect >0), whereas bars pointing inward of the black circle indicate decreased levels in AMD (effect <0). Each estimate represents the difference in the outcome variable (i.e. AMD risk) per standard deviation increase/decrease in the scaled-transformed metabolite values. The positive and negative effect size translates to metabolic increases and decreases, respectively, in individuals with AMD in comparison to the control group. Colors indicate groups and subgroups of metabolites separated by an empty space between bars. Groups and subgroups of metabolites are described in Table S2. The prefixes indicate the size of particles: L, large; M, medium; S, small; XS, very small. The metabolite abbreviations are described in Table S1.

MR Analyses

Three methods of 2-sample MR (MR Egger, inverse variance weighted, and weighted median MR) were performed to evaluate the causal relationships between 325 metabolite biomarkers and AMD. This led us to identify 19 metabolites most likely causal for AMD in patients of European ancestry (Fig S9). These metabolites showed significant (FDR <0.05) causal effects on AMD for all 3 MR methods (Fig 10, Table S4 and Fig S11). We identified 5 putative causal metabolite groups where increased metabolite concentrations likely cause AMD, including subgroups of metabolites that contain ratios of large VLDL and medium HDL (Table S4). Conversely, decreased ratios of 14 metabolites showed evidence of increasing risk of AMD. A list of these metabolites, the direction of effect, and the number of genetic instruments used in MR analyses are summarized in Table S3.

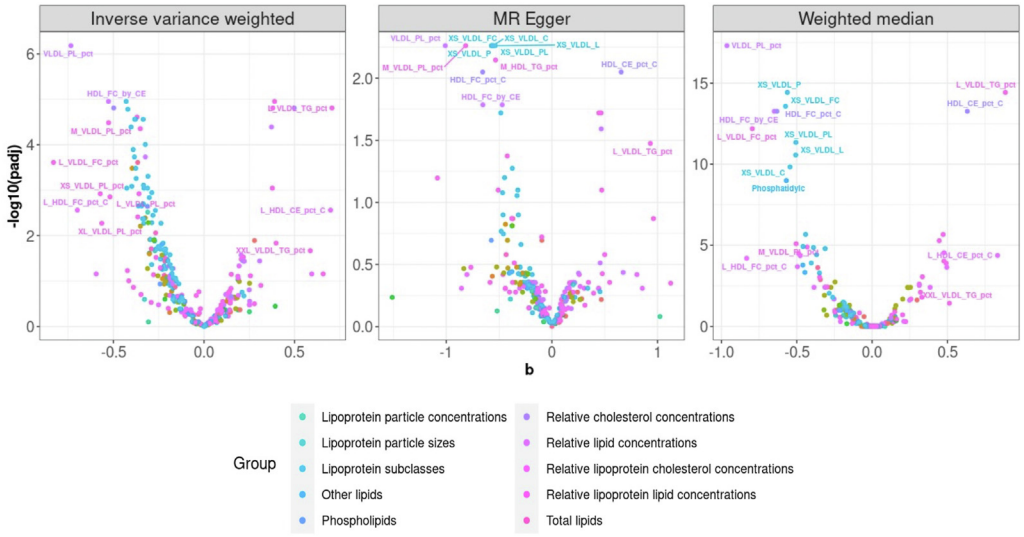


Figure 10. Mendelian randomization (MR) analyses on 325 metabolites using 3 MR methods. Significant metabolites resulting from corrected *P* values (false discovery rate [FDR] <0.05) and an effect size >0.5 (b > 0.5 or b < −0.5) are labeled. Colors represent 21 groups of metabolites. Only metabolite groups with ≥1 member with an absolute effect >0.5 are included in the legend. Positive b values indicate potential causal factors where higher metabolite plasma concentrations increase age-related macular degeneration (AMD) disease risk. Negative b values indicate metabolites where lower concentrations increase AMD risk.

Validation of MR Results

To identify potentially misleading causal estimations in MR analyses, sensitivity analyses (pleiotropy analysis and heterogeneity test) were performed to evaluate the robustness of the 19 MR-identified metabolites that most likely cause AMD in European ancestry.

The MR-Egger intercept test showed no evidence ($P < 0.05$) of a significant pleiotropic variant among the selected genetic instrument variants for 19 metabolites (Table S5).

Cochran's Q statistic was used to test the heterogeneity of SNP effects for the identified 19 metabolites (Table S6). There was evidence of significant heterogeneity of effects across all metabolites, which may also be seen from the forest plots (Fig S12) and funnel plots (Fig S13). However, the estimates obtained from individual SNP leave-one-out tests (Fig S14) were highly consistent. Further, the consistency of MR effect estimates using the different methods, does not suggest the MR estimates are highly biased.

AMD Risk Prediction Models

The classifier was constructed first from only age and sex (baseline model) and second from the combination of metabolites, age, and sex iterating on 52 subsets of samples with 1:1 case-control ratios (due to a high imbalance between cases and controls).

The performance of XGBoost showed age as the most significant predictor likely overpowering the contribution of metabolites in AMD risk prediction (Fig S15A). Apart from age as the primary predictive factor, amino acids including phenylalanine, glycine, histidine, and members of relative lipoprotein: lipid subclasses were among those metabolites with the highest predictive values (Table S7). The averaged receiver operating characteristic-area under the curve value showed no improvement in the baseline model compared to when metabolites were added to the model (Fig S15B).

Integration of Results

In this study, we identified 84 metabolites that differ in AMD patients compared with non-AMD controls in the UK Biobank cohort. The MR analysis of 325 metabolites identified 19 metabolites with likely causal effects for AMD. Our efforts to incorporate the metabolites into a prediction model showed no improvement compared with the baseline model (including only age and sex; Table S8).

To identify those metabolites that are both disease biomarkers and likely causal for AMD, we looked for metabolites identified in both analyses in this study. Seven metabolites including the percentage of phospholipids to total lipids in medium VLDL, and 6 lipid components that contain very small VLDLs, exhibited lower levels in AMD patient plasma in addition to having causal effects (listed in Fig 16). In our MR analysis, these metabolites showed negative effects (beta values) that suggest decreased levels of the metabolites contribute to an increased risk of disease; therefore, lower levels of these metabolites likely cause AMD (Fig 16A). Notably, we confirmed a negative correlation between the harmonized effects of SNPs governing AMD risk and the concentrations of these 7 metabolites (Fig 16B), further supporting the observation of the lower levels of these metabolites in AMD patients.

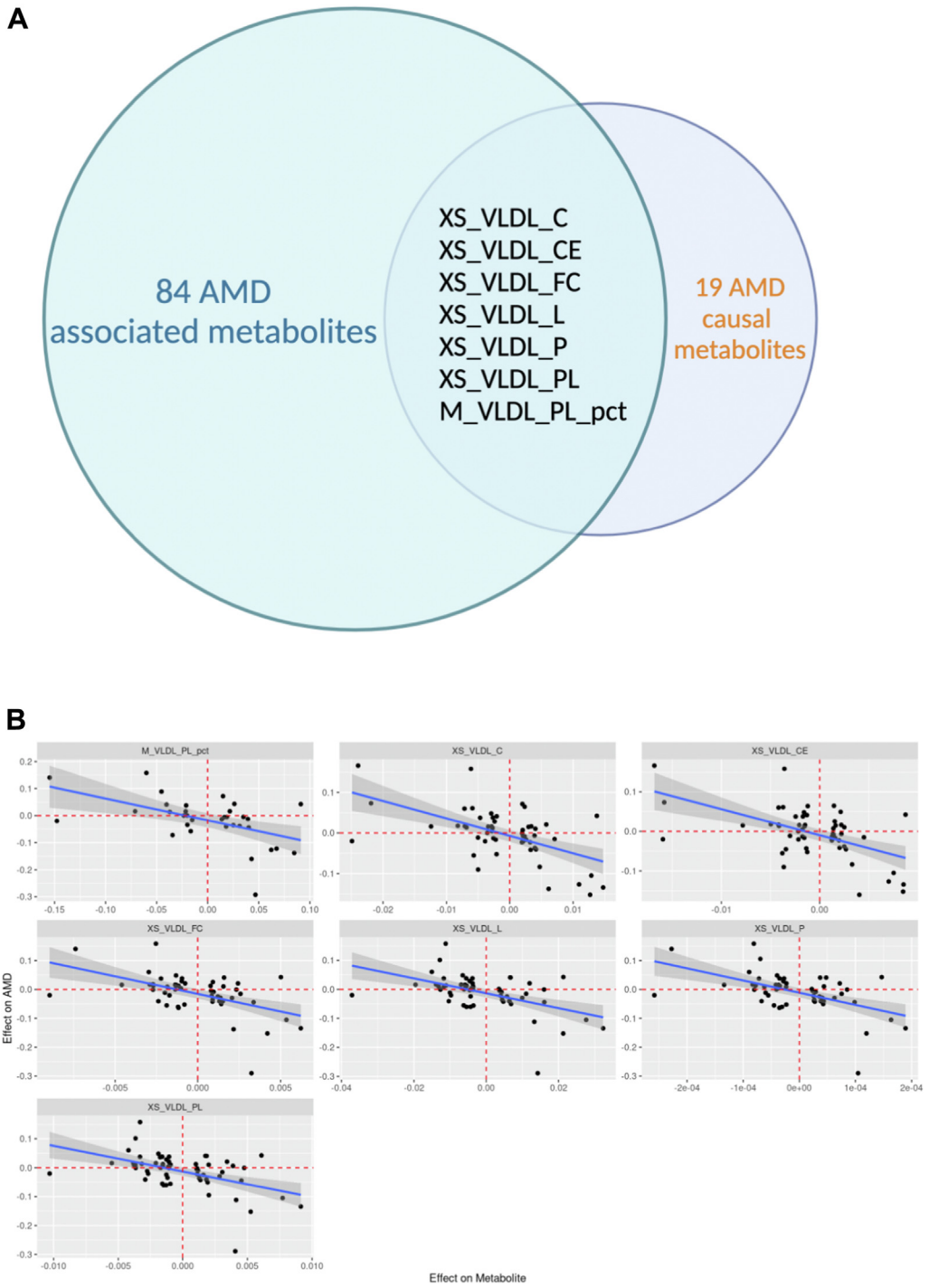


Figure 16. Metabolites that are both disease biomarkers and likely causal for age-related macular degeneration (AMD). **(A)** A Venn diagram summarizing the findings in the 2 facets (association and Mendelian randomization [MR] analyses) of this study. A total of 84 biomarkers demonstrated statistical significance (false discovery rate [FDR] < 0.05) in the association analysis (facet 1). Nineteen metabolites reached the significance threshold (FDR < 0.05) across all 3 MR methods of which 7 biomarkers exhibited lower levels in individuals with AMD. These 7 metabolites exhibited lower levels in AMD patient plasma, in addition to having causal effects that suggest lower genetically predicted levels of these metabolites appear to increase AMD risk. **(B)** Effect size comparisons between AMD-risk single nucleotide polymorphisms (SNPs) and SNPs associated with metabolite levels. Each dot represents a SNP. Each panel represents a metabolite and contains the SNPs that were found to have a genome-wide significant effect on that metabolite. The x-axis captures the effect sizes of the SNPs on a given metabolite while the y-axis captures the effect of the same SNPs on AMD risk. The blue line represents a regression line between the two. Metabolites causally affecting the disease are expected to have correlated effect sizes. Metabolite descriptions as follows: XS_VLDL_PL: phospholipids in very small very low-density lipoprotein (VLDL); XS_VLDL_P: concentration of very small VLDL particles; XS_VLDL_L: total lipids in very small VLDL; XS_VLDL_FC: free cholesterol in very small VLDL; XS_VLDL_CE: cholesteryl esters in very small VLDL; XS_VLDL_C: cholesterol in very small VLDL; M_VLDL_PL_pct: phospholipids to total lipids in medium VLDL percentage.

Discussion

In this study, we leveraged the UK Biobank dataset to investigate the role of circulating metabolites in AMD in 3 distinct facets: (1) the associations of plasma metabolites in AMD patients compared with non-AMD controls, (2) the likely causal impacts, and (3) predictive values of these biomarkers.

While previous studies have examined metabolite associations with AMD, this study places a strong emphasis on clinically relevant metabolites, including metabolite ratios and derivatives. Additionally, we leveraged a substantial sample size to enhance the identification of genetic instruments for MR analysis in these metabolites.

The association analysis of metabolites revealed 84 metabolites linked to the risk of AMD. These metabolites are enriched in subclasses of lipoproteins and lipid components. These results are in line with the effects shown in other observational studies conducted on AMD patients.[6,14,47] In a recent study, Han et al highlighted 155 metabolites (prominently lipid-related plasma metabolites) that were associated with advanced AMD.[14]

Using the NMR platform, Acar et al conducted a study on 2267 AMD patients and 4266 controls and identified 60 metabolites associated with AMD including HDL and VLDL lipoprotein particles, fatty acids (total fatty acids, monounsaturated fatty acid, and saturated fatty acid), amino acids, and citrate.[6] Among these, we were able to test associations of 55 metabolites and replicated findings for 10 of those metabolites including apolipoproteins (apolipoprotein B), 2 subgroups of cholesterol, and 7 subgroups of lipoprotein subclasses (4 medium VLDLs and 3 small VLDL particles). The effect sizes of these metabolites in our study and Acar et al's are compared in Fig S17. Specific differences in our findings and the study by Acar et al are amino acids which showed significant associations in their study but were not significant in our study. In contrast, we observed strong associations with various subgroups of very small VLDL ratios, small and large low-density lipoproteins, and members of intermediate-density lipoprotein which were not reported by Acar et al. These variations could arise from participant fasting status in both studies. The UK Biobank collected nonfasting venous blood samples, while Acar et al incorporated a mix of fasting and nonfasting states during blood sample collection for association meta-analysis.[6] Furthermore, the UK Biobank measured metabolic biomarkers from plasma samples, whereas Acar et al utilized 5 cohorts with access to plasma, serum, or both.[6]

Next, we investigated the causal effects of metabolites and pinpointed 19 metabolites that most likely promote AMD. Two groups of metabolites including lipoproteins and relative lipoprotein: lipid concentrations consist of 68% of these likely causal metabolites. Members of lipoprotein subclasses that contain very small VLDL particles were highlighted as likely causal metabolites in AMD.[26]

Mendelian randomization applications by Han et al utilizing a different platform identified 96 metabolites with causal associations that are enriched in lipids.[14] We adopt a stringent 2-step approach, involving a more rigorous linkage disequilibrium r^2 threshold for constructing genetic instruments and the implementation of 3 MR methods, to enhance the robustness of our MR findings. This methodological rigor contributes to a lower number of identified associations compared with previous studies.

The low number of metabolites that overlap between the significant causal metabolites and highly associated metabolites in this study may arise for several reasons, such as the likely bystander effects of the single metabolite associations. Moreover, genetically predicted effects of metabolites in AMD perhaps have a lower dynamic range whereas measured metabolite levels are likely to be impacted by a variety of environmental and/or technical factors that are inevitable in measuring the metabolites. Additionally, blood metabolite levels may not reflect levels in the retina which is the primary disease tissue in AMD.

Surprisingly we found that no metabolites showed clinically relevant predictive power in identifying AMD using our machine-learning approach. Age consistently overshadowed the contribution of metabolites in the prediction of AMD, possibly diminishing their impact on receiver operating characteristic-area under the curve evaluations.

We recognize the caveats in this study, notably its retrospective nature, which results in a lack of current information regarding the status of both AMD patients and non-AMD controls as they have aged. In particular, underrepresentation of AMD diagnoses in the UK Biobank cohort (approximately 2%) compared to what is expected (~8%),[48] likely biased toward late-stage disease, with many cases of early-stage AMD likely currently undiagnosed as per the clinical reporting in UK Biobank. Furthermore, this study is constrained to reports of International Classification of Diseases 10th Revision codes within the UK Biobank dataset, limiting investigation into widely used standardized grading in retinal disease epidemiology and regional variations in disease progression risk.[49] The manually graded retinal imaging dataset of UK Biobank by a recent study[50] would potentially ease the usage of this data for researchers, once it becomes available.

We exclusively used European subjects in our study cohort; therefore, our findings may vary across different ancestries. Additionally, non-AMD controls have not been checked for any other diseases that could potentially confound metabolite profiles in controls. We also acknowledge that the possible effect of lipid-lowering medications on metabolic profile is not included in this study, limited by incomplete data in the UK Biobank for the entire cohort subset. Moreover, the skew towards later-stage and more severe AMD has not been taken into account in this study. Given previous demonstrations of AMD disease-stage specific metabolic profiles,[14] testing the predictive values of metabolites in a heterogeneous group of AMD patients may limit the sensitivity of the results (in particular prediction models) in this study. Furthermore, the metabolite data from the UK Biobank cohort is derived from nonfasting blood samples, a factor that could have implications for the development of an optimal prediction model.

A uniform sample size of fasting metabolites matched for age and sex, along with carefully selected control samples free from underlying health conditions, may enhance the efficacy of implementing metabolite profiles in AMD

patients in the clinic. Combining current AMD risk factors, circulating metabolic biomarkers, imaging, and genetic risk factors may enhance AMD incidence/progression risk prediction, something that is yet to be investigated. This may explain why, whilst we were able to identify individual metabolites as having predictive power as well as in some cases, likely causative roles, this failed to translate into overall predictive power, suggesting the effect sizes in the presence of age effect were too weak to enhance the overall predictive model.

Despite the extensive evidence for the association of lipids with AMD development and progression,[5] their roles are obscured due to their extremely complex biological processes. Our findings highlight the detailed subclasses of lipoproteins (both measured and derivative) that should be explored further to identify the specific members that functionally promote AMD. Our 3-tiered analysis gives greater confidence in pursuing these findings than do previous studies based solely on individual metabolite association studies. While the findings in this study are in line with the effects shown in other studies conducted on AMD patients, validation of our results in an independent dataset will further refine the causal roles of these subgroups of metabolites in AMD.

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HUMAN SUBJECTS: Human subjects were included in this study. The study was approved by the Walter and Eliza Hall Institute of Medical Research Human Research Ethics Committee (HREC project number 17/09LR). Ethics approval was obtained by the Northwest Multi-centre Research Ethics Committee and our research adhered to the tenets of the Declaration of Helsinki (UK Biobank research ethics approval). Informed consent was obtained from all study participants and all participants were free to withdraw from the study at any time.

No animal subjects were included in this study.

Author Contributions:

Conception and design: Farashi, Bonelli, Ansell, Bahlo

Data collection: Farashi, Bonelli, Jackson, Ansell, Bahlo

Analysis and interpretation: Farashi, Bonelli, Jackson, Ansell, Bahlo

Obtained funding: N/A

Overall responsibility: Farashi, Bonelli, Jackson, Ansell, Bahlo, Guymer

Abbreviations and Acronyms:
AMD = age-related macular degeneration; **ApoE** = apolipoprotein E; **CETP** = cholesterol ester transfer protein; **FDR** = false discovery rate; **GWAS** = genome-wide association studies; **HDL** = high-density lipoprotein; **LIPC** = hepatic lipase; **MR** = Mendelian randomization; **NMR** = nuclear magnetic resonance; **SNP** = single nucleotide polymorphism; **UK** = United Kingdom; **VLDL** = very low-density lipoprotein; **XGBoost** = extreme gradient boosting.

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